Annual Report 2003

Project SB-2

Conservation and Use of Tropical Genetic Resources

Formerly know as: **Assessing and Utilizing Agrobiodiversity through Biotechnology**

CIAT
For Internal use only

November, 2003

Project SB-2: Conservation and Use of Tropical Genetic Resources

(formerly known as Assessing and Utilizing Agrobiodiversity through Biotechnology)

PROJECT OVERVIEW

PROJECT DESCRIPTION

Objective: To preserve the Designated Collections and employ modern biotechnology to identify and use genetic diversity for broadening the genetic base and increasing the productivity of mandated and selected nonmandated crops.

Outputs:

1. Improved characterization of the genetic diversity of wild and cultivated species and associated organisms.
2. Genes and gene combinations used to broaden the genetic base.
3. Increase efficiency of breeding program using genomics tools
4. Mandated crops conserved and multiplied as per international standards.
5. Germplasm available, restored, and safely duplicated.
6. Designated Collections made socially relevant.
7. Strengthen NARS for conservation and use of Neotropical plant genetic resources.
8. Conservation of Designated Collections linked with on-farm conservation efforts and protected areas.

Milestones:

2004 High throughput screening of germplasm bank and breeding materials implemented, using microarray technology. Al tolerance in *Brachiaria* characterized. Gene discovery for drought tolerance in bean for nitrification in brachiaria initiated. Marker-assisted selection for ACMV and whitefly resistance initiated. Transgenic rice resistant to a spectrum of fungal diseases. Development of insertion mutagenesis population in rice, using Ac/Ds. Gene flow studies for bean and rice completed. Links with conservation efforts in protected areas and on farms established. Germplasm collections regenerated. Initiation of DNA banks for core collections. Safe-duplication and restoration continued. Biosafety field testing of transgenic cassava initiated.

2005 Efficient transformation system devolved for cassava. Bean with high iron and zinc tested and transferred to CIAT Africa program for bioavailability testing. Survey of cassava germplasm for beta carotene. SNP markers developed for bean and implemented for MAS. Targeted sequencing of cassava genome. Isogenic of QTL in rice developed and tested. Gene expression studies. Technology transfer for rapid propagation system to NARS. Testing of Ac/DS population for gene identification.

2006 Scaling up of marker assited selection and transformation established for rice bean and cassava. High trough put screening for selected tropical fruits initiated. Marker assisted selected for multiple traits implemented in beans, rice and cassava. Target genes for drougt identified and tested in beans. High iron and zinc bean lines developed through markers assisted selection released for field testing. Beta carotene cassava tested in Colombia, Brazil and selected countires in Africa.

Users: CIAT and NARS partners (public and private) involved in germplasm conservation and crop genetic improvement and agrobiodiversity conservation; AROs from DCs and LDCs, using CIAT technologies.

Collaborators: IARCs (IPGRI through the Systemwide Genetic Resources Program, CIP, and IITA through root and tuber crop research, IFPRI through biofortification proposal and CATIE); NARS (CORPOICA, ICA, EMBRAPA, IDEA, INIAA, INIFAP, UCR, INIAs); AROs (IRD, CIRAD, Danforth Center, CAMBIA, NCGR, and universities—Cornell, Yale, Clemson, Kansas State, Bath, Hannover, Rutgers, Ghent, Gembloux); biodiversity institutions (A von Humboldt, INBIO, SINCHI, Smithsonian); corporations and private organizations.

CGIAR system linkages: Saving Biodiversity (40%); Enhancement & Breeding (55%); Training (4%); Information (1%).

CIAT project linkages: *Inputs to SB-2:* Germplasm accessions from the gene bank project. Segregating populations from crop productivity projects. Characterized insect and pathogen strains and populations from crop protection projects. GIS services from the Land Use Project. *Outputs from SB-2:* Management of Designated Collections (gene banks); genetic and molecular techniques for the gene bank, crop productivity, and soils (microbial) projects. Identified genes and gene combinations for crop productivity and protection projects. Propagation and conservation methods and techniques for gene banks and crop productivity projects. Interspecific hybrids and transgenic stocks for crop productivity and IPM projects.

CIAT: SB-2 PROJECT LOG FRAME (2004-2006)

PROJECT: CONSERVATION AND USE OF TROPICAL GENETIC RESOURCES
PROJECT MANAGER: JOE TOHME

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Goal To contribute to the sustainable increase of productivity and quality of mandated and other priority crops, and the conservation of agrobiodiversity in tropical countries.	CIAT scientists and partners using biotechnology information and tools in crop research. Genetic stocks available to key CIAT partners.	CIAT and NARS publications. Statistics on agriculture and biodiversity.	
Purpose To conserve the genetic diversity and ensure that characterized agrobiodiversity, improved crop genetic stocks, and modern molecular and cellular methods and tools are used by CIAT and NARS scientists for improving, using, and conserving crop genetic resources.	Information on diversity of wild and cultivated species. Mapped economic genes and gene complexes. Improved genetic stocks, lines, and populations.	Publications, reports, and project proposals.	Pro-active participation of CIAT and NARS agricultural scientists and biologists.
Output 1 Genomes characterized of wild and cultivated species of mandated and nonmandated crops and of associated organisms.	Molecular information on diversity of mandated and nonmandated crops species, and related organisms. Bioinformatic techniques implemented. QTLs for yield component in rice, for nutrition traits in beans and cassava, and for nitrification and Al tolerance in *Brachiaria*.	Publications, reports, and project proposals. Germplasm. Availability of a laboratory information management system (LIMS).	Availability of up-to-date genomics equipment, and operational funding.
Output 2 Genomes modified: genes and gene combinations used to broaden the genetic base of mandated and nonmandated crops.	Transgenic lines of rice and advances in cassava, beans, *Brachiaria*, and other crops. Cloned genes for iron, zinc and drought traits Cloned genes and preparation of gene constructs. Information on new transformation and tissue culture techniques.	Publications, reports, and project proposals. Germplasm.	IPR management to access genes and gene promoters. Biosafety regulations in place.
Output 3 Collaboration with public- and private-sector partners enhanced.	CIAT partners in LDCs using information and genetic stocks. New partnerships with private sector.	Publications. Training courses and workshops. Project proposals.	Government and industry support national biotech initiatives.

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Narrative Summary	**Measurable Indicators**	**Means of Verification**	**Important Assumptions**
Output 4 Mandated crops conserved and multiplied as per international standards.	Germination rates for long-stored materials. Cost per accession/year, compared with other gene banks.	Visits to GRU substations and conservation facilities.	Absence of uncontrolled diseases. Quarantine greenhouse space available at different altitudes.
Output 5 Germplasm available, restored, and safely duplicated.	Number of germplasm requests received and satisfied annually. Users received germplasm and data. Users asked for novel germplasm and data.	Visits to multiplication plots. Reports on requests and delivery. Number of core collections multiplied and shipped.	Agreement with CIAT holds.
Output 6 Designated Collections made socially relevant.	Landrace diversity restored to farmers. Farmers use new varieties. Breeders use novel genes.	Germplasm catalogs. Plant variety registration logs. National catalogs.	International collecting possible. Quarantine matters cleared.
Output 7 Strengthen NARS for conservation and use of Neotropical plant genetic resources.	NARS germplasm collections conserved. Number of trainees trained at CIAT. Number of universities and NARS using training materials.	Country questionnaires. Courses registered. Distribution and sales of training materials.	NARS and networks willing to cooperate.
Output 8 Conservation of Designated Collections linked with on-farm conservation efforts and protected areas.	Number of case studies and pilot *in situ* conservation projects.	Project documentation.	NARS interested in conservation efforts. Farmers interested in conservation efforts.

CONTENT

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OUTPUT 1. Characterizing genomes of wild and cultivated species of mandated crops and associated organisms

Activity 1.1 Characterization of genetic diversity

1.1.1. Gene flow studies in the bean plant model

Special Project, supported by BMZ, Germany
D.G. Debouck[1], R. Araya[2]
[1] SB-1 Project , [2]University of Costa Rica

Field work

The field work is part of the monitoring of wild forms of common bean, genetically compatible with the cultivated form (landraces, commercial cultivars). It includes: the mapping of all populations in one geographic area (either through collection consultations or direct visits), the spotting of observed cases of gene flow, and the study of the conditions by which gene flow is created and maintained.

During this field work (December 2002- January 2003), we were interested in 1) searching more populations of wild common bean in the Valle Central of Costa Rica, 2) verifying the stability of wild-weed-crop complexes spotted in previous field works (1987, 1998), 3) finding more 'intermediate' materials that would deserve study by molecular markers (see section 3).
The methodology followed was the one defined elsewhere to look for wild *Phaseolus* species (Debouck 1988).

The results of the field work can be seen in the following table and figure.

Número	Especie	Fecha d/m/a	Provincia, Distrito	Sitio cercano	Coordinates
2111	vulg s	15/12/2002	San José, Aserrí	Aserrí	84°07'W 9°52'N 1550 m
3106	vulg s	13/12/2002	Alajuela, Carrizal	Chagüite	84°10'W 10°06'N 1510 m
3132	vulg s	14/12/2002	Alajuela, Zarcero	Zarcero	84°23'W 10°10'N 1610 m
3133	vulg s	14/12/2002	Alajuela, Sabana Red.	Sabana Redonda	84°14'W 10°07'N 1380 m
3134	vulg s	15/12/2002	San José, San Gabriel	Tranquerillas	84°07'W 9°48'N 1500 m
3135	vulg s	15/12/2002	San José, Tarbaca	Chirogres	84°06'W 9°48'N 1480 m
3136	vulg s	15/12/2002	San José, San Miguel	Sn Miguel Desamp.	84°04'W 9°51'N 1370 m
3137	vulg s	16/12/2002	San José, San Antonio	Bebedero	84°10'W 9°54'N 1600 m
3138	costar	16/12/2002	San José, San Antonio	Bebedero	84°10'W 9°54'N 1700 m
3139	costar	16/12/2002	San José, Vista de Mar	Vista de Mar	83°58'W 9°58'N 1790 m
3140	vulg s	17/12/2002	Cartago, San Rafael	Parque Iztarú	83°58'W 9°54'N 1750 m
3141	leptos	17/12/2002	Cartago, San Rafael	Parque Iztarú	83°58'W 9°54'N 1640 m
3142	costar	18/12/2002	Cartago, San Nicolás	Río Taras	83°55'W 9°55'N 2000 m
3143	vulg s	18/12/2002	Cartago, San Rafael	Hda. Tres Ríos	83°59'W 9°54'N 1500 m
3144	costar	16/01/2003	Cartago, San Rafael	Cerros Carpintera	83°59'W 9°54'N 1630 m
3145	xantho	15/01/2003	San José, San Antonio	Bebedero	84°10'W 9°54'N 1650 m
3146	leptos	15/01/2003	San José, Aserrí	Piedra de Aserrí	84°07'W 9°52'N 1550 m
3147	vulg s	15/01/2003	San José, Tarbaca	El Tigre	84°06'W 9°49'N 1450 m
3148	vulg s,w	15/01/2003	San José, San Miguel	El Manzano	84°05'W 9°49'N 1370 m
3149	Sp. (X)	16/01/2003	Cartago, San Nicolás	Quircot, sitio 31	83°56'W 9°54'N 1520 m
3150	vulg c	16/01/2003	Cartago, San Nicolás	Quircot, sitio 26	83°56'W 9°54'N 1560 m
3151	vulg w	16/01/2003	Cartago, San Nicolás	Quircot, sitio 29	83°56'W 9°54'N 1510 m
3152	vulg c	16/01/2003	Cartago, San Nicolás	Quircot, sitio 29	83°56'W 9°54'N 1510 m
3153	vulg c	16/01/2003	Cartago, San Nicolás	Quircot, sitio 29	83°56'W 9°54'N 1510 m
3154	vulg w	17/01/2003	Cartago, San Nicolás	Quircot, sitio 26	83°56'W 9°54'N 1560 m
3155	vulg s,w	17/01/2003	Cartago, San Nicolás	Quircot, sitio 26	83°56'W 9°54'N 1560 m
3156	vulg s,w	17/01/2003	Cartago, San Nicolás	Quircot, sitio 30	83°56'W 9°54'N 1530 m
3157	Sp. (X)	17/01/2003	Cartago, San Nicolás	Quircot, sitio 30	83°56'W 9°54'N 1530 m
3158	Sp. (X)	17/01/2003	Cartago, San Nicolás	Quircot, sitio 30	83°56'W 9°54'N 1530 m
3159	xantho	13/01/2003	Alajuela, Carrizal	Chagüite	84°10'W 10°06'N 1510 m

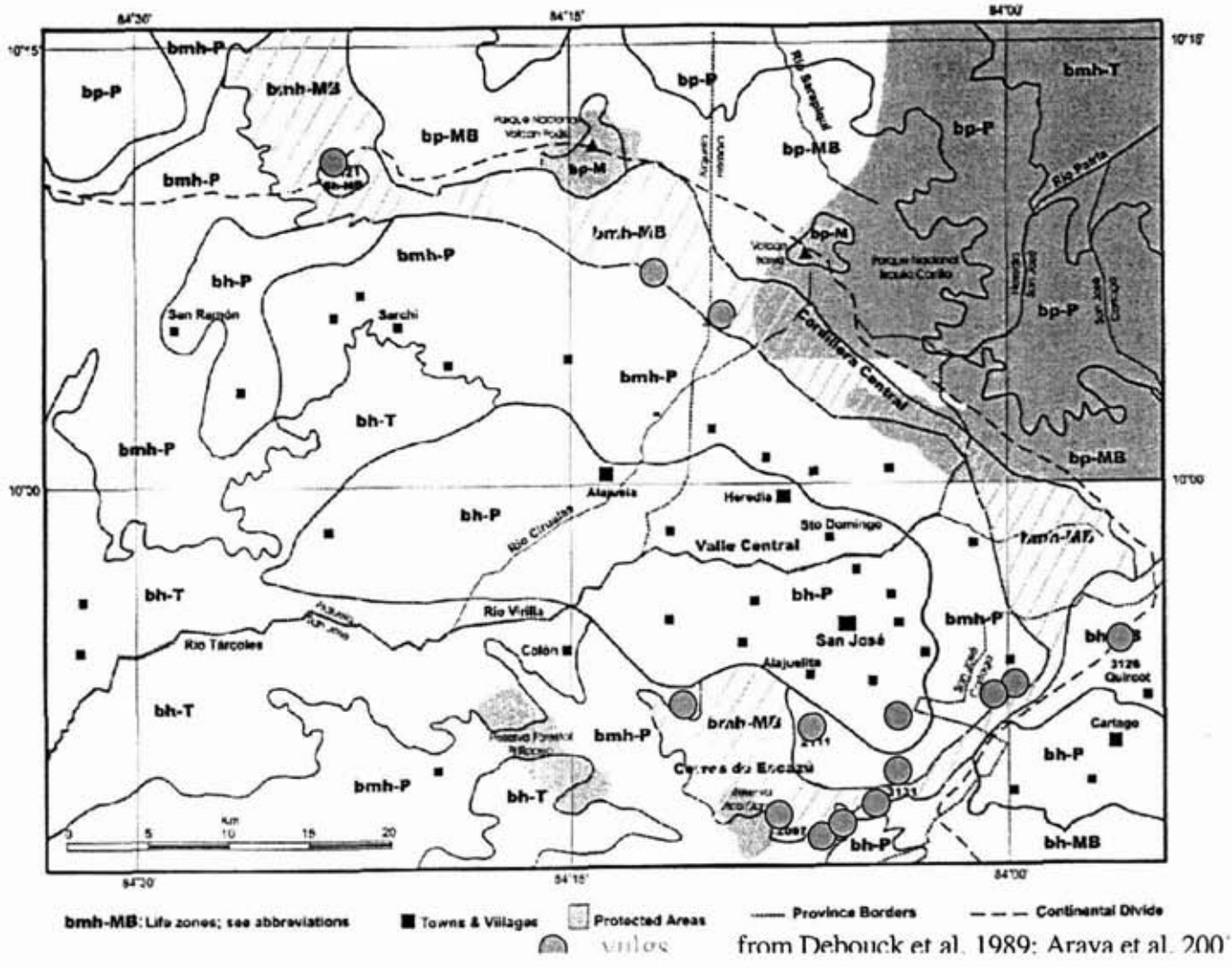

Figure 1 – Map of Valle Central with all populations known for wild *P. vulgaris*.

The number of known populations for wild *Phaseolus vulgaris* L. has been doubled as compared to previous works (Debouck et al. 1989; Araya et al. 2001), and currently covers most of the Central Valley.

The field work has revealed the low permanency of wild-weed-crop complexes once the planting of bean crop has been discontinued, as in Aserrí and Tarbaca, and to a lower extent Quircot. Of equal importance for the permanence of a complex is the maintenance of a 'weedy' environment: if farmers are using herbicides as in some plots in Quircot, wild and weedy forms disappear. If the original humid forest vegetation is allowed to come back as in Tres Ríos, complexes and populations of wild forms tend to regress as well. If farmers are switching to cash crops (such as the year-bean, *P. dumosus*) instead of maintaining traditional landraces, then the complexes regress. Interestingly, the field work has revealed natural hybrids with *P. costaricensis*, perhaps of value in future breeding. Finally, it seems important to report that while farmers know about the presence of the wild and intermediate forms, in our case study site (= Quircot) they do not longer make selections in the segregating populations resulting from natural hybridizations. This situation is in contrast with that observed in certain parts of the Colombian and Peruvian

Andes (Beebe et al. 1997). There, in the late 1980s and 1990s, farmers were still actively selecting forms of interest to them in the hybrid swarms resulting from natural hybridizations between wild and cultivated forms and among weedy and cultivated forms. Our interviews with farmers at Quircot would indicate that the proximity of markets and access to new sources of variability would not incite to make use of the variants generated through the w-w-c complexes.

We plan to extend the field observations in December 2003-January 2004, in order to validate the above described conditions allowing the permanency of the complexes.

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1.1.2 Studies of gene flow under field station conditions

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In this part, gene flow is studied in the field in Costa Rica under conditions at the experimental stations of Alajuela (840 m) and Fraijanes (1850 m). The design (plot planted with beans, of 15 x 15 m) has been standardized and is used planting after planting at these stations. Two subplots of white-flowered and white-seeded bean variety are planted on both sides of a plot with lilac-flowered and black-seeded bean variety. The white-seeded variety cumulates recessive characters such as green hypocotyl and green stems, while the black-seeded variety has dominant characters such as purple hypocotyl and colored internodes. Intensity of gene flow is measured by countings of purple hypocotyls on seedlings from seed harvested in the plots with recessive lines. If gene flow through pollen carried by bees or other insects has occurred, the seed harvested on the white-flowered lines is hybrid, and with purple hypocotyl being dominant over the green recessive hypocotyl, these hybrids can be easily picked up quickly after germination.

The % of outcrossing has been of 0.06 and 0.03% at Alajuela for two trials, while it has been of 0.03% at Fraijanes for one trial. There is a marked effect of the dominant wind at Alajuela, while it is less marked at Fraijanes. Main pollinators observed were bees (*Apis*)

and carpenter bees (*Xylocopa*). There is also a marked distance effect, since hybrid plants are observed in rows 1-10, and mainly in rows 1-5 (row 1 is the closest to the pollen donor line, while row 10 is at the extreme, or 6 m from the border of the pollen donor line).

1.1.3 Studies of gene flow with help of biochemical and molecular markers

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[1]Universidad Nacional, [2] SB-2 Project ; [3] SB-1 Project

We present here evidence on gene flow between wild and cultivated forms of common bean in Costa Rica in addition to our previous work (González-Torres et al. 2003).

Seeds were collected from natural populations in the Central Valley of Costa Rica as previously reported (González-Torres et al. 2003). We focus on 226 'weedy' or 'intermediate' materials initially selected on morpho-agronomic characteristics, which phenotype is inherited from possible hybridization between wild and cultivated materials. A similar procedure has been used by Papa & Gepts (2003). The analyses were conducted on: 1) morpho-agronomic evaluation; 2) biochemical analysis of phaseolin by SDS-PAGE (Gepts et al. 1986), and isozymes: diaphorase (DIA) and peroxidase (PRX) according to Ramírez et al. (1987), and 3) molecular marker analysis: eight microsatellite primers reported by Gaitán-Solis et al. (2002), and cpDNA polymorphisms by PCR- RFLPs following the protocol of Chacón-Sánchez (2001).

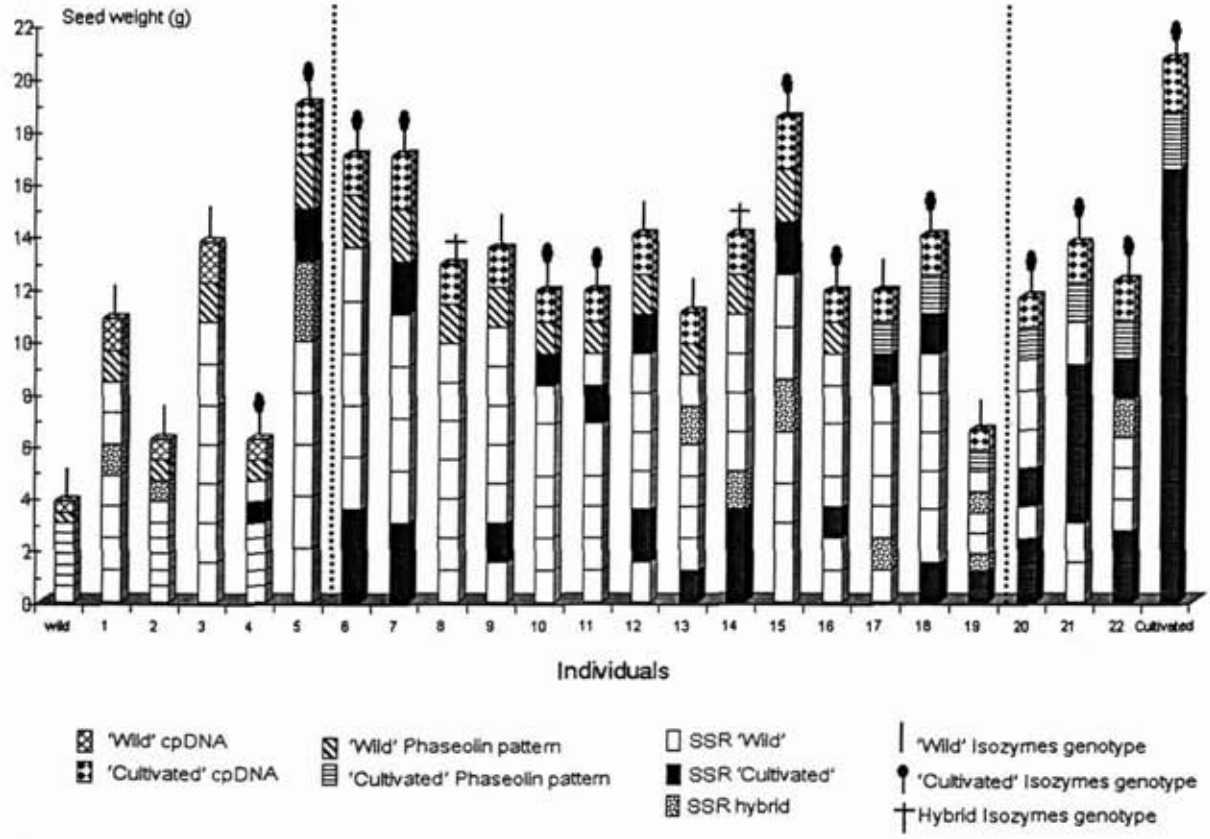
The wild populations showed mainly two phaseolin patterns, S-4 and S (Table: morphological, biochemical and molecular markers used and No. individuals analyzed for each parameter). In cultivated materials, the phaseolins T, Sb and S-4 were also observed although in low frequency.

Biological status	Seed Average Weight (g)	Phaseolin Type	Isozymes		Microsatellites		cpDNA haplotypes
			Pattern[1]	Allele[2]	Primer	Allele	
Wild	6 **N=443**	"S-4" "S" **N=402**	DIA –1 **N=227**	PRX 100 **N=204**	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 **N=134**	160 *80* 162 110 163 146 137 122	G, H **N=97**
Weedy	13 **N=226**	"C" "CH" "H" "S" "X-7"[3] "S-4" **N=191**	DIA-1 DIA-2 DIA-4 **N=170**	PRX 100 PRX 98 **N=170**	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 **N=142**	160, 177 *80* 162, 183 110, 106 163, 189 146, 150 137, 174 122, 135	G, H J, K, L **N=100**
Cultivated	23 **N=188**	"S" "X-7" "CH" **N=186**	DIA –2 DIA –4 **N=150**	PRX 98 **N=150**	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 **N=35**	177 *80* 183 106 189 150 174 135	J, K, L **N=33**

[1] According to Sprecher (1988); [2]according to Koenig & Gepts (1989); [3] Phaseolin pattern to be checked.

The figure is a representation of markers used on a selection of individuals; bar height shows the weight (g) of 100 seeds. The shortest bar represents mainly wild characteristics and the longest bar is a description of cultivated materials. The other bars show exchange among individuals of the following markers: shared SSR alleles, change in cpDNA haplotypes, seed weight, isozymes and phaseolin patterns. In individuals 1 and 2, all the evaluated parameters are "wild" and they have a hybrid SSR locus, which suggests a recent crossing of wild material with pollen of cultivated material. Seed size of individual 3 could be a phenotypic consequence of more than one past event of gene flow from cultivated material into the wild form, because all evaluated parameters are "wild" including hypocotyl color (purple), purple flower, 85 days to flowering and growth habit IV. Besides, its F2 displays a weight of 10.3 g, which suggests that it has kept "wild" characteristics and acquired a "cultivated" seed size. Individual 8 has hybrid isozymes, "wild" microsatellites and phaseolin, but it has a "cultivated" chloroplast haplotype. Individual 9 has the same characteristics as individual 8 but it has "wild" isozymes. These materials may represent cases of repeated gene flow of cultivated materials crossed with wild forms. Individual 14 is hybrid (PRX enzyme and one SSR locus), meaning that it comes from recent flow of "wild" pollen into a cultivated form. The evaluation of 22 cases from Costa Rica indicates that all materials are indeed product of a hybridization showing that the methodology implemented in the selection of the intermediate materials was the

appropriate one. Papa et al. (2003) found in intermediate materials of Mexico that the contribution of cultivated parental population was significantly higher than the wild parental one. So far, for these materials of Costa Rica, we have found a more important gene flow from wild material into the cultivated type.



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1.1.4 Genetic diversity assessment of Caribbean common bean germplasm

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Introduction

The introduction of beans to the Caribbean was postulated to have occurred from northern South America along the "Arawak arc" of Leeward Islands long before the Spanish Conquest. The Caribbean was also know to have been influenced by the pre-Colombian cultures of Central America. Therefore, the Caribbean was a transition zone between the two regions and was likely to have had a mix of bean germplasm even before the time of the colonies. Later, as a trading center and way station for the Europeans, the Caribbean likely received new crops and varieties from all over Latin America. This rich heritage makes the Caribbean even today a probable center of secondary diversity for beans. Caribbean nations and societies meanwhile have undergone rapid changes in the past fifty years, which have led to the abandonment of agriculture in many places there. Where agriculture holds on, such as the interior of Hispaniola (Dominican Republic and Haiti) farm-size is small and land pressure is intense leading to environmental degradation and emigration from rural areas. Given all this it is interesting to document and preserve the genetic diversity of beans that are still left in the Caribbean. At the University of Puerto Rico, the bean program has collected traditional bean types at local markets or from farmers, most of which were classified as "Andean" because of their seed size and color class. The UPR researchers have conducted a genetic diversity analysis of this germplasm using RAPDs and comparing this germplasm to other "Andean" genotypes from the Dominican Republic and CIAT. Last year we reported the Phaseolin types found in this germplasm and the probable Andean x Mesoamerican hybrid origin of many of the accessions with "Andean" seed types found in the Caribbean. The objective of this work was to validate these results by characterizing the same germplasm set for microsatellite diversity.

Methodology

A total of 129 entries of common bean genotypes were genotyped as described in 2002 Annual Report. These included a total of 112 traditional varieties (or selections thereof) from the Caribbean (65 from Dominican Republic; 18 from Haiti, 26 from Puerto Rico, and 1 each from Cuba, Panama and Jamaica). In addition, 16 check varieties were included of which 8 were advanced breeding lines from CIAT (DRK57, AFR735, CAL96, AFR619, AFR699, DOR364, A36, AND109), 1 was an advanced breeding line from the University of Puerto Rico (UPR9443-4), 3 were varieties from Colombia (ICA Palmar,

ICA Pijao and Calima); 2 were varieties from the USA (Redhawk and Montcalm), 1 was a variety from Peru (Blanco Laran) and another was a germplasm accession that has been extensively characterized at CIAT (G19833, Northern Peru). DNA was extracted from the young trifoliate leaf of a single seedling from each accession using a ammonium acetate extraction technique. Microsatellite PCR reactions and polyacrylamide gel electrophoresis were as described previously. Silver-stained gels were dried overnight and scanned for data analysis.

Results and Discussion

A total of 27 microsatellite markers (19 genomic and 8 gene-based markers) were selected to evaluate the genotypes (Table 1). A minimum of one marker was selected per chromosome and the rest were chosen based on genomic representation and good amplification. Only single copy microsatellites were evaluated and in all cases single bands were called. Band sizing and allele calling was done based on comparisons to 25 bp size standard ladders that were used two times per gel. Heterozygosity was not evaluated given that only a single plant per accession was sampled. The total number of alleles evaluated across all 27 markers was 118 and on average each marker revealed 4.4 alleles each. The number of alleles and discrimination power (D) values were significantly higher for genomic microsatellites (6.1 alleles, 0.622 D value) than for gene-based microsatellites (4.3 alleles, 0.513 D value) based on unpaired t-test (at $P<0.05$). The most polymorphic markers in terms of number of alleles or high D values were GATs91 (12 alleles, 0.889 D value), BM152 (12 alleles, 0.782 D value) and BM143 (10 alleles, 0.780 D value). The least polymorphic markers in terms of either low number of alleles (3 alleles or less) or low D values ($D<0.500$) were BM142, BM155, BMd10, BMd15, BMd26, BMc5, BMy4, GATs11B and GATs54 of which five were gene-based and the others were genomic.

Microsatellite analysis uncovered two major groups among the Caribbean germplasm: an Andean group that was similar to the controls G19833 and Calima; and a Mesoamerican group that was similar to the controls ICA Pijao and DOR364 (Figure 1). These two genepools were related at a 0.18 Dice similarity value.

There were a greater number of accessions and a higher overall diversity within the Andean group (79 accessions, 0.47 to 0.99 Dice similarity values) than within the Mesoamerican group (49 genotypes, 0.65 to 0.99 Dice similarity values) of this dendrogram. Microsatellite markers were ideal markers to distinguish the fine-scale relationships within these genepools. In the case of the Caribbean germplasm with an Andean affinity, all the landraces and breeding lines were more similar to the Colombian control genotype Calima (red mottled cream), than to the Peruvian control genotype G19833 (red mottled yellow). This was expected since many of the Caribbean Andean genotypes are red mottled like Calima and probably derived from the Northern Andes rather than from Central Andean areas of South America such as Peru.

Table 1. Diversity assessment of Caribbean germplasm across individual microsatellites.

Type of marker	Microsatellite marker	Map Position	Range in Allele sizes	No. of Alleles	Diversity Value (d)
Genomic	**AG1**	CR2	124-142	**3**	**0.649**
	BM139	CR2	84-120	**6**	**0.754**
	BM140	CR4	160-200	**5**	**0.573**
	BM142	CR2	154-156	**2**	**0.560**
	BM143	CR3	94-172	**10**	**0.780**
	BM152	CR2	106-144	**12**	**0.782**
	BM155	CR5	116-118	**2**	**0.484**
	BM160	CR7	184-264	**7**	**0.719**
	BM170	CR6	166-180	**4**	**0.569**
	BM175	CR5	158-174	**3**	**0.661**
	BM183	CR7	144-160	**4**	**0.626**
	BM184	CR9	150-164	**3**	**0.504**
	BM189	CR8	108-120	**4**	**0.585**
	BM205	CR7	136-142	**4**	**0.706**
	BMd33	NA	98-110	**4**	**0.534**
	GATs11	CR10	226-232	**4**	**0.677**
	GATs11B	CR10	116-118	**2**	**0.292**
	GATs54	CR3	102-104	**2**	**0.477**
	GATs91	CR2	218-268	**12**	**0.889**
Genic	**BMc5**	CR10	134-142	**2**	**0.506**
	BMd10	CR1	138-142	**4**	**0.302**
	BMd15	CR4	164-202	**2**	**0.528**
	BMd26	CR4	136-142	**2**	**0.532**
	BMy1	CR4	152-172	**5**	**0.706**
	BMy2	CR11	148-158	**4**	**0.582**
	BMy4	CR1	162-166	**3**	**0.384**
	BMy6	CR4	136-142	**3**	**0.566**
Total/Average	--	--	---	**118 (4.4)**	**0.590**

It was also noteworthy that the Caribbean genotypes with a Mesoamerican affinity were more closely related to each other than they were to the black seeded and small red seeded control genotypes DOR364 and ICA Pijao. This also might be expected given the Caribbean Mesoamerican genotypes represent distinct germplasm probably derived from inter genepool hybridization but isolated from the Central American germplasm from which they may have arisen. Such inter-genepool hybridization may explain how the red mottled seed coat color was transferred from the Andean genepool into Mesoamerican-like germplasm.

The germplasm assignments of the Caribbean genotypes were generally supported by the previous phaseolin analysis, with "T" phaseolin being predominant in the Andean germplasm group and "S" phaseolin being predominant in the Mesoamerican germplasm group, however it was notable that a three Caribbean genotypes that clearly were associated with the Andean group in the dendrogram (Haiti 661, Orocovis 1A and Vason) had "S" type phaseolin. These results suggested that some Caribbean Andean germplasm has had a hybrid inter-genepool origin and that "S" type phaseolin has been introgressed into an Andean genomic background. It was also interesting that these three genotypes are from Haiti, Puerto Rico and Dominican Republic showing that this process has occurred in several parts of the Caribbean. The other genotypes which showed Andean microsatellite fingerprint patterns and "S" phaseolin, and which therefore showed a possible inter-genepool origin (AFR298, Redhawk, Montcalm, UPR-94434 and Blanco Laran) came

from breeding programs (in CIAT, USA, UPR and Peru, respectively) where recombination could be expected to have been actively encouraged. The one genotype which showed "C" phaseolin (Naranjito 1, a landrace from Puerto Rico) fell in with the Andean germplasm set according to the microsatellite data. The "C" pattern is thought to be a hybrid of "S" and "T" phaseolins from the Mesoamerican and Andean genepools, respectively.

Another factor corroborating the genepool affiliations of the Caribbean genotypes was average seed weight. Overall average seed size was correlated with the germplasm group with which the genotype was affiliated whereby the Caribbean genotypes with an Andean affinity had an average seed size of 41.7 grams per 100 seed weight (g/100s) compared to the Caribbean genotypes with a Mesoamerican affinity which had an average seed size of 31.1g/100s. Interestingly, within the Andean group, those genotypes with "T" phaseolin versus those with "S" or "C" phaseolin had very similar average seed size of 38.2g/100s versus 37.3g/100s. By comparison those Caribbean genotypes with a Mesoamerican fingerprint pattern and confirmed "S" phaseolin had average seed size of 29.1g/100s that was significantly smaller than both the Andean genotypes with "T" phaseolin as well as those with "S" phaseolins. The one genotype which showed "C" phaseolin had seed size of 24.5 g/100s.

Conclusions

The microsatellite analysis uncovered relationships between the Caribbean landraces that may reveal the history of bean introduction into the Caribbean. It was notable that while accessions from the Eastern Caribbean (Puerto Rico) were all Andean (26 out of 26 genotypes, 100%), those from the Western Caribbean (Haiti) were predominantly Mesoamerican (16 out of 18 genotypes, 88.9%). Dominican Republic was a zone of intermingling of the two types of germplasm with about half the collection (34 out of 65 genotypes, 52.3%) being Andean and half Mesoamerican (32 out of 65 genotypes, 48.5%). Meanwhile the single genotypes from Cuba, Jamaica and Panama were Andean. Therefore there appears to be gradients from east to west in the penetration of Mesoamerican beans and from west to east in the penetration of Andean beans into the Caribbean which would agree with the hypotheses that A) Mesoamerican beans were introduced into the region from Central America, Mexico or the Yucatan, most likely via Cuba, although this needs to be confirmed with greater sampling B) Andean beans arrived from the South American mainland via the Leeward island chain and C) Mesoamerican beans began to overlap with Andean beans precisely on the island of Hispaniola. Within the Dominican Republic, Mesoamerican beans were prevalent in Vason and Hondo Valle collections, while Andean beans were prevalent in Panjor collections. Pacasas and Derrumba collections were mixed with both types of germplasm found in these sites perhaps indicating preference or adaptation characteristics. Relationship between breeding lines were also uncovered by the microsatellite analysis. Saladin 97 a recently released variety was related to Pompadour J a native landrace from the Dominican Republic. Interestingly, the improved lines JB178 and CIAS 95 as well as the landrace Pacasas 1A, all from the Dominican Republic were

more closely related to Colombian varieties Calima and ICA Palmar and the CIAT lines, DRK57, AFR735, AFR619, AFR699 than to the Andean landraces from the Caribbean. This confirms the recent emphasis placed in the INIAF breeding program on adopting and breeding the large-seeded red mottled grain type, brought in originally from Colombia and based on crosses with a native large-seeded type "Jose Beta" which clustered with other Andeans from Puerto Rico, these being somewhat distinct from the majority of Pompadour types.

Finally, the microsatellite fingerprinting was also useful for identifying potential duplicates in the collection. A total of six identical sets were found among the landraces: Orocovis 1C and Orocovis 2; Pompadour Jorgillo 17 and Pompadour Jorgillo 19; Gurabo 2, Gurabo 3 and Gurabo 6; and Panjor 4 and Panjor 8 among the Andeans. Meanwhile, Haiti 284 and Haiti 495; and HondoValle 10 and HondoValle 32 were potential duplicates among the Mesoamericans. Surprisingly UPR9443-4 and Montcalm were also identical in this analysis. The information generated by this study will be useful for the breeding of several Caribbean seed classes including the red mottled (Dominican Pompadour), pink striped (Jamaican Miss Kelly, Puerto Rican Colorado de Pais) and red speckled (Haitian Pompadour) types. The evidence of genepool mixing indicating possible past hybridization and introgression and points to the potential advantages of recombining Andean beans with Mesoamerican beans. Indeed in several breeding programs this recombination may have been encouraged as evidenced by genotypes like Montcalm, UPR9443-4, Redhawk which had Andean fingerprint but Mesoamerican "S" type phaseolin. The advantages of hybridization between the genepools for some Caribbean germplasm may have been in combining medium to large seed size of the Andean types, with greater adaptation to tropical lowland conditions of the Mesoamerican types. Hybrid progeny that fit local preferences and had these advantages were probably selected by the farmers in the region and as such, the germplasm of the Caribbean shows promise for breeding efforts that try to adapt Andean beans to lowland tropical climates.

Future Plans

- Compare the microsatellite results with the RAPD data and complete the microsatellite analysis with additional markers, especially using more gene-based microsatellites.
- Evaluate a larger number of Caribbean genotypes, especially a greater number of genotypes from Cuba and those from other Caribbean seed classes including the red mottled (Dominican Pompadour), light red kidney (Velasco Largo), pink striped (Jamaican Miss Kelly, Puerto Rican Colorado de Pais) and red speckled (Haitian Pompadour) types, that are held in the FAO-designated collection of common bean at CIAT for microsatellite diversity and compare to this first set.
- Determine whether the large seeded Caribbean germplasm traces back to the Nueva Granada race and whether several races of Mesoamerican beans contributed genes to the this germplasm.

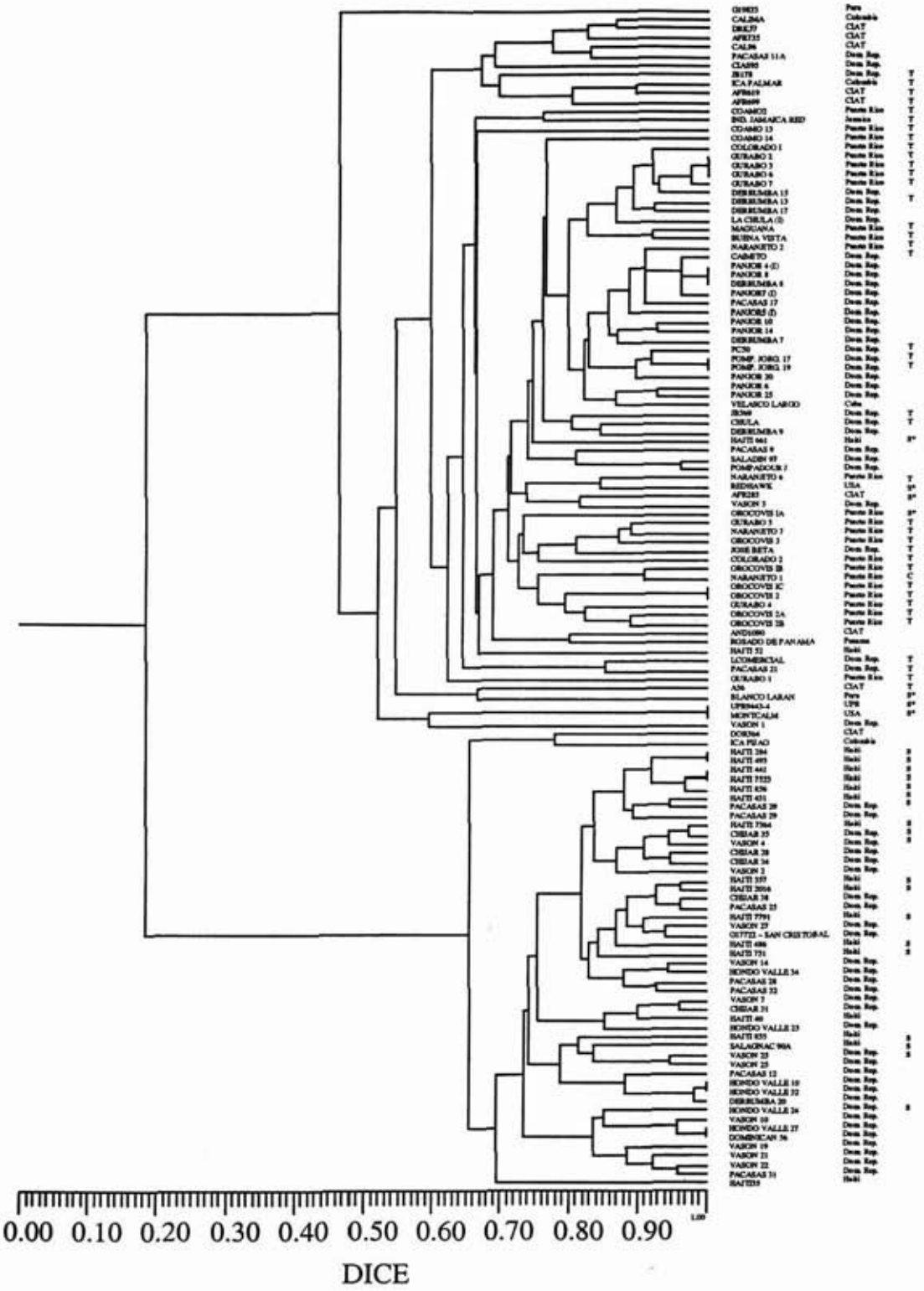


Figure 1. Dendrogram of relationships among Caribbean germplasm uncovered by microsatellite marker analysis.

1.1.5 Evaluation of microsatellite diversity in combined parental surveys

MW Blair, MC Giraldo, HF Buendia, E. Tovar
SB-2 Project – CIAT

Introduction

The objective of this work was to characterize microsatellite diversity in three sets of common bean genotypes that are parents of recombinant inbred line populations at CIAT and include wild and cultivated germplasm of common beans, both Mesoamerican and Andean, which have been used as parents in the bean breeding program.

Methodology

The 44 genotypes used in this study are described in previous CIAT annual reports (2000, 2001) and were the parents of over a dozen mapping populations being studied at CIAT for various traits. The genotypes were grouped in 3 parental surveys that were carried out separately with common controls (DOR364, G19833) included in each survey. The genotypes were evaluated for allelic diversity with up to 150 microsatellite markers (of which 65 were gene-based; 85 were genomic) depending on the survey to which they belonged (150 for survey I, 148 for survey II and 97 for survey III) as shown in Table 1. The markers were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The amplification conditions are given in other parts of this annual report. Markers that did not amplify (6 in survey I, 14 in survey II and none in survey III) were not considered further. The PCR products were resolved by electrophoresis for approximately one hour at 120 constant volts on silver-stained 4% polyacrylamide gels. Microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega). Null alleles were uncommon for both genomic and gene-based microsatellites but were scored as missing bands. The discriminating power (D) of each microsatellite was calculated.

Results and Discussion

In all three parental surveys, the average number of alleles and discriminating power was higher for genomic microsatellites (ranging from 3.7 to 5.4 alleles and 0.467 to 0.578 discriminating power when including monomorphic markers, and 4.4 to 6.3 alleles and 0.613 to 0.692 discriminating power when excluding monomorphic markers) than for gene-based microsatellites (ranging from 2.8 to 3.3 alleles and 0.370 to 0.481 discriminating power when including monomorphic markers, and 3.2 to 4.1 alleles and 0.484 to 0.642 discriminating power when excluding monomorphic markers) (Table 1). The highest diversity was registered in parental survey I, which contained a good mix of Andean and Mesoamerican cultivated genotypes as well as wild accessions, compared to the other two surveys. Parental survey II had the lowest diversity values, mainly because it was predominantly made up of Mesoamerican genotypes only. Parental survey III

Table 1. Average number of alleles and discriminating power (d) for genomic and genic microsatellites with and without monomorphic markers as evaluated in each of three parental surveys.

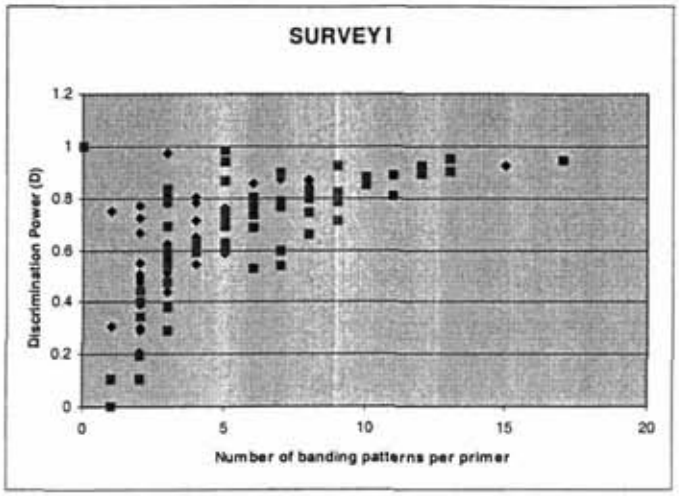
Parental Panel	Marker Class	No. of Markers				Average No. Alleles		Average Disc. power (d)	
		Total	Poly	Mono	NA	w/o Mono	w/ Mono	w/o Mono	w/ Mono
Survey I	genomic	85	66	13	6	6.29	5.42	0.692	0.578
	genic	65	48	16	0	4.06	3.30	0.642	0.481
	overall	150	114	29	6	5.35	4.47	0.671	0.535
Survey II	genomic	83	64	14	5	4.42	3.81	0.623	0.511
	genic	65	46	10	9	3.20	2.80	0.564	0.464
	overall	148	110	24	14	3.91	3.38	0.598	0.491
Survey III	genomic	59	45	14	-	4.53	3.69	0.613	0.467
	genic	38	29	9	-	3.83	3.16	0.484	0.370
	overall	97	74	23	-	4.26	3.48	0.563	0.429

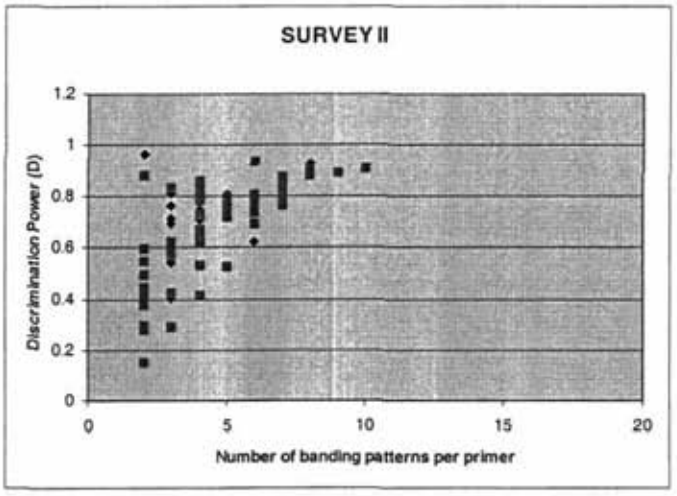
contained a mix of Andean and Mesoamerican genotypes but no wild accessions. When allele number is plotted against discrimination power (d), the higher diversity of genomic versus gene-based microsatellites is evident (Figure 1). Similarly, one can observe the different amount of diversity assessed in each of the three parental surveys. As shown in Figure 1, the discrimination power of each microsatellite was positively correlated with the number of alleles produced at the locus (r=0.686 to 0.803).

Future plans

- Cross survey comparison of microsatellite diversity by correlating allele sizes found in the respective genotype panels.

- Assembling of microsatellite fingerprint data into the AceDB database, BeanGenes that were described in last year's annual report.





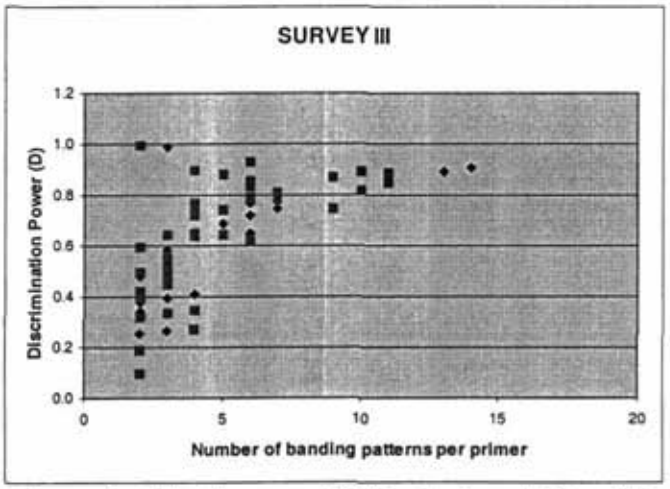


Figure 1. Relationship between discrimination power (D) of each microsatellite and the number of alleles detected with each microsatellite in three sets of parental genotypes evaluated with a total of 150 markers, of which 85 were genomic microsatellites

1.1.6 Simple Sequence Repeat (SSR) Assessment of Genetic Diversity of Local Cassava Varieties from Guatemala

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Introduction

Two primary centers of diversity, one in South America and the other in Meso-America have been postulated for the genus Manihot (Roger and Appan 1973). Although several studies have demonstrated a likely South American origin for the cultivar (Allem, 1994; Fregene et.al 1994; Roa et al. 1997; Olsen and Schaal 1999), the diversity of cassava and its wild relatives in Memo- America is great enough to suggest a second center in Meso-America. Besides, the potential of Meso-American diversity in cassava improvement has not been properly assessed. Three recent studies of genetic diversity in land races from South America and Meso-America (Chavariaga et. al. 1999; Fregene et. al. 2002; Raji et. al. unpublished data) have revealed unique alleles in land races from Guatemala at a frequency high enough to suggest a Meso American center of cassava diversity. The results of the three studies were based upon 6, 4, and 13 Guatemalan land races. The small sample size of the previous study could distort the allele frequencies and lead to wrong conclusions. A larger collection and SSR characterization of land races from Guatemala was therefore planned to confirm preliminary data of a Meso-American center of diversity and to secure the largely untapped diversity in Guatemala before it becomes extinct. In addition, a selection from the Guatemalan collection will be crossed to CIAT elite parents to evaluate the utility of the Meso-American diversity in cassava breeding.

The present study was to confirm the high genetic differentiation between cassava land races from Guatemala and Nigeria, Brazil, and Colombia. If the uniqueness of the Guatemalan germplasm is confirmed, genetic crosses to CIAT's elite breeding lines will be made to test hybrid vigor and delineate heterotic pools. Plant materials are a collection of cassava from all over Guatemala and a representative group used in previous studies from Nigeria, Colombia and Brazil to confirm earlier results. It is hoped that results of the uniqueness and the utility of the Guatemalan germplasm will give collection and conservation of this germplasm in regions of Meso-America high priority (Azurdia and Gomez 2002)

Methodology

A collection of cassava land races was carried out all over Guatemala in May last year (Azurdia and Gomez 2002). A total of 128 accessions were collected in the departments of Baja Verapaz, Quiche, Huehuetenango, Alta Verapaz, San Marcos, Escuintla y Santa in Guatemala. See Guatemalan study on the MOLCAS web site

(http://www.ciat.cgiar.org/Molcas) for names of accessions. For comparison with results of previous studies, DNA from 6, 11 and 12 cassava land races from Nigeria, Colombia y Brazil respectively were included, making 4 samples for the analysis of genetic diversity and differentiation. DNA from the Guatemalan accessions was isolated at the Facultad de Agronomia, Universidad de San Carlos de Guatemala using a micro-prep protocol of the Dellarporta (1983) methodology and transferred to CIAT. DNA from the other accessions was obtained from previous studies at CIAT.

A set of 36 SSR markers, carefully chosen to represent a broad coverage of the cassava genome with moderate to high polymorphism information content (PIC) and robust amplification, were used in this study. SSR markers, PCR amplification, polyacrylamide gel electrophoresis, and silver staining used in this study have been described elsewhere (Fregene et al 2002). The allele data was captured using the program "Quantity One" (Bio-Rad Inc) and entered directly into EXCEL (Microsoft Inc) for statistical analysis. Statistical analysis on the raw SSR data include: genetic distance analysis using a of a distance matrix based upon 1-proportion of shared alleles (1-PSA), principal component analysis (PCA) and cluster analysis (UPGMA) of the distance matrix, and parameters of genetic diversity and differentiation.

Results

A total of 33 SSR markers were analyzed in the 128 accessions from Guatemala that includes an accession of the wild relative M.aesculifolia. Unique alleles were observed in the accessions from Guatemala for the markers SSRY 12 (0.14), SSRY 20(0.383), SSRY 34(0.006), SSRY 38(0.063), SSRY 51(0.1), SSRY 59(0.014), SSRY 63(0.014), SSRY 69(0.023), SSRY 82(0.007), SSRY 103(0.24), SSRY 108(0.043), SSRY 135(0.13) y SSRY 147(0.013). In parenthesis are the frequencies of the observed alleles. The first and second principal components of the PCA, based on upon the genetic distance 1-proportion of shared alleles, are shown in Fig 1. Accessions from Guatemala form two groups, one that clusters along with land races from Brazil, Nigeria and Colombia in a broad group and a second group that is clusters separately. The only sample from M. aesculifolia is located far away from both clusters. The results observed confirms previous observation of a high genetic differentiation of between certain groups of cassava land races from Guatemala and these from other parts of Latin America and Africa (Fregene et al. 2003)

Assessment of genetic diversity was based on samples of cassava land races from the 4 countries, with an addition that accessions from Guatemala were divided into two groups G1 and G2 based on clustering from PCA of genetic distances and UPGMA of F_{ST} data. Table 1 summarizes the parameters of genetic diversity observed for accessions from the 5 samples. Genetic diversity, as assessed by the average gene diversity (H_E) was high in the accessions analyzed 0.5422 ±0.2468. The population with the highest diversity was Colombia followed by the cluster G2 and that with the lowest was the cluster of the Guatemalan accessions clustered separately from other accessions. Average number of alleles was 3.8+/-0.0358 for all accessions. Average number of alleles per locus was

highest in the cluster G2 of Guatemalan land races, 5.2, and the lowest in the Nigerian land races, 2.9. A break down of genetic diversity parameters by individual SSR markers can be seen in Table 2.

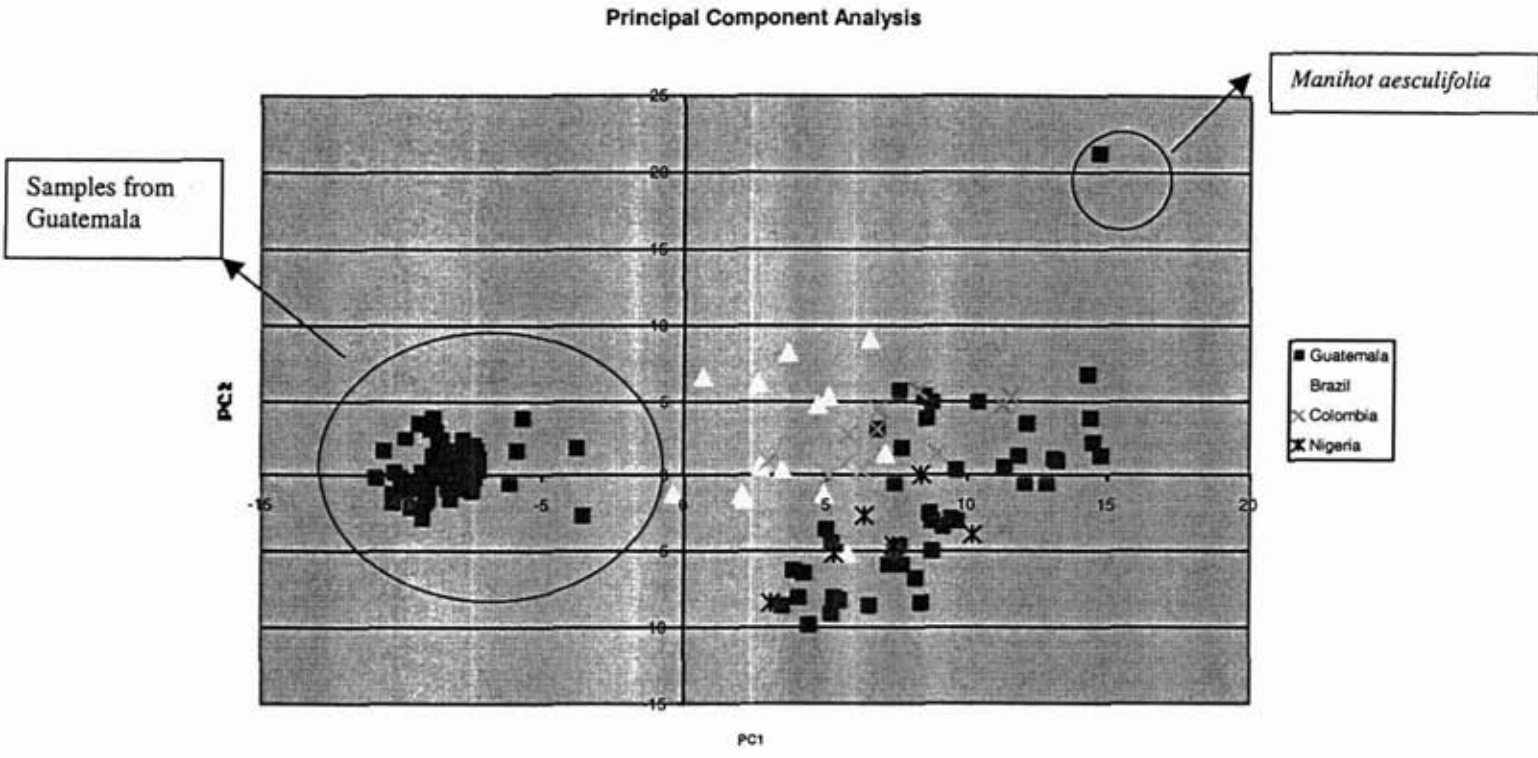

Fig.1. Principal component analysis of genetic distances between cassava accessions from Guatemala, Brazil, Colombia and Nigeria.

A UPGMA cluster analysis of the genetic distance data also produced 2 clusters of the Guatemalan accessions similar to that found with the PCA (data not shown). In addition, 2 sub groups were found within the group G1 that clustered away from the majority of accessions. A UPGMA of a pair-wise analysis of genetic differentiation (F_{ST}) again confirmed the separation of a group of accessions from Guatemala (Fig 2) as observed with the PCA and UPGMA analysis of genetic distances. The geographical distribution of accessions in cluster G1 can be observed in Figure 5. The distribution of accessions closely mirrors the distribution of 2 wild Manihot species in Gutemala, namely *Manihot rhomboide* and *Manihot aesculifolia* (Figure 3). The majority of accessions in sub group A are found in western Guatemala and they overlap, as regards geographical origin, with *Manihot rhomboide*. On the other hand, genotypes from sub group B are found mostly in the Eastern part of the country together with natural populations of *Manihot aesculifolia*.

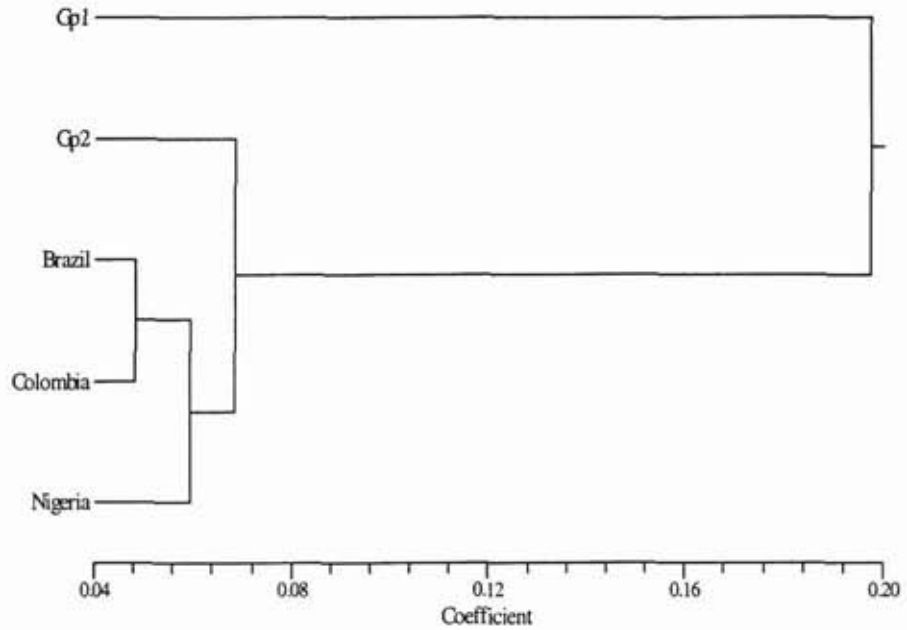

Fig. 2: UPGMA tree of pair-wise F_{ST} data calculated between samples from the four different countries.

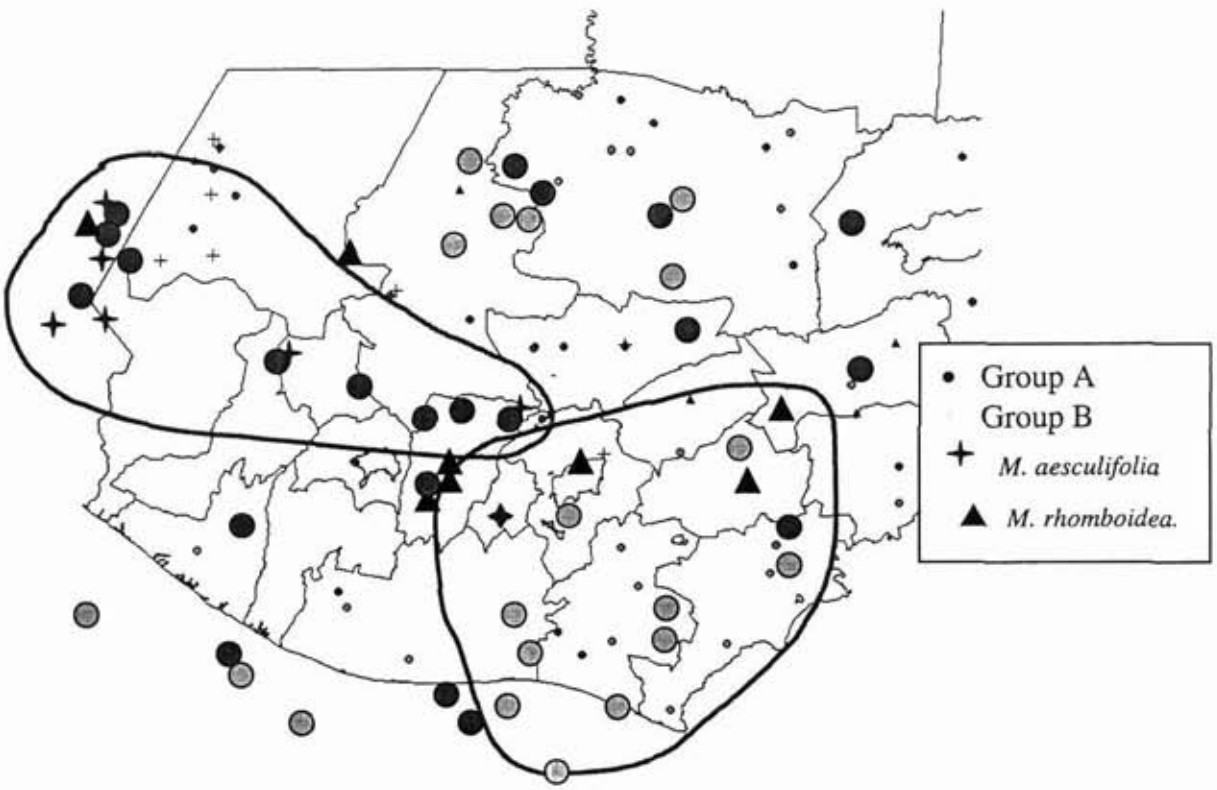

Fig 3: Distribution of accessions from groups A and B, of G1 and *Manihot aesculifolia* and *Mahihot rhomboide* in Guatemala .

The origins of highly differentiated samples of cassava germplasm from Guatemala can be explained by independent domestication events in populations of different Manihot species that yet exist or are now extinct. They can also be explained by an introgression from *Manihot* species in certain regions that overlap in geographical spread with cassava. Cassava is an allogamous crop and natural cross pollinate between cassava and populations of wild *Manihot* species has been demonstrated Wanyera et al (1993).

The highly differentiated landraces from Guatemala may represent heterotic pools, like those for maize (Shull 1952). A principal reason for this study was to assess genetic diversity in cassava landraces as a first step to delineating heterotic pools for a more systematic improvement of combining ability via recurrent reciprocal selection (Keeratinijakal and Lamkey 1993). The heterotic patterns found in maize populations at the turn of the century is the basis of a very successful maize hybrid industry and has raised maize yields 500% since 1928 (Shull 1952; Tomes 1998). A high level of genetic differentiation, as revealed by molecular markers, was later found between these populations (Melchinger et al. 1990). It is noteworthy that accessions from sub group A and B, for example accession 405, 478, 332, and 729 have excellent agronomic characteristics (data not shown)

Table 1. Intra-population and inter-population estimates of genetic diversity parameters of cassava and races from different agro-eclogies of Guatemala, Brazil, Colombia, Nigeria

Pop.	n	No. de Loc.	No. of loci Pol.	Percent Pol. Loc.	Average ofof alleles/Loc.	No.Average of allles/Loc. Pol.	No. HO	HE	HEc_p
G1	24	33	28	84.8	2.8	3	0.6273	0.41	0.419
G2	74	33	33	100	5.2	5.2	0.5895	0.6066	0.6107
BRA	12	33	32	97	4	4.1	0.5562	0.5745	0.6013
COL	8	33	33	100	4.1	4.1	0.5912	0.6111	0.6555
NIG	4	33	31	93.9	2.9	3.1	0.648	0.5085	0.6067
	mean	33	31.4	95.15	3.8	3.87	0.6024	0.5422	0.5786
	std	6.28	0.98	0.91	0.0358	0.0845	0.0918	0.2468	

Ho: observed heterozygosity ; He: Average gene diversity ; Hec_p: Average gene heterozygosity corrected for small samples sizes

Table 2. Parameters of Genetic diversity, Ho, Hs, Ht, Dst, Gst and Gst' (correction for differences in sample size) by SSR locus

Locus	Ht	Hs	Dst	Gst	Dm	Rst
SSRY4	0.7596	0.6843	0.0753	0.0991		
SSRY9	0.61	0.5767	0.0333	0.0546		
SSRY12	0.7061	0.6427	0.0634	0.0897		
SSRY19	0.7698	0.652	0.1177	0.153		
SSRY20	0.8235	0.7471	0.0764	0.0928		
SSRY21	0.6691	0.6035	0.0655	0.0979		
SSRY34	0.6554	0.4869	0.1685	0.2572		
SSRY38	0.1665	0.1585	0.008	0.0477		
SSRY51	0.7177	0.6324	0.0854	0.1189		
SSRY59	0.7533	0.5214	0.2319	0.3078		
SSRY63	0.6556	0.5402	0.1155	0.1761		
SSRY64	0.7151	0.5325	0.1826	0.2554		
SSRY69	0.7428	0.6926	0.0502	0.0676		
SSRY82	0.8154	0.6638	0.1517	0.186		
SSRY100	0.7673	0.6648	0.1025	0.1336		
SSRY102	0.2887	0.2534	0.0354	0.1225		
SSRY103	0.5395	0.4988	0.0407	0.0755		
SSRY105	0.687	0.5074	0.1796	0.2615		
SSRY106	0.5847	0.5173	0.0674	0.1153		
SSRY108	0.6123	0.4013	0.211	0.3446		
SSRY110	0.3628	0.3502	0.0126	0.0346		
SSRY127	0.5545	0.5353	0.0192	0.0346		
SSRY135	0.6644	0.6218	0.0426	0.0641		
SSRY147	0.3403	0.3132	0.0271	0.0796		
SSRY151	0.7632	0.7046	0.0587	0.0769		
SSRY155	0.6983	0.6141	0.0843	0.1207		
SSRY161	0.6889	0.5989	0.09	0.1306		
RY164	0.7527	0.6476	0.1052	0.1397		
RY169	0.6498	0.5982	0.0516	0.0794		
SSRY171	0.3128	0.2801	0.0326	0.1044		
SSRY179	0.5225	0.5018	0.0208	0.0397		
SSRY180	0.6236	0.565	0.0587	0.0941		
SSRY181	0.6305	0.5831	0.0473	0.0751		
Mean	0.6244	0.5422	0.0822	0.1252	0.0822	0.1516
Std	0.1619	0.1397	0.0589	0.0791		
95%	CI	0.5627	0.4793	0.064	0.1009	
95%	CI	0.6743	0.587	0.1035	0.1514	

Ho Average observed heterozygosity within country
Ht Total Heterozygosity in the entire data set
Hs Gene diversity within country averaged over the entire data set
Dst Average gene diversity between populations
Gst Coefficient of gene differentiation

Conclusions

A previous study of the assessment of genetic diversity of cassava land races in 14 South and Central American and African countries revealed a number of unique alleles in accessions from Guatemala and suggests a second center of diversity in Guatemala (Fregene et al 2003). Meso America is a center of diversity for many other food crops including common beans, maize, amongst others. This study shows unique alleles from Guatemala for a higher number of SSR markers and provides additional evidence for possible independent domestication of cassava in Meso America. However, an introgression with *Manihot* species that overlap with the geographical origins of these accessions makes it impossible to rule out introgression with these species. Further studies are required to clarify which is the most likely scenario via the collection and characterization of wild *Manihot* species the eastern and western parts of Guatemala. Additional future activities include a diallel cross of theses excellent land races from Guatemala and other regions (initial experiments are described below in activity 8) .

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1.1.7 Simple Sequence Repeat (SSR) Assessment of Genetic Diversity of Local Cassava Varieties from Ghana and Predictability of Heterosis

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Introduction

Cassava is the number one provider of calories in Ghana. The Portuguese first introduced cassava from Brazil to Ghana during the 16th and 17th centuries (Jones, 1959). In the then Gold Coast, the Portuguese grew the crop around their trading ports, forts and castles. It was a principal food eaten by both the Portuguese and the slaves. By the second half of the 18th century, cassava had become the most widely grown crop of the people of the coastal plains (Adams 1957). The spread of cassava from the coast into the hinterlands was very slow. It reached Ashanti region, Brong Ahafo and the northern Ghana, mainly around Tamale in the 1930. Until the early 1980s,the Akans of the forest belt preferred plantain and cocoyams and sorghum and millet in the north. Cassava became firmly established in most areas after the serious drought of 1982/83 when all other crops failed completely (Korang-Amoakoh,Cudjoe and Adams 1987). Cassava ranks first in the area under crop cultivation and utilization. It contributes 22% of the agricultural gross domestic product AGDP compared to 5% for maize, 2% for rice, and 14% for cocoa (Al-Hassan, 1989;Dapaah, 1996). According to the Ghana Living Standards Survey (GLSS), 83% of 1.73 million sampled households were found engaged in cassava production. The spread of cassava into the upper west and upper east of Ghana is an indication of growing trend in cassava production through area expansion(MOA, 1990).

In the traditional bush-fallow system, some cassava plants are allowed to grow during the fallow period, which is long enough to allow cassava to flower and set seeds. The usual out crossing habit of cassava leads to the production of numerous volunteer hybrids and a

heterozygous gene pool, which creates phenotypic diversity. Desirable new hybrid combinations from volunteer seed are often selected by farmers and propagated. This process creates pools of new land races, which are adapted to the different agro-ecological zones of Ghana. Farmers have done selection for desirable traits for over 500 years. Hence the landraces possess higher frequencies of genes required for adaptation to biotic and abiotic stresses, food quality characteristics than unadapted materials. Vegetative propagation also leads to the accumulation of pest and diseases and good varieties susceptible to these biotic stresses disappear. These factors lead to a fairly high turnover of varieties and have implications for gene pool structure of cassava in any center of diversity. Selection is one of the principal factors at work in cassava's gene differentiation in Africa. High heterosis for yield components, starch, and number of roots have been observed in cassava, and hence considered a promising method of genetic improvement (Easwari Amma and Sheela, 1996). Heterotic groups identified in maize in the early 20th century (Shull et al 1953) have been the basis of a very successful hybrid seed industry.

Objectives for the study were:

The objective for this study is to assess the genetic diversity and differentiation in Ghanaian landraces
To detect heterotic patterns in the collection and between the Ghanaian collection and land races from other countries and regions.
To generate hybrids between the Ghanaian land races and genotypes from putative heterotic groups and select together with farmers superior hybrids from the crosses.

Methodology

In January 2002 a collection of cassava land races from all the agro ecological zones in Ghana was done. A total of 45 villages visited during the collaborative study on cassava in Africa (COSCA) were visited. Another 28 villages, important for cassava production, were also visited. Farmers were assembled and asked to share information on cassava varieties grown by them, characteristics of their varieties, and reasons for keeping them. Farmers volunteered to give mature cassava stems, which were labeled. A total of 320 land races were collected. For the list of genotypes, passport data and characteristics please see the MOLCAS home page (http://www.ciat.cgiar.org/molcas)

Fresh young leaf samples of the accessions were collected on ice and used for DNA extraction. An amount of 0.1g of the fresh young leaf was ground in liquid nitrogen and the DNA extracted using the Qiagen kit. The extraction was carried out in IITA, Ibadan, Nigeria. The DNA was carried in absolute ethanol to CIAT. DNA quantification was done using the fluorometer. The DNAs were diluted to 10ng/ul and used for SSR reactions. A sub-set of 36 SSR markers, two from each of the 18 linkage groups of the cassava genome, was employed to obtain an estimate of genetic diversity and differentiation in the land races. PCR amplification, automated gel analysis and date collection were as descried by Fregene et al (2003). Genetic distance, based upon the proportion of shared alleles

(PSA), obtained from the raw allele size data using the computer microsat (Minch 1993, http://www.lotka.stanford.edu/microsat.html). Distances between the accessions were subjected to principal component analysis (PCA) using JMP (SAS Institute 1995) to obtain a structure of relationship between the land races. Parameters of genetic diversity and differentiation were calculated from allele data using the computer packages GENSURVEY (Vekeman et al 1997) and FSTAT (Goudet 1990).

Results

A total of 320 landraces were collected including 18 genotypes with yellow roots. Farmers who responded were predominantly women. Among the land races some were very early bulking with maturity at 3-9 months after planting. The various local names given suggest a lot of useful traits farmers had associated with the varieties. Woody stems were cut to 20-30cm sizes and planted in plastic pots in a nursery. These were sent to the field after 4weeks and planted in an irrigated field at the Ashiaman office of the Ghana Irrigation Authority. A copy of the collection was packaged and sent to IITA. Accessions were planted in single rows at 1m x 1m spacing with improved varieties as checks.

Data from a total of 33 of 36 SSR loci, 3 markers gave poor quality data, was used to derive estimate of genetic diversity and differentiation genetic distances between individual genotypes. The average number of alleles for each locus was close to 5 and is similar to that found for a study of land races from Nigeria, Tanzania and 7 Neo-tropical countries (Table 1). The probability that 2 randomly selected alleles in a given accession are different, average gene diversity, was 0.5245±0.0045 and it is lower than that found in the found for the previous study. Average gene diversity was comparable across all regions with an exception of the central region and central savannah region. Genetic diversity parameters, including total heterozygosity (Ht) and genetic differentiation (Gst) ranged widely across markers. Genetic differentiation, as estimated by F_{ST} (theta), was very low for samples between regions, overall 0.04, with the exception of some accessions from Northern Ghana that showed moderate to high genetic differentiation. (data not shown). The results found here support previous findings that agricultural practices and the allogamous nature of cassava produces a large pool of volunteer seedlings that natural and human selection acts upon to maintain a high level of diversity and low differentiation (Doyle et al. 2001; Fregene et al. 2002).

Table 1 Intra-population and inter-population estimates of genetic diversity parameters of cassava land races from different agro-eclogies of Ghana

Population	N	#loc.	#loc_P	PLP	K	K_P	HO_p	HE_p	HEc_p
Ashanti	11	33	30	90.9	3.9	4.1	0.5285	0.5017	0.5262
Brong Ahafo	37	33	31	93.9	5.4	5.6	0.5012	0.5267	0.5339
Central	8	33	29	87.9	3.3	3.7	0.5082	0.4701	0.4999
Eastern	27	33	30	90.9	5.4	4.9	0.5322	0.5123	0.5223
Coastal Sav.	4	33	27	81.8	2.9	3.4	0.5404	0.467	0.5405
Greater Accra	10	33	29	87.9	3.7	4	0.5016	0.5065	0.5336
Volta	28	33	31	93.9	5.3	5.5	0.5133	0.5735	0.5839
Northern I	109	33	31	93.9	6.9	7.2	0.5369	0.5779	0.5806
Northen II	53	33	31	93.9	6.3	6.5	0.5479	0.5851	0.5908
mean				90.57	4.69	4.98	0.5234	0.5245	0.5457
std deviation				4.13 1	0.36	1.31	0.0176	0.045	0.0317

PLP: Percentage of polymorphic loci at the 5% level within accessions
K: Mean number of alleles per locus within accessions
K_P: Mean number of polymorphic alleles per locus within accessions
Ho_p: observed heterozygosity
HE-p: Average gene diversity
HEc_p: Average gene heterozygosity corrected for small samples sizes

Genetic distances between all pairs of individual accessions was calculated by the 1-proportion of shared alleles (1-PSA) and presented graphically by a principal coordinate analysis (PCA) (Fig1). The PC1 and PC2 accounted for 26% and 16% of the total variance respectively. The PCA shows loose clustering of the land races by region but of note is the sub-structure of some land races from Northern Ghana. A similar sub-structure in accessions from Nigeria and from Tanzania was observed in earlier studies. The presence of a defined sub-structure in the genetic relationship of cassava land races from Africa appears to be a common feature of cassava germplasm in a number of countries but it is yet to be understood the underlying factors for the groupings. UPGMA cluster analysis of F_{ST} estimate of genetic differentiation amongst land races was able to group the land races into loose clusters according to agro-ecologies, with a group of genotypes from the Northern region sub-structure being the most differentiated (Fig 2). (Fregene et al.2002). At least 63 duplicates or closely related accessions were identified in the collection.

Table 2. Parameters of Genetic diversity, Ho, Hs, Ht, Dst, Gst and Gst' (correction for differences in sample size) by SSR locus

LocName	Ho	Hs	Ht	Dst	Dst'	Ht'	Gst	Gst'
SSRY4	0	0.346	0.439	0.093	0.104	0.45	0.212	0.23
SSRY5	0.499	0.474	0.481	0.006	0.007	0.481	0.014	0.015
SSRY9	0.463	0.582	0.587	0.005	0.006	0.587	0.009	0.01
SSRY12	0.704	0.597	0.598	0.001	0.001	0.598	0.001	0.001
SSRY19	0.811	0.738	0.764	0.026	0.029	0.766	0.034	0.037
SSRY20	0.79	0.73	0.76	0.03	0.033	0.764	0.039	0.043
SSRY21	0.596	0.487	0.514	0.027	0.03	0.517	0.053	0.058
SSRY34	0.479	0.428	0.425	-0.004	-0.004	0.424	-0.009	-0.01
SSRY38	0.043	0.08	0.082	0.001	0.002	0.082	0.018	0.02
SSRY47	0.429	0.668	0.739	0.071	0.079	0.747	0.096	0.106
SSRY51	0.79	0.694	0.751	0.057	0.063	0.757	0.076	0.084
SSRY52	0.752	0.603	0.616	0.014	0.015	0.618	0.022	0.025
SSRY59	0.152	0.639	0.701	0.062	0.069	0.708	0.089	0.098
SSRY63	0.445	0.484	0.519	0.036	0.04	0.523	0.069	0.076
SSRY64	0.725	0.67	0.689	0.02	0.022	0.691	0.028	0.031
SSRY69	0.557	0.552	0.568	0.016	0.018	0.57	0.028	0.031
SSRY82	0.846	0.84	0.858	0.018	0.02	0.86	0.021	0.024
SSRY10	0.725	0.779	0.798	0.019	0.021	0.8	0.024	0.027
SSRY10	0.007	0.01	0.009	0	0	0.009	-0.037	-0.041
SSRY10	0.804	0.76	0.764	0.004	0.004	0.764	0.005	0.005
SSRY10	0.829	0.761	0.768	0.008	0.009	0.769	0.01	0.011
SSRY10	0.422	0.361	0.373	0.012	0.014	0.375	0.033	0.036
SSRY11	0.279	0.272	0.274	0.002	0.002	0.274	0.008	0.009
SSRY12	0.814	0.629	0.653	0.024	0.026	0.656	0.036	0.04
SSRY14	0.08	0.083	0.086	0.002	0.003	0.086	0.029	0.032
SSRY15	0.689	0.79	0.806	0.015	0.017	0.807	0.019	0.021
SSRY15	0.072	0.595	0.632	0.037	0.041	0.636	0.059	0.065
SSRY16	0.528	0.658	0.668	0.01	0.011	0.669	0.014	0.016
SSRY16	0.258	0.316	0.321	0.004	0.005	0.321	0.013	0.015
SSRY17	0.544	0.555	0.591	0.037	0.041	0.596	0.062	0.068
SSRY17	0.845	0.726	0.776	0.05	0.056	0.781	0.065	0.071
SSRY18	0.672	0.532	0.535	0.003	0.004	0.536	0.006	0.007
SSRY18	0.605	0.705	0.761	0.055	0.062	0.767	0.073	0.08
Overall	0.523	0.55	0.573	0.023	0.026	0.575	0.04	0.045

Ho Average observed heterozygosity within country
Ht Total Heterozygosity in the entire data set
Hs Gene diversity within country averaged over the entire data set
Dst Average gene diversity between populations
Gst Coefficient of gene differentiation.

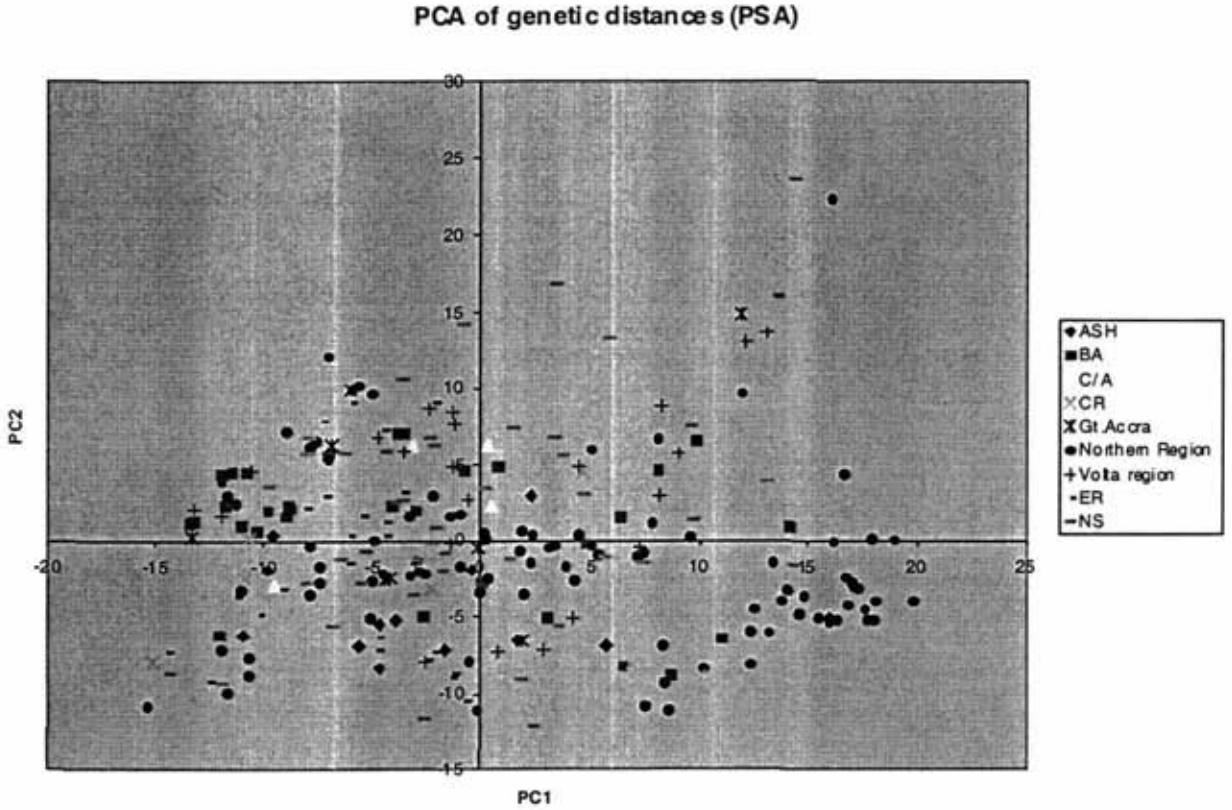

Fig. 1: Principal component analysis of genetic distances, based 1- proportion of shared alleles, between cassava accessions from Ghana.

One of the principal reasons for this study was to assess genetic diversity in cassava land races as a first step to delineating heterotic pools for a more systematic improvement of combining ability via recurrent reciprocal selection. Based upon clusterings obtained above, genotyopes representative of the clusters were selected as parents for a diallel experiment to search for heterotic patterns and established in a crossing block at the CRI experimental station in Wenchi. Genotypes from other parts of Latin America, for example Guatemala, that cluster away from African accessions were also shipped from CIAT to Ghana for the study of heterotic patterns. The Latin American genotypes were multiplied, hardened and planted in the same crossing block where the land races are currently planted.

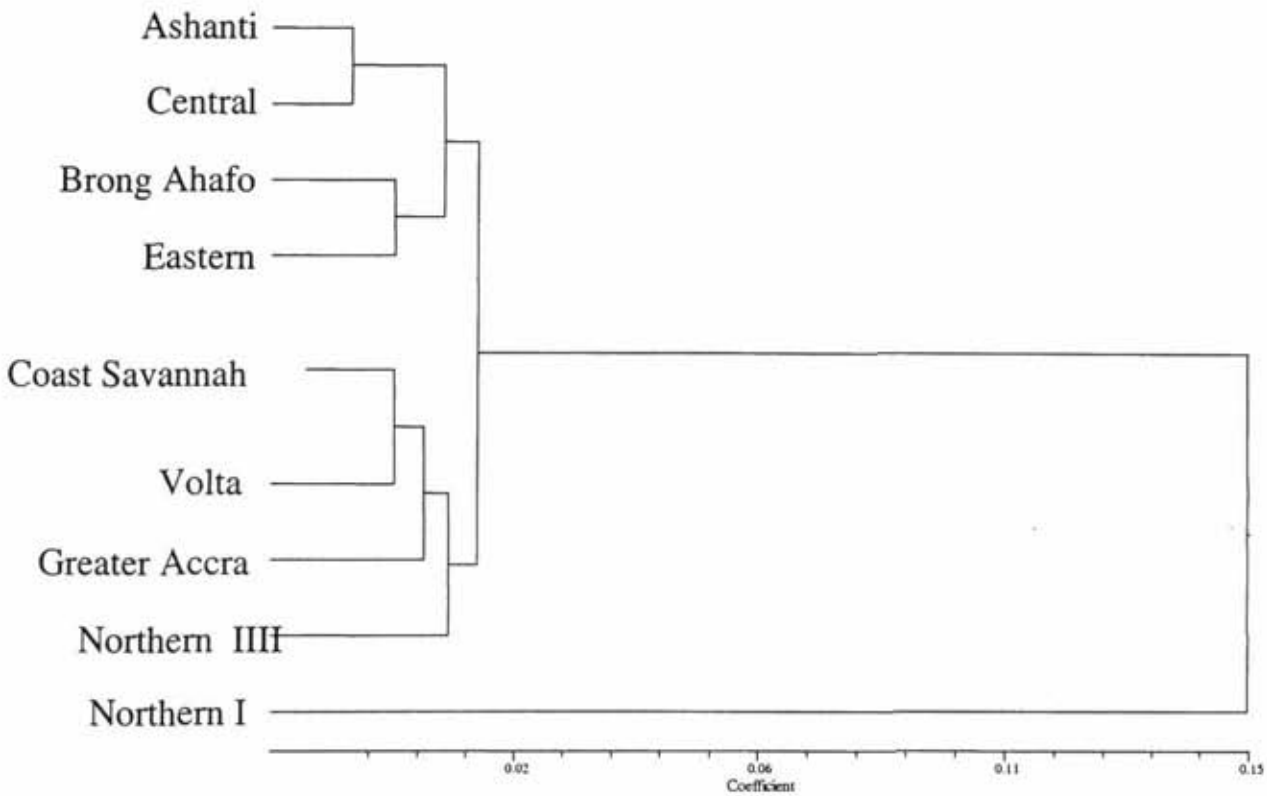

Fig. 2: UPGMA tree of pair-wise F_{ST} data calculated between samples from different regions of Ghana.

Conclusions

A total of 320 land races were collected and established at the University of Legon and IITA, Ibadan. Genetic diversity and differentiation in the collection was assessed using SSR markers. Discovery of a sub group within the land races, as observed from PCA of genetic distances (proportion of shared alleles) and UPGMA of pairwise F_{ST} between the different regions, that may represent heterotic groups. To test heterotic patterns present within the collection or between the collection and Latin American accessions, selected genotypes were planted in a crossing block for making genetic crosses.

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1.1.8 Simple Sequence Repeat (SSR) Assessment of Genetic Diversity of Local Cassava Varieties from Uganda

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Introduction

Cassava is grown mainly for its starchy roots and the leaves and forms a staple by an estimated half of Uganda's population (Bua, pers.comm, 2001). Cassava was introduced into Uganda after 1850 by Europeans and Arabs (Langlands, 1970). Because of its excellent adaptability to erratic rainfall and low fertility soils, it became a major dietary staple, a famine reserve crop and a source of cash to many small-scale farming communities. The impact of a cassava mosaic disease (CMD) epidemic in Uganda in the late 80's and early 90's on genetic diversity grown by small farmers is thought to be significant. In a survey carried out in 2000 in Mukono, Soroti and Apac districts in Uganda, the impact of the CMD epidemic on cassava diversity was clearly seen via the loss of previously well-known varieties (Kizito and Gullberg 2002, unpublished data). But it was also observed that additional genetic variability had arisen from the use of volunteer seedlings by some farmers in Mukono district. Traditional farming systems of slash and burn followed by 3-15 years of fallow have been known to encourage the allogamous nature of cassava producing a large pool of volunteer seedlings that natural and human selection acts on to produce new varieties which maintains a high diversity, for instance in Tanzania (Fregene et al, 2003). We assessed the genetic diversity and differentiation based on SSR markers of landraces from all over Uganda and a small subsection from Latin America and other African countries. The objectives of this study were to: assess the genetic diversity and differentiation of cultivars within and between different districts in Uganda; also to determine how the Uganda cassava diversity compares with the total genetic diversity of species within Africa and the cassava collection after the CMD epidemic.

Methodology

A total of 257 local cassava varieties were collected accessions September through to December 2002 in 17 districts that lie between latitudes N02° 12[1] and S 00° 44[1], longitudes E029° 56[1] and E034° 21[1], and altitudes of 4451ft and 2177ft above sea level in Uganda. Three counties on average in each district were selected at random and fields with mature crop were sampled from every 7-10 km along the roads that traversed each of the counties. In each farmer's field the different varieties were identified according to their

morphological characteristics as well as by the name given by the farmer. Where no single variety dominated, plants of the co-dominant varieties were sampled, labelled to be planted and maintained in NAARI. Another 20 accessions, representatives of diversity, were selected from Tanzania, and 18 accessions South America from a previous study of cassava accessions from Tanzania and the neo-tropics (Fregene et al. 2003). In addition to this, 20 from the Ghanaian germplasm bank, 20 from Nigeria and 20 from Guatemala was included. In all, 350 accessions were studied. DNA isolation was from young leaf tissue harvested by the CTAB method (Doyle & Doyle, 1987) at Med Biotech Laboratories, Kampala. A subset of 36 SSR markers with high polymorphism information content (PIC) routinely used for diversity analysis in cassava was source of markers, the 36 markers have been chosen to represent a wide coverage of the genome. PCR amplification, automated gel analysis and date collection were as descried by Fregene et al (2003). Genetic distance, based upon the proportion of shared alleles (PSA), obtained from the raw allele size data using the computer microsat (Minch 1993, http://www.lotka.stanford.edu/microsat.html). Distances between the accessions were subjected to principal component analysis (PCA) using JMP (SAS Institute 1995) to obtain a structure of relationship between the land races. Parameters of genetic diversity and differentiation were calculated from allele data using the computer packages GENSURVEY (Vekeman et al 1997) and FSTAT (Goudet 1990).

Table 1: Genetic diversity within groups of cassava landraces classified according to country of origin. Standard deviations (SD) were estimated by jackknifing over loci (200 replications). H_t, H_s, D_{st}, and G_{st}[a] are given over loci and over groups (country populations).

Population	Sample Size Loci[b]	No.of loci pol. loci	No.of Of pol[b]. /locus	Percent Alleles pol[b]	Mean no. alleles/ .locus	Mean no.	H_o[c]	H_e[d]	H_{e-p}[e]
UGANDA	198	35	33	94.3	5.2	5.4	0.5530	0.5454	0.5468
COLOMBIA	5	35	33	94.3	3.3	3.4	0.5081	0.5363	0.5963
BRASIL	3	34	33	97.1	2.8	2.8	0.5735	0.5069	0.6304
PERU	3	35	33	94.3	2.7	2.8	0.5810	0.5218	0.6619
GUATEMALA1	7	35	33	94.3	2.5	2.6	0.5290	0.3908	0.4219
GUATEMALA2	11	35	34	97.1	3.8	3.9	0.5274	0.5640	0.5906
TANZANIA	19	35	32	91.4	3.9	4.1	0.5658	0.5386	0.5536
NIGERIA	20	35	33	94.3	3.9	4.0	0.5002	0.5002	0.5131
GHANA	19	35	33	94.3	4.2	4.4	0.5429	0.5542	0.5694
Mean				94.59	3.59	3.71	0.5423	0.5176	0.5649
Std				1.70	0.86	0.89	0.0285	0.0519	0.0698

	H_t	H_s	D_{st}	G_{st}
Mean	0.6305	0.5635	0.0670	0.1078
Std	0.1696	0.1606	0.0332	0.0502
95%CI	0.5713	0.5083	0.0566	0.0916
99%CI	0.6827	0.6135	0.0767	0.1235

[a] H_t= total heterozygosity in the entire data set; H_s= heterozygosity within country averaged over the entire data set; D_{st}= average gene diversity between populations; G_{st}= coefficient of gene differentiation.
[b] pol. =polymorphic [c]H_o= average observed heterozygosity within country; [d]H_e= average expected heterozygosity within country
[e]H_{e-p}= average expected heterozygosity within country corrected for small sample sizes (Nei, 1978)

Results

A total of 35 of 36 SSR loci was used to provide estimates for the genetic diversity and differentiation of 350 accessions of cassava accessions from Uganda and eight other countries, one eliminated for being monomorhphic. The number of alleles observed at each locus in the data set ranged from 2 to 12 alleles per locus over the 35 loci. The average gene diversity, H_e, for the entire 350 accessions was more than half 0.5649± 0.0698, average gene diversity of accessions from Uganda was 0.5530 (Table 1). However, only 1% (G_{st}=0.0192± 0.0511) of within district variation in Uganda was due to differentiation. On the other hand, 10% (G_{st}=0.1078±0.0502) of the overall heterozygosity (H_t=0.6305± 0.1696) in all the country accessions could be attributed to differentiation among the samples from both Africa and Latin America, revealing that germplasm within Uganda is at the moment quite uniform. When accessions from Uganda were divided according to districts, the least values for average gene diversity were observed in Lira and Luweero districts, 0.4011 and 0. 4219 respectively, while Kasese stands out with the highest value of 0.6208. This affirms earlier findings of higher varietal diversity in the western and southwestern districts of Uganda as opposed to those in the eastern districts (Otim-Nape et al, 2001). The relatively high level of genetic diversity observed on the whole in this study is unexpected considering Uganda has reported two major cassava mosaic disease (CMD) epidemics before. The most recent CMD epidemic being in the last 10-15 years, since 1988, affected drastically the cassava varietal composition and saw a decrease in area planted to cassava at its peak between 1990-1994 (Otim-Nape et al, 2001). This finding continues to demonstrate the fact of active involvement of Ugandan farmers in continuous testing and the adaptation of new planting materials to their unique situations. The importance of volunteer seedlings in the dynamics of cassava diversity has been demonstrated in traditional farming systems of the Makushi Indians from Guyana (Elías et al, 2001).

Genetic distance between accessions based on 1- proportion of shared alleles (1-PSA) was calculated and presented graphically by a principle coordinate analysis (Fig. 1). The PC1 and PC2 components accounts for about 39% and 10% of the total variance respectively. The PCA reflects a loose separation between accessions from Africa and the Neotropics as has been shown in earlier studies (Fregene et al. 2003). No distinct substructure was found amongst the Ugandan accessions except for accessions from Nakasongola district. Sub-structures in diversity have been reported for earlier studies for accessions from Ghana, Tanzania and Nigeria. Genetic differentiation averaged over all loci estimated by F_{st} (theta) was 0.103± 0.009 (jackknifing) and 0.082± 0.126 calculated by bootstrapping at 99% confidence interval (table 4). This agrees with previous diversity studies in Tanzania (Fregene et al, 2003). Pair wise calculations of F_{st} (theta) over all loci between pairs of country landraces and Uganda also revealed lower differentiation between African countries compared with Latin American countries, the lowest being between Ghana and Uganda (0.039) and the highest being between a group of accessions from Guatemala group and Nigeria (0.2631).

A dendogram of landraces for UPGMA of pair wise F_{st} estimates separates the African from Neotropical accessions with the group of accessions from Guatemala being the most genetically differentiated (Fig.2). These results agree with some previous studies on which a high differentiation has been observed among certain cassava groups cultivated in Guatemala and those in other parts of Latin America and África (Fregene et al, 2003; CIAT, 2003). Of particular interest to cassava breeding programs is the group (G1) from Guatemala, Accessions from G1 from Guatemala is a representation of the region East and South of Guatemala and it may represent a heterotic group based on differences in allele frquncies. The phenomenon of heterosis or hybrid vigor is an important factor in improvement of heterozygous crops such as cassava, and in cases like the corn, the patterns found in these populations at the beginning of the XX century have been the base of a very sucecssful industry of hybrid corn, elevating productivity by more than 500% (Shull, 1952; Tomes, 1998).

Conclusions

Characterization of the cassava landraces of Uganda with 35 markers and estimation of genetic diversity and differentiation

Assessment of the genetic relationship between different countries of Africa and Latin America.

Evidence of high differentiation between some land races from Guatemala and the rest of the countries that may represent heterotic pools.

Evidence of low impact in cassava diversity after of epidemic of CMD and low structure in cassava landraces of Uganda.

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1.1.9 Simple Sequence Repeat (SSR) Assessment of Genetic Diversity of Local Cassava Varieties from Sierra Leone

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Introduction

Cassava is an important stapleμg crop in Sierra Leone, second only to rice in importance. Civil strife in the recent past has lead to an erosion of genetic resources and loss of some very valuable germplasm. A collection was conducted by the National Program in collaboration with IITA in the Southern and Eastern provinces where cassava is or more importance to the populace, to safe guard local varieties for the future. The germplasm collection from Sierra Leone is being held at IITA Ibadan pending when peace fully returns to the country and the collection can be returned to the National root crop program. The collection was analyzed with SSR markers as part of MOLCAS efforts to characterize diversity found in local African varieties compared to what exists in Latin America.

Methodology

Forty villages in the Eastern and Southern province of Sierra Leone were visited in 2001 for the collection of cassava germplasm. In each village farmers were invited to share their most important varieties, between 3 and 4 varieties were collected from each village. The stakes of all accessions collected was established in the field at IITA. DNA was isolated from leaf tissues using a DNA isolation kit (QIAGEN Gmbh) at IITA and carried to CIAT for SSR analysis. A subset of 36 SSR markers with high polymorphism information content (PIC) routinely used for diversity analysis in cassava was source of markers. The markers have been chosen to represent a wide coverage of the genome. PCR amplification, automated gel analysis and date collection were as descried by Fregene et al (2003). Genetic distance, based upon the proportion of shared alleles (PSA), obtained from the raw allele size data using the computer microsat (Minch 1993, http://www.lotka.stanford.edu/microsat.html). Distances between the accessions were subjected to principal component analysis (PCA) using JMP (SAS Institute 1995) to obtain a structure of relationship between the land races. Parameters of genetic diversity and differentiation were calculated from allele data using the computer packages GENSURVEY (Vekeman et al 1997) and FSTAT (Goudet 1990).

Results

A total of 127 local cassava varieties were collected in from 40 villages. Thirty three SSR markers were analyzed in 98 accessions from Sierra Leone, the remainder accessions were not available at the time of SSR analysis. The average number of alleles for each locus was close to 5 and is similar to that found for a study of land races from Nigeria, Tanzania and 7 Neo-tropical countries (Table 1). Genetic distances between all pairs of individual

accessions was calculated by the 1-proportion of shared alleles (1-PSA) and presented graphically by a principal coordinate analysis (PCA) (Fig1). The PCA shows a sub-structure in diversity of the local varieties as have been observed in other collections from Africa. The presence of a defined sub-structure in the genetic relationship of cassava land races from Africa appears to be a common feature of cassava germplasm in a number of countries but it is yet to be understood the underlying factors for the groupings. The sub structure observed in the PCA was the basis of further analysis of genetic diversity parameters carried out.

Average number of alleles per SSR locus in the collection was roughly 4 and average gene diversity, was 0.5749±0.0690 it is comparable to that found in previous studies in several African countries. Genetic differentiation, as estimated by F_{ST} (theta), between the groups ranged from 0.088 to 0.140 which is low to moderate differentiation. The results found here support previous findings that agricultural practices and the allogamous nature of cassava produces a large pool of volunteer seedlings that natural and human selection acts upon to maintain a high level of diversity and low differentiation (Doyle et al. 2001; Fregene et al. 2002).

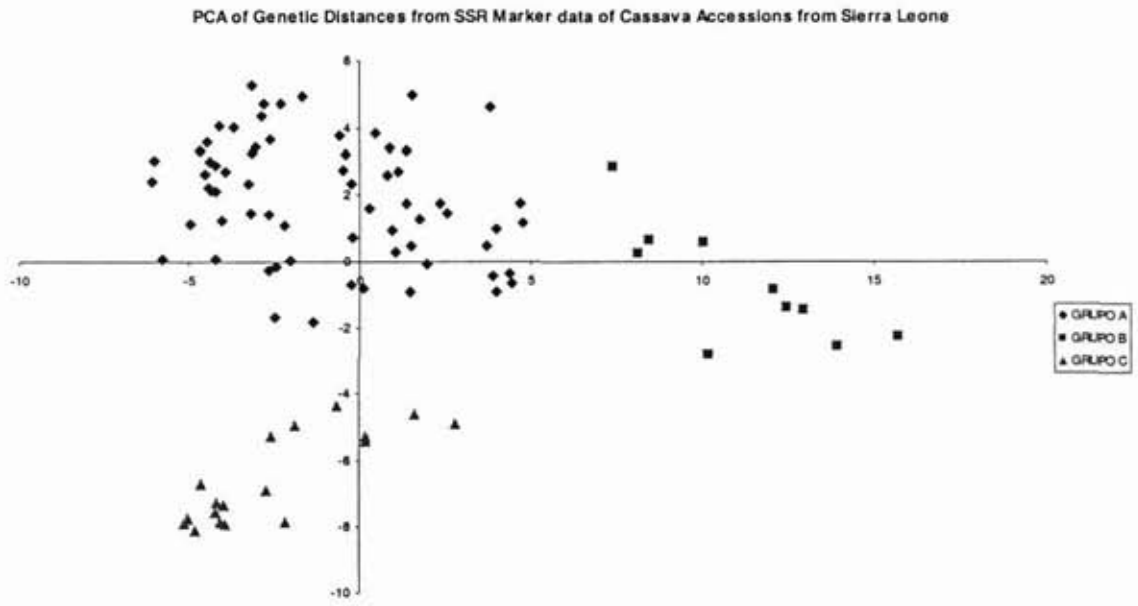

Figure 1. PCA of Genetic Distance based 1-poportion of shared alleled (PSA) of local varieties from the Southern and Eastern districts of Sierra Leone

A dendogram of landraces for UPGMA of pair wise F_{st} estimates separates the African from Neotropical accessions with the group of accessions from Guatemala being the most genetically differentiated (Fig.2). These results agree with some previous studies on which a high differentiation has been observed among certain cassava groups cultivated in Guatemala and those in other parts of Latin America and África (Fregene et al, 2003; CIAT, 2003). Of particular interest to cassava breeding programs is the group (G1) from Guatemala, Accessions from G1 from Guatemala is a representation of the region East and South of Guatemala and it may represent a heterotic group based on differences in allele frquncies. The phenomenon of heterosis or hybrid vigor is an important factor in improvement of heterozygous crops such as cassava, and in cases like the corn, the patterns found in these populations at the beginning of the XX century have been the base of a very sucecssful industry of hybrid corn, elevating productivity by more than 500% (Shull, 1952; Tomes, 1998).

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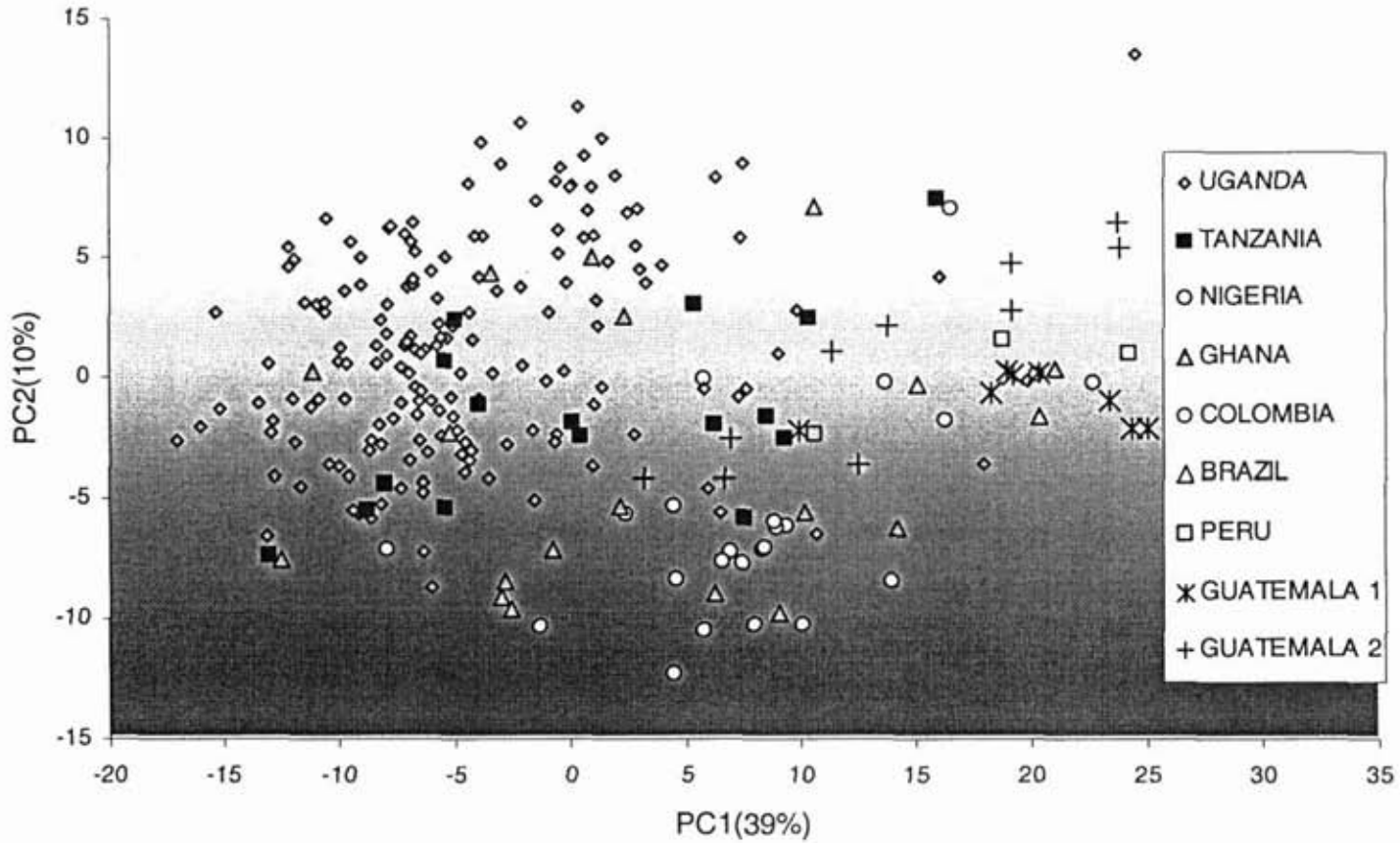

Figure 4. PCA of Genetic Distance (1-PSA) of local cassava varieties within the different districts in Uganda

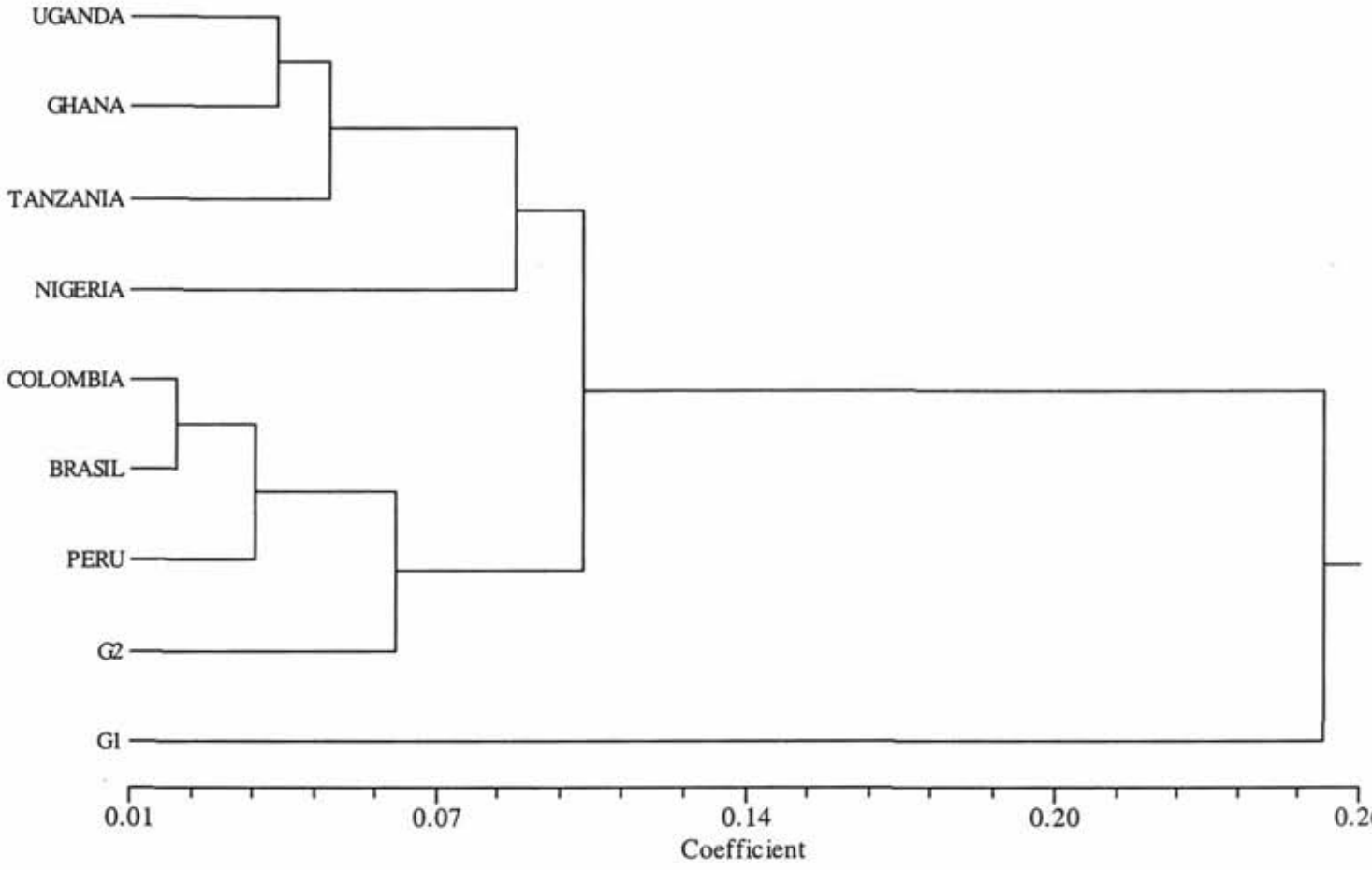

Figure 2. Unweighted pair group method with arithmetic averaging (UPGMA) dendogram of the pairwise fixation index (Fst) between cassava landraces, grouped by country and by source.

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Table 1 Genetic diversity within groups of cassava landraces classified according to country of origin. Standard deviations (SD) were estimated by jackknifing over loci (200 replications). H_t, H_s, D_{st}, and G_{st}[a] are given over loci and over groups (country populations).

Population	n	#loc.	#loc_P	PLP	K	K_P	HO_p	HE_p	HEc_p	Fis_p
A	60	33	32	97.0	4.2	4.3	0.6425	0.5639	0.5686	-0.1391
B	9	33	33	100.0	4.3	4.3	0.6747	0.6100	0.6469	-0.0607
C	16	33	30	90.9	2.9	3.1	0.7355	0.4930	0.5092	-0.4542
mean	3 pop.			95.96	3.83	3.93	0.6843	0.5556	0.5749	-0.2180
std				4.63	0.80	0.72	0.0472	0.0589	0.0690	0.2083

[a] H_t= total heterozygosity in the entire data set; H_s= heterozygosity within country averaged over the entire data set; D_{st}= average gene diversity between populations; G_{st}= coefficient of gene differentiation.
[b] pol. =polymorphic
[c] H_o= average observed heterozygosity within country
[d] H_e= average expected heterozygosity within country
[e] $H_{e\text{-}p}$= average expected heterozygosity within country corrected for small sample sizes (Nei, 1978)

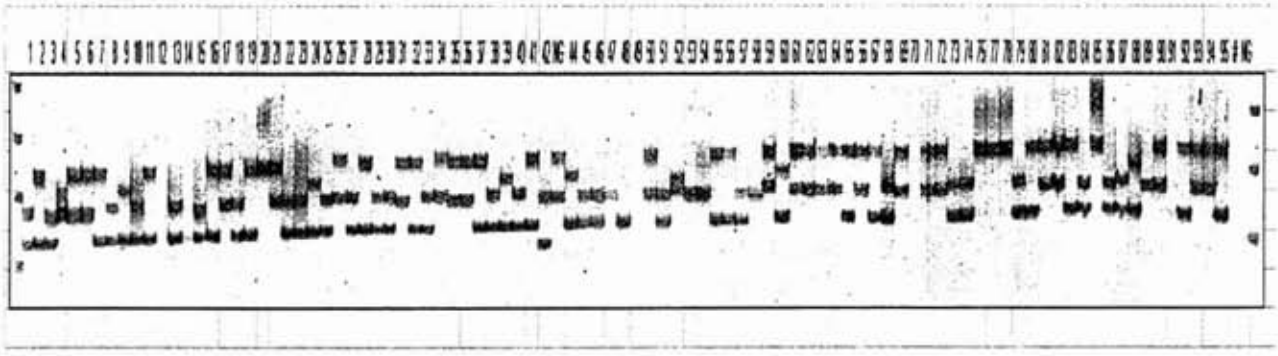

Figure 1. Silver stained acrylamide gel picture of the Sierra Leonean accessions analyzed with SSR marker SSRY 82

Conclusions

The SSR analysis of genetic diversity of local cassava varieties from Sierra Leone reveals the same pattern of high average gene diversity, low differentiation, and a pronounced sub-structure. Future perspectives include tracing the lineages of some of the lines in the 3 clusters found to get a better idea of the impact of germplasm development/introduction and adoption in the country.

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1.1.10 Simple Sequence Repeat (SSR) Assessment of Genetic Diversity of Local Cassava Varieties from Cuba

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[1]INIVIT, Cuba; [2] CIAT
Funding: Cassava Biotechnology Network (CBN)

Introduction

Cassava is an important crop of modern tropical economies and an attractive one for millions off resource poor farmers found in the tropics (Best and Henry 1994). Recently a second center of diversity have been postulated in Central America based on SSR markers (Monte et al. 2003), in addition to the one in Brazil (Olsen and Schaal 1999). However, the potential of diversity in the second center, particularly in the Caribbean is not well documented. Recently SSR markers have been utilized to study the diversity of cassava from different countries (Fregene et. al. 2003). SSR markers are particularly attractive to study genetic diversity due to their abundance in plant genomes, high levels of polymorphisms and adaptability to automation. These studies revealed a high amount of diversity in accessions from several neotropical countries, a low level of genetic differentiation between country samples, with the exception of a group of accessions from Guatemala, and sub-structure in diversity of accessions from some African countries.

SSR markers can contribute to a better understanding of genetic diversity present in a collection of local cassava varieties held in Cuba to permit a more rational conservation and use of diversity on the island. We present here preliminary results of SSR study of genetic diversity of cassava from Cuba compared to a sub-set of accessions from Africa, South and Central America

Methodology

A total of 94 accessions were selected from a collection of cassava held at INIVIT in Cuba, selection criteria was the economic importance and origin in Cuba. A set of 54 clones from Africa and the Neotropics, 12 from Nigeria, 10 from Tanzania, 12 from Guatemala, and 20 from South America, representative of a large set of accessions from these countries used in previous SSR studies (Fregene et al 2003) were included for comparisons. A third set of 13 improved genotypes from CIAT with traits of agronomic interest were added. DNA from all accessions was obtained using the Dellaporta et al. method (1983). Concentration and quality of the DNA was checked by flourometry and agarose gel electrophoresis respectively. The DNA samples were diluted to a working concentration of 10ng/ul for subsequent PCR amplification.

PCR amplification, automated gel analysis and date collection were as descried by Fregene et al (2003) and Mba et al. (2003). Statistical analysis to be conducted include calculations of pair-wise genetic distance, based upon the proportion of shared alleles (PSA), using the computer microsat (Minch 1993, http://www.lotka.stanford.edu/microsat.html). Distances between the accessions will be subjected to principal component analysis (PCA) using JMP (SAS Institute 1995) to obtain a structure of relationship between the land races. Other analysis are estimation of parameters of genetic diversity and differentiation, calculated from the raw SSR allele data using the computer packages GENSURVEY (Vekeman et al 1997) and FSTAT (Goudet 1990).

Results

A total of 15 SSR markers have been analyzed until now in all accessions. A high number of alleles and level of polymorphisms have been observed in all SSR markers analyzed until date (Fig 1). Evaluation of the remainder 21 SSR markers is ongoing and will be completed by October. Also ongoing is reading of the gels and determination of allele sizes using the program Quantity one. This raw SSR data will be used for subsequent analysis as described in the methodology.

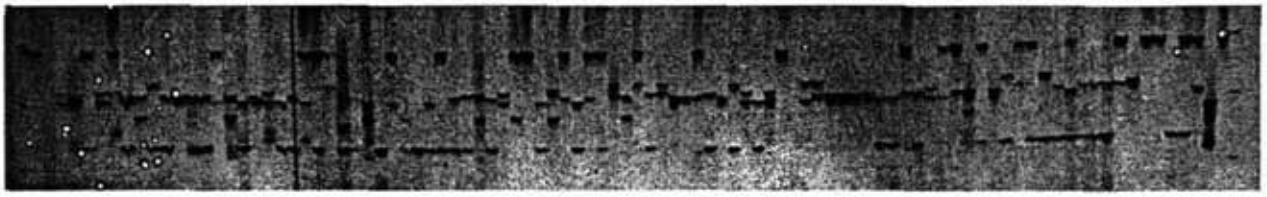

Figure 1. Polyacrilamide gel of a PCR amplification of cassava accessions from Cuba, Nigeria, Tanzania, Guatemala, and South America using the SSR primer SSRY51.

Conclusions

A SSR study of genetic diversity of cassava in Cuba has been initiated. The outcome of the study is expected to provide insights on the significance of the Caribbean region as a center of diversity for cassava and to guide rational conservation and plant improvement efforts.

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1.1.11 Molecular characterization of rice and red rice using microsatellites and its relation with the morphological seed traits

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[1] SB2, [2] IP4 . GTZ, Germany. Project No. 99.7860.2-001.00

Introduction

The genus *Oryza* (AA genome) contains two cultivated species of rice, *Oryza sativa* L and *Oryza glaberrima* Steud and five wild species including *Oryza rufipogon* Griff., the ancestor of rice, and one of them (*O. glumaepatula*) native of Central and South America (Oka and Chang, 1961;Vaughan and Tomooka, 1999). Red rice (*Oryza sativa f. spontanea*) is a weedy rice with red pericarp and dark-colored grains, commonly found in rice fields. It is the same species as the cultivated, having similar morphologicals traits at vegetative phase that makes difficult to dintinghuish them in the field at early life cycle, but at maturity they are taller, with profuse tillering, seed shattering and dormancy favoring its persistence in the field. Most published reports have classified red rice populations into two major groups based on hull color of mature seeds, which are strawhull and blackhull ecotypes (Smith et al., 1977; Sonnier, 1978 cited by Noldin, 1999). According to Langevin *et al.* (1990), the red rice can be grouped in ecotypes with characters alike cultivated rice or wild rice (Oka and Chang, 1961). Other researches indicate that red rice shows intermediate characteristics between wild rice *O. rufipogon* and cultivated *indica* or *japonica* varieties of *Oryza sativa* L. (Oka, 1988 cited by Bres-Patry *et al.* 2001). Another hypothesis is that weedy rice may evolve through the degeneration of domesticated rice, as weedy types of rice, where wild rice is not present (Vaughan *et al.* 2003).

Several population studies have focused on the genetic structure of red rice. These studies have related groups of individuals with morphological seed traits such as color of awn and hulls. A molecular analysis of 26 red rice accessions collected in Uruguay showed three groups: The first containing seeds with blackhulls, purple apiculus and long awn and the second showing seeds awnless or short awn and greyed yellow color apiculus and hulls. The third group included commercial varieties analyzed and some red rice accessions similar to the commercial varieties (Rodriguez *et al.*, 2001). Avozani *et al.* (2001), analyzed 36 red rice accessions with RAPDs markers and found six groups, the most remarkable were the first with only red rice accessions, mainly with awned seeds, and the fifth group including commercial varieties and some individuals similar to varieties in seed traits. Gealy *et al.* (2002) used microsatellite markers to distinguish among red rice, rice cultivars, and red rice–cultivated rice hybrids derivatives (RC hybrids) and the cluster analysis suggested that there were three distinct genotypic groups. The first group consisted of awnless, strawhull red rice types. The second group had primarily awned, blackhull red rice. The third group was composed of RC hybrids and rice cultivars. Preliminary work conducted by our research group (Gonzalez et al., 2002, and SB2 Annual

Report 2002) using 148 accessions of red rice collected in farmers fields in Colombia, 9 commercial Latin American rice varieties, sixteen homozygous Cica 8 transgenic lines, four hand-made hybrids between these transgenic lines and three varieties, and the AA wild species *O. barthii, O. glaberrima, O. glumaepatula,* and *O.rufipogon*, and 50 microsatellites indicated that is possible to discriminate the diversity of red rice by microsatellites associated with plant morphological traits as found in other studies, but also associated with plant development (phenology) characteristics such flowering. Microsatellite cluster analyses discriminated awn from awnless red rice, and within those groups distinguished early to intermediate flowering types from late flowering. Some red rice variety types were clustered with commercial varieties, and some morphologically like wild species were clustered with *O. rufipogon*. This year we report results from a multiple correspondence analysis including five additional microsatellites markers.

Materials and Methods

Plant Material and Genetic Analysis using Microsatellite Markers. The plant material used and procedures followed for the molecular characterization using microsatellites were the same as previously described in SB-02 Annual Report 2002 (González et al., 2002). The plant material was divided in four groups: Group 1 represented by the 148 red rice accesions, Group 2 included the rice commercial varieties (Coprosem, Oryzica 1, Cimarron and Fedearroz 50), Group 3 was composed by *O. rufipogon* accession from Malasia; and Group 4 by *Oryza glaberrima* and two wild species *Oryza barthii* and *O. glumaepatula* (accession from Costa Rica). The PCR products were resolved on silver-stained polyacrylamide gels and microsatellites alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

Statistic analysis. Allelic frequencies were calculated for all materials analyzed. Pearson chi square test was used to evaluate the association between specific microsatellites alleles with black brown awn, apiculus and hulls (BBAAH). Two multiple correspondence analyses (MCA) was conducted. The first analysis only included the molecular markers data (MCA-M), and the second analysis included both the molecular and seed morphological data (MCA-MSM). The Pearson chi-square and MCA are tests apply to establish the significance of associations between categorical variables. The Pearson chi-square test is based on expected frequencies in a two-entry data set, whereas MCA is a modeling technique to analyze associations in multi-entry data set. All analyses were conducted using SAS software (SAS, 1989).

Results and Discusión

Distribution of alleles in four rice groups. The number of alleles per locus ranged from 5 to 12 (average 10.4 alleles per locus). Figure 1 showed a total of 146 alleles obtained in this study. Group 1, composed by the red rice accessions, contained 110 alleles of which 56 alleles (51%) were specific to this group and were not present in the Groups. These results indicated that the red rice population is highly diverse and contained the highest

number of specific alleles respect to the other Groups. In contrast to Groups 2 and 4, it is interesting to note that *O. rufipogon* (Group 3) do not have specific alleles sharing all of them with red rice, and some of them with either the varieties (Group 2) or the other wild species (Group 4). However, *O. rufipogon* is represented in this analysis by just one accession. It will be important to determine if this pattern is obtained when a broader range of accessions are included. (Figure 1).

Association between specific microsatellites alleles with black brown awn, apiculus and hulls (BBAAH). Pearson chi-square test showed a highly significant association (Chi-squares from 18.4 to 61.2; $p = 0.0001$) between 7 alleles (2, 7, 17, 23, 53, 90 and 115 derived from 7 microsatellites markers) and BBAAH traits (Table 2). These markers are distributed in chromosomes 1,3,5,7 and 12 respectively (Table 2). These alleles were specific of red rice and/or *O. rufipogon* (Figure 1). A limited number of chromosomal regions (1, 3, 4, and 7) enclosing most of the genes/QTLs identified in a natural hybrid between a *japonica* variety and red rice collected from a rice field was previously found to be associated with key morphological differences between red rice and rice (Bres-Patry *et al.* 2001). We found that 4 of the 7 alleles highly significantly associated with BBAAH traits were located in chromosomes 1, 3, 4 and 7, in addition of other two alleles present in chromosomes 5 and 6 not previously reported (Table 2). The Pearson test also showed a high association between the absence of allele number 95 and BBAAH traits (Chi-square 15.61, $p = 0.0001$, Table 2). This allele was found in the red rice and rice varieties groups (Figure 1).

Multiple Correspondence Analysis (MCA). The multiple correspondence analyses using molecular data (MCA-M) generated five groups of which three grouped all red rice accessions (Figure 2A). The wild species *O. barthii* and *O. glaberrima* clustered together in the fourth group, and *O. glumaepatula* in the fifth group (Figure 2A). The three groups that enclosed all red rice accessions were analyzed in more detail and the analysis was complemented with seed morphological traits (MAC-MSM) (Figure 2B). First group (Group V) clustered 86 red rice individuals with the rice varieties (Figure 2B). Group V was composed by 58% of red rice accessions, including awnless or greyed yellow-awn red rice (43 and 56 % respectively), and 98 to 99 % red rice with greyed yellow hulls and apiculus alike the varieties. Most of the red rice variety biotypes are in this group. Group OR clustered ten red rice (7%) with *O. rufipogon* (Figure 2B). This group included red rice with awn (91%) and with brown apiculus (91%) and brown hulls (82 %). Group R (Figure 2B) was composed by the remaining 51 red rice accessions (35%) that did not fall in neither of the other two groups, and was characterized by individuals with awnless (41%), greyed yellow awn/hull (37%) or brown awn/hull (22%) seeds. Greyed yellow apiculus and hulls were registered in more than 63% of individuals, whereas the 22% of red rice showed brown hulls and apiculus.

In the MCA-M analysis, variability could be explained by 17 microsatellites alleles. Of these 15 alleles are specific to *O. barthii*, *O. glaberrima* and *O. glumaepatula,* one allele is shared by *O. rufipogon* and red rice group, and the other allele is shared by red rice and

rice varieties (Figure 1). In MCA-MSM analysis, variability was explained by the presence of 16 microsatellite alleles and four seed morphological traits (black brown awn, apiculus and hulls, absence of greyed yellow apiculus). Of these 16 alleles, 11 alleles were derived from the MCA-M analysis (Figure 1) and the other five alleles from the Pearson chi-square analysis (Table 1).

Conclusions

Specific microsatellites alleles were identified distinguishing rice varieties, red rice accessions and wild species. These alleles could be useful for studying red rice genetics, dispersion of red rice genotypes, degree of hybridization between red rice and cultivated rice, and genetic introgression and persistence of domesticated genes in red rice and wild species populations. The addition of seed morphological traits complemented the molecular analysis facilitating the discrimination of three main groups within the red rice population. One group included red rice similar to commercial varieties, another group alike *O. rufipogon* accession, and the third group with intermediate traits.

Future Activities

- Red rice types similar to *O. rufipogon* based upon the morphological and molecular characterization should be subjected to taxonomic classification to elucidate if they are introduced accessions of the Asian wild species.

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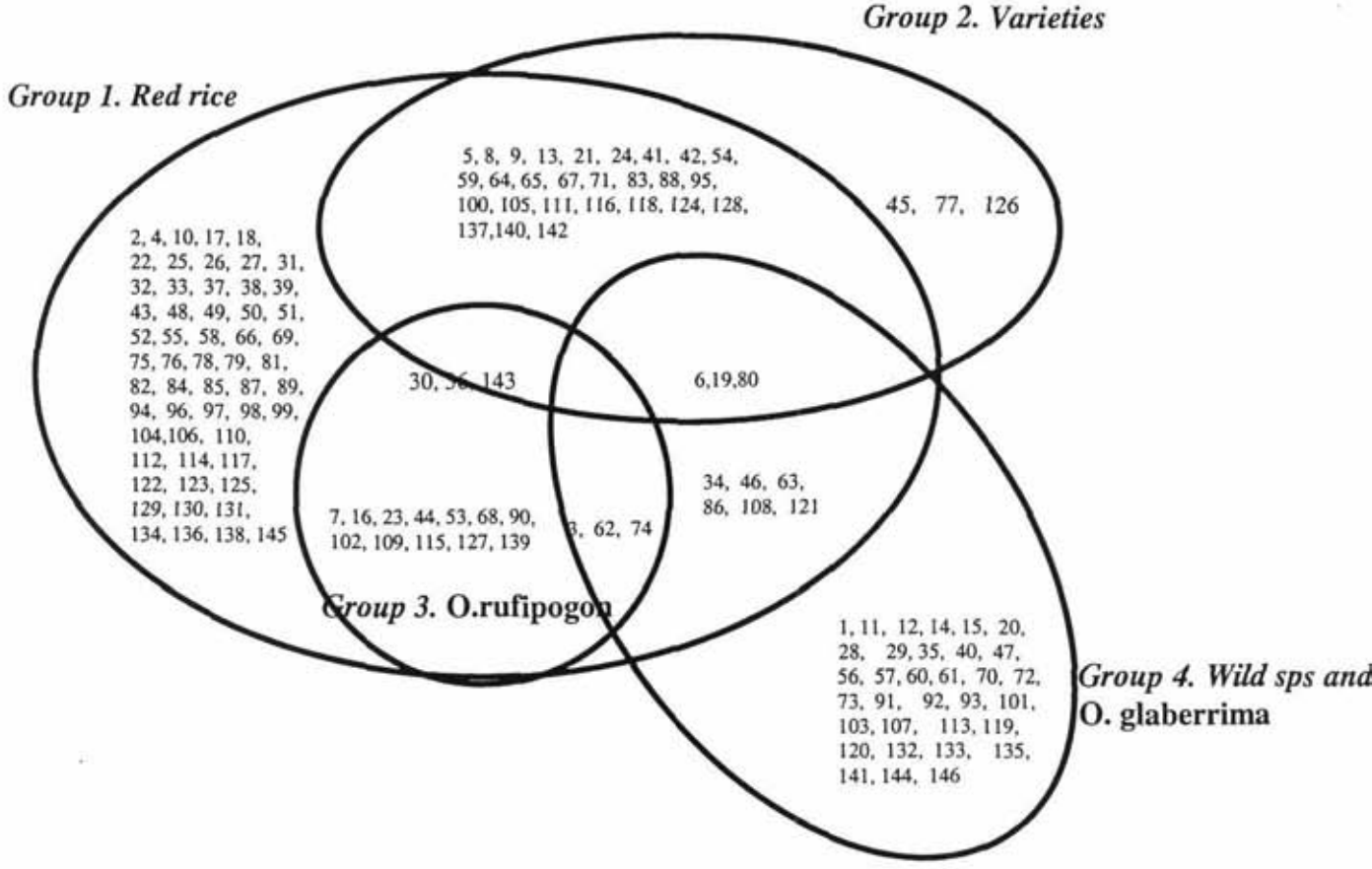

Figure 1. Venn diagram showing the microsatellites alleles in each rice group.

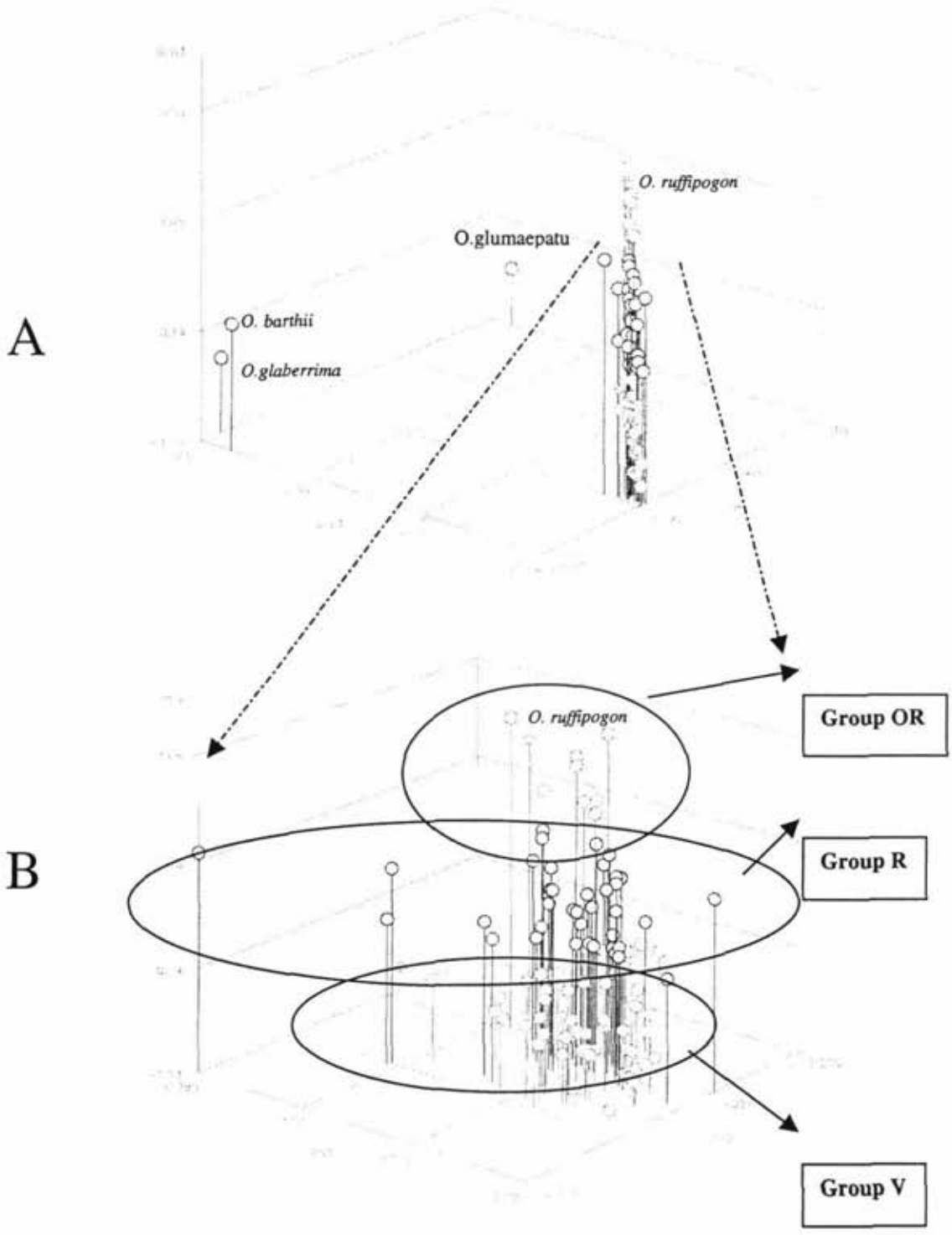

Figure 2. MAC using molecular marker data set for all the materials (A), and combining molecular data and seed morphological traits for red rice (B)

Table 1. Association between specific microsatellites alleles with brown awn, apiculus, and hulls (BBAAH)

Alleles	Chromosome	Chi-square	Probability	Presence BBAAH
2	3	25.47	0.0001	Yes
7	5	38.98	0.0001	Yes
17	12	33.57	0.0001	Yes
23	12	61.26	0.0001	Yes
53	1	18.44	0.0001	Yes
90	7	44.79	0.0001	Yes
95	7	15.61	0.0010	Not
115	1	32.51	0.0001	Yes

1.1.12 Assessment of combinatory ability between red rice and rice under greenhouse conditions

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[1] SB2, [2]IP4. Funding from GTZ, Germany. Project No. 99.7860.2-001.00

Introduction

Cultivated rice, *O. sativa* L., is an autogamous plant, with a low out crossing rate of 0-1% (Roberts *et al.* 1961). In contrast, red rice (*Oryza sativa* f. *spontanea*) a weedy relative of the crop, characterized by a red pericarp and dark-colored grains, shows out-crossing ranging from 1% to 52% hybridization rate (Langevin *et al.* 1990). Several biological, genetic and environmental factors affect the level of outcross compatibility. Among those the effects of temperature, humidity, genotype, flower morphology, stigma receptivity, pollen viability, pollen germination and development of pollen tube had been studied in detailed for most species (Jensen and Salisbury, 1988). Red rice seeds shatter readily and possess dormancy characteristics, which favors the persistence of the weed in rice field. These characteristics in addition to the vigorous growth and other plant traits make this weed highly competitive respect to rice, and a potential candidate as gene receptor from the cultivated species. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species. Last year we reported on the morphological, phenological and molecular characterization of 152 plants collected from rice farmers fields in Tolima and Huila Departments in Colombia, and its

corresponding first and second self-progeny. Based on that characterization 6 red rice types, including the scope of diversity present in the collection, were selected as candidates to conduct the studies on gene flow. As complementary step, the level of compatibility with transgenic and non-transgenic rice was determined using hand-made cross. Below is summarized results obtained from greenhouse and field grown plants. The hybrids generated will be used a controls in a parallel study optimizing the use of molecular marker to trace gene flow at large scale in the field.

Materials and Methods

Hand-made crosses. Crosses were made following procedures as described by Sarkarung (1996) with some modifications (Jaime Carabalí, personal communication, CIAT Project IP4). Six red rice biotypes (1-3-4, 1-21-3, 4-12-2-, 5-38-5, 5-36-4, and 5-48-2), the F_3BC_1 line derived from the Cica 8 transgenic line A3-49-0-12-3 (carrying the NS3 gene for RHBV resistance and *uid-A* gene for gus expression) backcross to variety Cica 8, the non-transgenic variety Cica 8 (control), and rice line commonly known as Purple (IRRI accession) were used. The variety purple is characterized by having purple tillers and leaves, and grains with purple apiculus and pericarp. Preliminary inheritance analysis in crosses with other varieties had indicated that the NS3 and *uid-A* gene are inherited following a simple Mendelian segregation. In rice, anthocyanins are encoded by few dominant genes (Reddy, A.R. 1996). The use of these traits would facilitate tracing the hybrids and inheritance in subsequent generations and ease the adaptation of molecular markers for assessing gene flow at large scale. These plants were grown to maturity under either greenhouse or field conditions. Reciprocal and self-crosses were made between the different materials. Un-emasculated, self-pollinated plants were included as controls. The percentage of seed setting, abortive crosses and dead flowers were evaluated. Pollen viability of pollen donor plants was determined by fluorescence microscopy.

Floral structure. Spiklets of field grown plants were fixed with a solution of 3:1 ethanol: glacial acetic acid for 24 hr, and dissected under a dissecting scope. Ten spiklets per material from the central part of the panicles were evaluated by measuring the length of pistil (ovary, stile and stigma), anthers, and total length of the flowers.

Results and discussion

In general, higher out crossing rates were observed when rice was used as male parent (pollen donor) and red rice as a female parent (pollen recipient) (Table 1). A lower hybridization rate was noted in the reciprocal crosses (using red rice as a pollen donor), which was significant with the line Purple. Unexpectedly, higher crossing rates were noted in the hand-made crosses when using greenhouse-grown materials respect to those from the field in spite that the field pollen-donor plants showed more than 90% of pollen viable, and more than 80% fertility based on the seed setting from spontaneous self cross in the field. It seems that panicles from materials grown in the field may had been affected by stress during handling, in addition to the potential loss of pollen during the transportation

of panicles from the field to the crossing house, which may explain these differences. The highest out crossing rates, between 40% and 59%, were noted with the red rice 1-21-3, 4-12-2-, 5-36-4, and 5-38-5 from the greenhouse (Tables 1). Red rice 5-48-2 showed intermediate hybridization rates (12% to 20%) both from the greenhouse and from the field materials (Table 1). Unfortunately, several crosses could not be made with the line Purple in the greenhouse because of poor development due management problems. Crosses made with the transgenic line ranged from 5% to 12% with the field materials, and with Cica 8 from 17% to 60% with the greenhouse plants (Tables 1). These results are in agreement with those reported by Langevin et al. (1990) on hand-made crosses using red rice collected from the Southern USA showing hybridization of 1 % to 52 % depending on the variety used. Flower or seed abortion was only noted when red rice was used as female parent, suggesting some level of incompatibility or higher susceptibility to hand manipulation.

Analyses of floral structures indicated that flower morphology of the transgenic line and Cica 8 is similar to those of red rice (Figure 1) and Table 2. The presence of anthocianins was noted on the stigma of the line Purple, and it was also more plumose and spongy respect to red rice, Cica 8 and the transgenic line. The ovary of Purple was shorter and the style was longer respect to the other materials (Figure 1). The pistil of Purple was below the anthers in contrast to the other materials that were at the same level, suggesting morphology more prompt to self-pollination in the case of Purple. (Figure 1).

Future Plans

Due to the different hybridization rates obtained with field-grown materials respect to those of the greenhouse, new crosses will be made with plants grown in pots in the greenhouse and the field, in order to avoid the stress that panicles suffer during detachment from the tillers as previously done with field-grown plants. Additional crosses and floral morphology analyses will be done using other commercial rice varieties to evaluate potential effect of genotype on compatibility level with red rice and differences in flower morphology. This study is complementary to the assessment of gene flow currently conducted in the field under farm conditions.

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Table 1. Hybridization rates with hand-made crosses between six red rice biotypes, the F_3BC_1 transgenic Cica 8 A3-49-60-12-3/Cica 8 line and the line Purple grown in the field or greenhouse [1].

	Panicles from the field				Panicles from the greenhouse			
	Male Parent		Female Parent		Male Parent		Female Parent	
Red rice	T	P	T	P	NT	P	NT	P
1-3-4	0.00	10.8	0.00	0.81	17.1	37.5	Nd	1.4
4-12-2	4,97	0,52	3,78	0.26	37.2	Nd	Nd	Nd
1-21-3	12,40	7,50	15.00	Nd	58.8	Nd	4.8	Nd
5-38-5	0.00	3.48	0.00	2,38	51.9	Nd	29.7	Nd
5-36-4	1,23	0.00	8,17	0.00	41.7	Nd	Nd	Nd
5-48-2	11,60	0,74	14,80	Nd	19.9	Nd	15.1	Nd
T	0,39	Nd	0,39	Nd	Nd	Nd	Nd	Nd
P	Nd	0,48	Nd	0,48	Nd	Nd	Nd	1.7

[1]Total of two to eight panicles were used per cross. T: F_3BC_1 transgenic Cica 8 A3-49-60-12-3/Cica 8 line. P: Purple line. NT: Non-transgenic Cica 8. Nd: Not determined.

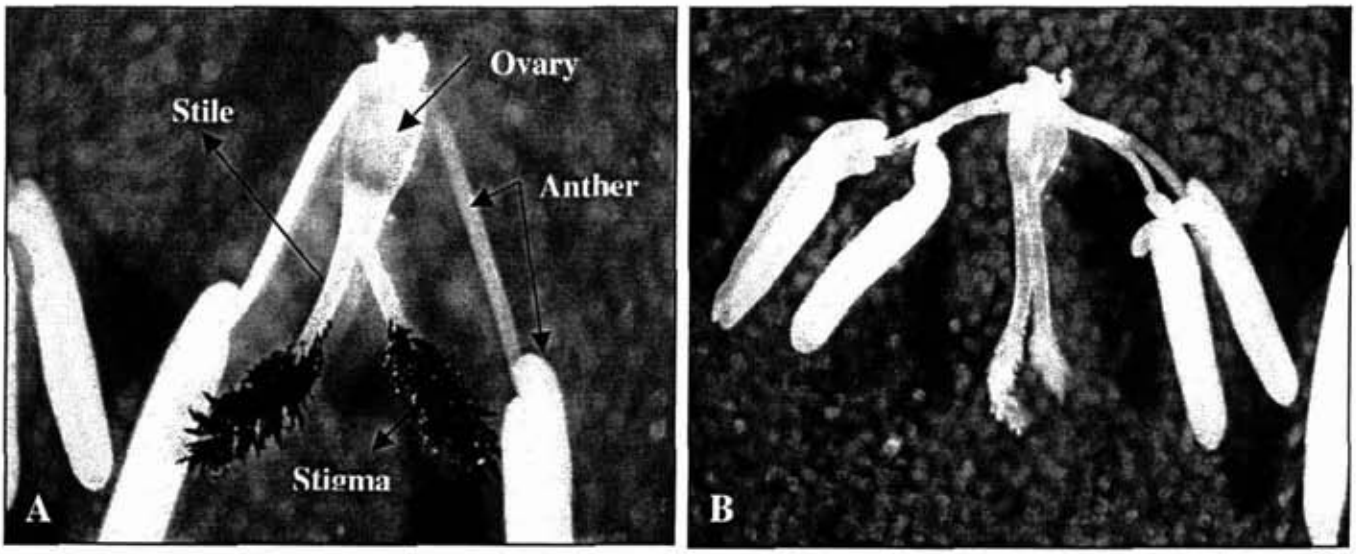

Figure 1. Floral morphology of Purple (A) and transgenic line (B).

Table 2. Length mean values of pistil and anthers from flowers of red rice, transgenic line, variety Cica 8 and line Purple.

Length[1] (mm)	Red rice				Line transgenic		Varieties	
	4-12-2	5-36-4	1-21-3	5-48-2	60-4-5/ FB007-19[2]	T	Cica 8	Purple
Ovary	0.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
Style	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Stigma	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1
Pistil	2.6 ± 0.1	2.5 ± 0.1	2.5 ± 0.2	2.0 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.2 ±0.1
Anthers	3.3 ± 0.3	3.9 ± 0.8	3.4 ± 0.2	3.2 ± 0.1	3.6 ± 0.2	3.5 ± 0.5	3.3 ± 0.2	4.0 ± 1.0
Stigma respect anthers	0.7	1.4	0.9	1.2	1.1	0.9	0.9	1.8

[1]Ten flowers (mm) per each material. Numbers refer to the mean ± Standard Error. [2]An additional transgenic line was included for comparison.

1.1.13 Assessment of gene flow from transgenic and non-transgenic rice into red rice under experimental field conditions

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[1] SB2, [2] IP4 . GTZ, Germany. Project No. 99.7860.2-001.00

Introduction

A careful assessment of potential impacts of gene flow from transgenic plants on population genetics of natural crop plant biodiversity is needed in other to design strategies for the safe deployment and durable use of these crops in the tropics. Genes from rice varieties may transfer quickly into red rice (1% to 52% hybridization rate)(Langevin *et al.* 1990). However, most of the hybridization rate estimates have been done under temperate conditions. Gene flow rates lower than 1% was reported between herbicide-resistant transgenic line and a non-transgenic Mediterranean *japonica* rice varieties (Messeguer *et al.*, 2001). Similar rates were reported by Noldin *et al.* (2001) and Zhang *et al.* (2003) between red rice and resistant herbicide transgenic line which contains the *bar* gene. Current studies showed that gene flow rates reached about 3 % when *O. rufipogon* and a commercial variety were sown together (Song *et al.*, 2003). This rate is higher than those reported previously [Messeguer *et al.* (2001), Noldin *et al.* (2001), and Zhang *et al.* (2003)]. Last year we presented a detail morphological and molecular characterization of a red rice population collected from rice farmers field in Colombia. Based on these results

some red rice accessions were selected to conduct gene flow analysis and identify indicators for easy tracing and monitoring of genetic introgression from rice into red rice, and persistence of domesticated genes in the weedy population under field conditions throughout subsequent generations. Here we present preliminary analyses of two field experimental designs to trace genetic introgression from transgenic and non-transgenic rice into red rice using the *NS3* (encoding for RHBV resistance in transgenic rice) and *gus* transgenes, the presence of anthocyanins in the leaves, tillers, and grain apiculus as morphological markers. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species.

Materials and Methods

Comparison of agronomic traits for red rice, transgenic lines and rice varieties. Seedlings of F3BC1 Cica 8 transgenic line A3-49-60-12-3/Cica 8-2, F3 Cica 8 transgenic lines A3-49-60-4-5/Fedearroz 50-19-1 and A3-49-60-4-5/Fedearroz 50-19-1-2, six red rice biotypes[1], and eight commercial rice varieties were transplanted in the field in three replicates of 42 plants per replicate. Morphological and phenological (days to flowering) characterization was conducted in order finalize the selection of the red rice candidates to be used in the gene flow studies. These red rice materials were pre-selected to include the phenotypic and genetic diversity present in the red rice population based on last year results on the analyses of qualitative and quantitative traits by principal coordinate and component tests respectively, agronomic traits, and molecular characterization by microsatellites markers at the same time some genotypes were included because they showed close overlap in flowering with rice varieties as well as similar height. These red rice types were also susceptible to rice hoja blanca virus (RHBV) as indicated by RHBV evaluations conducted in the field. The transgenic lines were chosen because they showed resistance encoded by the NS3 transgene, and contained the *gus* and *hph* (hygromycin resistance) marker genes. The rice line commonly known as Purple (IRRI accession) characterized by having purple leaves, tillers, and grain apiculus, was used to trace the inheritance of anthocyanins as a morphological marker (control) facilitating the identification of hybrids since anthocyanin production in rice is encoded by dominant gene(s).

Experimental field designs to trace gene flow from transgenic and non-transgenic (Purple line) into red rice. Seeds of the experimental genotypes were sown in a nursery under the field conditions at different dates to ensure flowering overlap between rice (pollen donor) and red rice (pollen receptor). The expression of *gus* gene was assayed histochemical in seedlings after 15 days later. Plants with *gus* expression were selected for the gene flow study. Two experimental field plots were used. The first design (multiple-square assay) consisted in square plots where rice and red rice were planted intermingled simulating

[1] Some biotypes were progeny of second or third generation of self pollination from original material collected in farmers fields

farmers field conditions. The second design (concentric circles assay) was used to measure gene flow distance affected by wind.

For the multiple-square assay, seedlings of 25 to 30 day-old were transplanted in plots of 1.8 m X 1.8 m. Each plot contained 81 plants, 16 of which corresponded to one red rice type (20%). The plants were planted at a distance of 0.2 m between plants, and at 5 meters between plots. Each plot was surrounded with a biological barrier of sweet corn of 1.8 m of height planted at 2.5 m from the border of each plot. A complete randomized block design, with four replicates per red rice type and pollen donor source (transgenic line or Purple line) was used (Figure 1). In order to ensure flowering overlap between rice (pollen donor) and red rice, pollen donor plants from at least two different sowing dates were planted in each plot. Plants in each plot were scored throughout the life cycle to maturity. Average wind speed and wind direction during flowering were recorded.

For the concentric circle assay two designs were used. In both cases, plants from both transgenic lines and Purple line were inter-planted within a circle at the center of the plot. Total of 176 pollen donor plants were planted in concentric 7 circles respect to the center at a distance of 0.25 m between plants. Between 220 and 262 plants red rice plants composed by a equal amount of four red rice types (5-38-5, 1-21-3, 5-36-4 and 1-3-4) were planted at 0.3 m between plants, in four concentric circles respect to the pollen source using a Statistic Latin Square Design (Figure 1). The first design, the first circle was 0.5 m from the pollen source, and the fourth circle was at 1,25 m. In the second design, the first circle was at 1 m from the pollen source and the fourth circle at 1,75 m.

Likewise for the other assay, pollen donor plants of at least two different sowing dates wee used to ensure flowering overlap with red rice.

Results and discussion

Red rice showed variation in stem (SC) and leaves (LC) color (Table1). Stem color ranged from light green (score 3) to green (score 6), and leaf color varied from green (score 6) to dark green (score 8). Color was defined using The Royal Horticultural Society color scale (1966). Red rice accessions 1-21-3, 1-3-3, 4-12-2 and 5-38-5 showed erect or intermediate growth habit (GH) likewise most rice commercial varieties (scores 3-5). In contrast red rice 5-36-4 and 5-48-2 showed decumbent habit (score 7-9). In relation to flowering, the Ryan-Einot-Gabriel-Welsch multiple range test ($p>0.005$) discriminated three groups: early flowering (red rice 1-21-3, 1-3-4, and 5-36-4, and the Purple line) with a mean value 92 to 95 day-after-sowing (DAS); intermediate flowering (red rice 5-38-5, 4-12-2, and 5-48-2, and all the rice varieties and transgenic lines A3-49-60-12-3/Cica 8-2, A3-49-60-4-5/FB007-19-1) with a mean value of 99 to 110 DAS); late flowering (the transgenic line A3-49-60-4-5/FB007-19-2) with a mean value of 123 DAS. About 71 % red rice accessions flowered earlier than the variety Cica 8 and the transgenic line A3-49-60-12-3/Cica 8-2 (Table 1), and most red rice flowered earlier than and the transgenic line A3-49-60-4-5/FB007-19-2. No significant difference was noted in the number of tillers between

the red rice, the transgenic lines and rice (Table 1). About 70% of the red rice was as tall as the transgenic line A3-49-60-12-3/Cica 8-2, and the Purple line, whereas the varieties Cica 8, Fedearroz 50 and Fedearroz Victoria 1 were taller than 70% of the red rice. Height differences seem not to be a critical point to prevent gene flow. Song et al. (2003) detected gene flow between species of *O. rufipogon* and the rice commercial variety Minghui - 63, which differed in 130 cm height (Table 1). Based on these results the transgenic line A3-49-60-12-3/Cica 8-2 was selected as one of the pollen donors for the gene flow studies.

About 60% of plants from the line A3-49-60-12-3/Cica 8-2 showed *gus* expression, indicating that the gene was still segregating in this F3BC1 generation. In order to have enough plants for the gene flow studies, a total of 4011 plants were evaluated, and 2246 plants showed *gus* expression (56%). Flowering was synchronous between the red rice; the Purple line and the transgenic line A3-49-60-12-3/Cica 8-2. Most treatments involving the Purple line and red rice 1-21-3, 1-3-4, 5-36-4 and 5-38-5 overlapped in flowering. The highest synchrony in flowering was noted between red rice 4-12-2 and the Purple line, with 87% of plants with flowering overlap. In the case of the transgenic line, flowering overlap with red rice of 56 to 77 % plants was noted. Even though the red rice 5-38-5 flowered earlier than the transgenic line, there was overlap towards the end of the flowering cycle of the red rice. Wind velocity ranged from 0 to 0,7 m/s, the maximum mean value of 1.6 m/s was registered from 10: 00 AM to 12 PM on cloudy days. Seeds from red rice, transgenic and Purple line were harvested keeping record of the plant location within each experimental plot.

Future plans

Progeny plants from the different treatments will be analyzed using specific microsatellites markers to identify hybrid plants, as well as by scoring *gus* expression and the presence of the *NS3* and *hph* genes by PCR (when using the transgenic line as pollen donor) or by the presence of anthocyanins in vegetative and reproductive tissues (when using the Purple line as pollen donor). These analysis will not only give an estimate of rate of hybridization between the different experimental types, but also the distance of gene flow, and will allow the optimization of an experimental approach to use molecular marker for tracing/ and monitoring genetic introgression from rice at large scale suitable for risk assessment in farmers fields and natural environments. Hybrids plants will be used to study genetic introgression dynamics and persistence of domesticated genes I recipient population over time. The information generated will be used to define management practices allowing a safety deployment of transgenic rice in the tropics.

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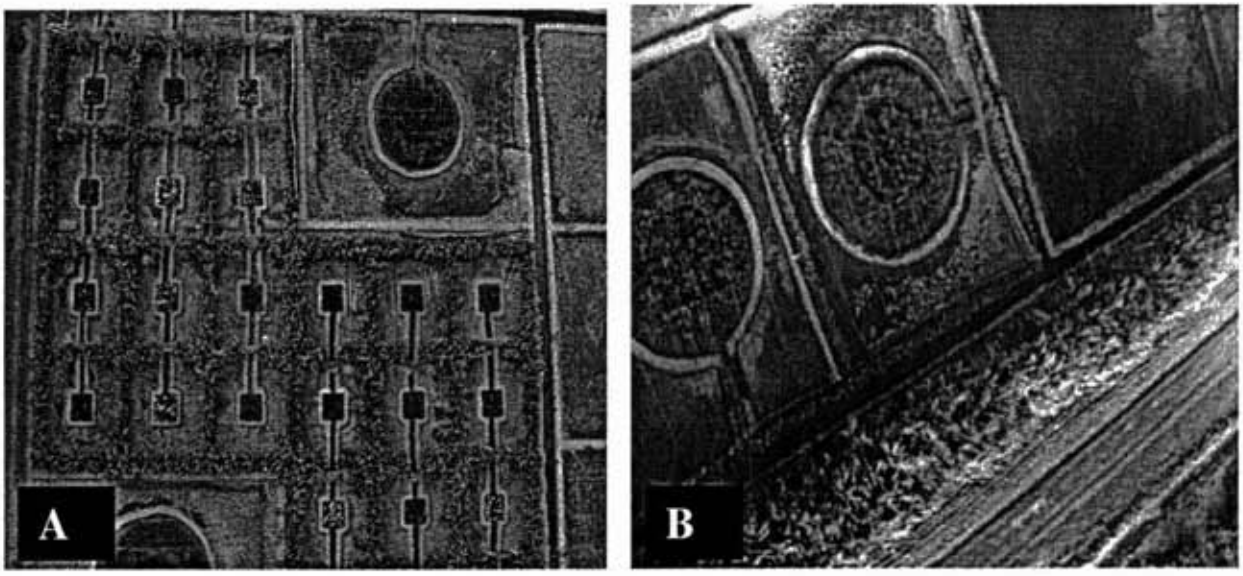

Figure 1. General view of field trials in (A) Square-plots assay, and (B) Concentric circle assay.

Table 1. Mean values of trains evaluated in the field

Line	*Genotype*[1]	SC[2]	LC[3]	GH[4]	Fl[5]	Tiller /plant	Plant height (cm)	Hight to highest panicle (cm)
Red rice	1-21-3	3	6	3	92f	19 a	102abcde	96abcd
	1-3-4	6	6	5	95ef	19ab	102abcde	91abcd
	4-12-2	3	8	3	105bcd	10abcd	95bcdef	94abcd
	5-36-4	4	8	9	100def	15abcd	94bcdef	80d
	5-36-4	4	8	9	95ef	17abc	97bcdef	84cd
	5-38-5	4	7	5	101cdef	15abcd	94cdef	90abcd
	5-48-2	6	7	7	107bcd	16abc	101bcdef	98ab
Transgenic	60-12-3/Cica 8-2 (GUS+)	4	8	5	110b	15abcd	91ef	85cd
	60-12-3/Cica 8-2 (GUS-)	4	8	5	111b	13abcd	86f	80d
	60-4-5/Fd50-19-1	7	8	1	98 def	8.7cd	104abcde	96abcd
	60-4-5/Fd50-19-2	7	8	1	124 a	7.7d	105abcd	93abcd
Rice varieties	Cica 8	4	8	5	105bcd	18ab	105abc	95abcd
	Purple	10	9	3	92f	12abcd	93def	87bcd
	Cimarron	5	8	3	101cdef	19ab	95bcdef	92abcd
	Coprosem	3	7	3	100cdef	10bcd	103abcde	95abcd
	Fedearroz 50	7	8	3	109bc	12abcd	111a	103ab
	Fedearroz 2000	7	8	1	100cdef	13abcd	99bcdef	98abc
	Fedearroz Victoria 1	5	7	1	104bcde	14abcd	107ab	104a
	Oryzica 1	3	6	5	99def	12abcd	100abcde	95abcd

Values followed by the same letter are not significantly different ($p=0.05$) Ryan-Einot-Gabriel-Welsch multiple range test. [1]Genotypes used: 6 red rice types. Transgenic lines 60-12-3/Cica 8-2 lines with (+) or without (-) *gus* expression. [2] SC= Stem color, [3]LC = Leaf color. Stem and leaf color range from 3= light green color, 4 -6= green color, 7-8= dark green color, 9= purple color, 10= dark purple color. [4]GH = growth habit, scored as 1=Erect, 3=Semi erect, 5=Intermediate, 7= opened, 9=decumbent, [5] Days to 50% plants flowering.

1.1.14 Genetic diversity in the multipurpose shrub legume *Flemingia macrophylla* and *Cratylia argentea*

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Little variation in nutritive value among *Cratylia* accessions was observed. CIAT 18674, 22375, 22406, 22408 and 22409 had higher dry matter yields in the dry and wet seasons than CIAT cv Veranera (CIAT 18516/18668). These accessions, collected in the states of Goias and Mato Grosso in Brazil, were selected for seed multiplication given that they had DM production higher than 3 t/ha per cut, good seed production capacity and equal or superior digestibility and crude protein content than Veranera.

Three semierect accessions CIAT 18437 from Indonesia, and 21083 and 21090 from Thailand were superior in comparison to CIAT 17403 (from Thailand). They had digestibility values >48% and dry matter yields >2 t/ha. More detailed analysis of two subsets showed that accessions with higher feed quality in terms of digestibility have lower fiber and condensed tannin contents than low-quality accessions.

For *Cratylia argentea* analysis of origin, agronomic morphological and molecular marker information did not identify correlations between the clusters obtained in the different approaches. In the case of *Flemingia macrophylla*, clustering obtained by molecular marker information correlated well with morphological information and grouped accessions according to their different growth types.

Introduction

The work of CIAT on shrub legumes emphasizes the development of materials to be utilized as feed supplement during extended dry seasons. Tropical shrub legumes of high quality for better soils are readily available, but germplasm with similar characteristics adapted to acid, infertile soils is scarce. *Flemingia macrophylla* and *Cratylia argentea* have shown promising results in such environments and hence work on these genera is part of the overall germplasm development strategy of the CIAT Forages team.

C. argentea is increasingly adopted and utilized, particularly in the seasonally dry hillsides of Central America, and more recently, the Llanos Orientales de Colombia. However, most research and development is based on only few accessions and hence activities to acquire and test novel germplasm of *C. argentea* is of high priority.

F. macrophylla also is a highly promising shrub legume with excellent adaptation to infertile soils. In contrast to *C. argentea*, whose adaptation is limited to an altitude below 1200 masl, *F. macrophylla* can successfully be grown up to altitudes of 2000 masl.

However, the potential utilization of *F. macrophylla* is so far limited by the poor quality and acceptability of the few evaluated accessions.

The project aims to investigate the genetic diversity within collections of *F. macrophylla* and *C. argentea* with three main objectives:

1) To identify new, superior forage genotypes based on conventional germplasm characterization/evaluation procedures (morphological and agronomic traits, forage quality parameters, including IVDMD and tannin contents)
2) To optimize the use and management, including conservation, of the collections. For this, different approaches to identify core collections for each species were tested and compared based on: (a) genetic diversity assessment by agronomic characterization/evaluation; (b) germplasm origin information; and (c) molecular markers (RAPDs).
3) To assist future germplasm collections on methodology, geographical focus and genetic erosion hazards.

Material and Methods

Agronomic characterization and evaluation. Space-planted, single-row plots in RCB design with three replications were established in Quilichao in March 1999 (*Cratylia argentea*, 39 accessions) and March 2000 (*Flemingia macrophylla*, 73 accessions). Additionally two replications were sown for seed production and morphological observations.

The following parameters were measured in the trials: vigor, height and diameter, regrowth, incidence of diseases, pests and mineral deficiencies, and dry matter yield during wet and dry seasons. For the analysis of nutritive value, crude protein content and *in vitro* dry matter digestibility (IVDMD) of the entire collections were analyzed. For the morphological evaluation, qualitative and quantitative parameters were measured, such as days to first flower, days to first seed, flower color, flowers per inflorescence, flowering intensity, pod pubescence, seeds per pod, seed color, leaf area, peduncle length, etc.

For *F. macrophylla*, a more detailed analysis of nutritive value was conducted of a representative subset (25 accessions), which included high, intermediate and low nutritive value accessions. The groups were selected based on crude protein content and IVDMD. The chemical analysis comprised fiber (NDF, ADF, N-ADF), extractable and bound condensed tannin (ECT, BCT) content and astringency (protein binding capacity). Monomer composition of the extractable condensed tannin fraction (procyanidin:prodelphinidin:proelargonidin ratio = C:D:P) was determined with a high-performance liquid chromatography system (HPLC). Due to extremely variable results both between laboratory replicates and among field repetitions, statistical analysis was not possible.

In order to give at least an idea of the monomer composition of extractable condensed tannins in *F. macrophylla* accessions, results from only five accessions, which were consistent between duplicates and among repetitions, are reported. Additionally, another subset of 10 accessions (9 high-quality accessions (18437, 18438, 21083, 21090, 21092, 21241, 21580, 22082, 22327) and CIAT 17403) was sampled 4, 6 and 8 weeks after cutting, to investigate the effect of age on digestibility as well as on protein, fiber and condensed tannin content and astringency.

Based on data referring to the morphological, agronomic and feed quality variation of all accessions a core collection will be created, using multivariate statistic tools (Principal Component Analysis and Cluster Analysis).

Analysis of available origin information. Based on ecogeographical information on origin of accessions, a core collection was created, hypothesizing that geographic distance and environmental differences are related to genetic diversity. The analysis was conducted with FloraMap™, a GIS tool developed by CIAT, which allows the production of climate probability models using Principal Component Analysis (PCA) and Cluster Analysis.

Genetic analysis by molecular markers (RAPDs).: Efforts made in genetic analysis showed that common manual DNA extraction methods did not work well with *F. macrophylla* and *C. argentea*. A modified protocol, which was used to extract DNA showed promising initial results. However, frequent degradation, contamination and partial digestion of DNA occurred, due to secondary plant compounds, probably polyphenols. In preliminary trials with a commercial extraction kit instead, the DNA purity was higher but partial digestion continued to be a severe problem. Various studies with amplified fragment length polymorphism markers (AFLPs), the method of choice, did not succeed and finally studies using this methodology could not be completed. Instead, random amplified polymorphic DNA (RAPD) markers, which do not require enzymatic digestion, were successfully employed.

A total of 47 RAPD 10-mer primers (Operon Technologies, Alameda, CA, USA) were screened as single primers for the amplification of RAPD sequences. Primers with highest levels of polymorphisms were repeated to test for reproducibility and those that produced polymorphic, distinct and reproducible bands were chosen for RAPD analysis. Multiple Correspondence Analysis (MCA) was performed on a matrix created based on the presence (1) or absence (0) of amplified bands. Subsequently, cluster analysis was performed on the coordinates obtained by MCA. Dendrograms were generated using UPGMA method. Nei's coefficient was used as estimator of similarity between accessions in order to generate between- and within-group similarity tables. Diversity was estimated using Nei's H and G_{ST} estimators.

Data analysis and synthesis. Individual and combined data analyses of all generated information was carried out using multivariate statistics. We have applied principle component analysis in all data sets (agronomic, morphological, geographical and

molecular). In addition, cluster analysis was performed and the resulting clusters were compared to identify similarities.

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Results and Discussion

Agronomic characterization and evaluation. Results from evaluations per season carried out for *Cratylia argentea* and *Flemingia macrophylla* indicated considerable phenotypic and agronomic variation in the collections studied. Data for *C. argentea* and *F. macrophylla* have been presented already in previous reports.

For *C. argentea,* IVDMD varied between 59 and 69% and crude protein content between 18 and 24%. Mean dry matter production was 2.2 (range 0.8 to 5.2) t/ha and 1.93 (range 0.6 to 3.3) t/ha in the wet and dry season, respectively. Dry season yields were relatively high and confirm the good adaptation of *C. argentea* to dry conditions.

There was a pronounced effect of season on some agronomic and quality traits. DM production was higher in the rainy season than in the dry season whereas ADF was higher in the dry than in the wet season. A season x genotype interaction was detected for IVDMD.

The cluster analysis dendrogram (Ward's Method) was truncated at the 6-group level. The detailed agronomic characteristics of each group are listed in Table A. Group 4 was the agronomically most promising cluster. It contained three accessions with the highest DM production (3.2 t/ha in the rainy and 2.4 t/ha in the dry season), CP content, regrowth and plant diameter values. The highest dry matter yields (2.4 to 3.8 t/ha) were recorded in accessions 22375, 22406 (Group 4), 18674, 22408 and 22409 (Group 6). Productivity of these accessions was higher than yields of the cultivar released in Costa Rica (cv. Veraniega) and Colombia (cv. Veranera) - an accession mixture of CIAT 18516/18668 (yield 1.9 and 3 t/ha). In addition to the higher yield, these accessions (18674, 22375, 22406, 22408 and 22409) also had equal or superior digestibility values (65 to 69%) and crude protein content (20 to 24%) in comparison to CIAT 18516/18668 (IVDMD 64 to 67%, CP 21 to 24%).

Based on high forage yield and good seed production potential we selected CIAT 18674, 22375, 22406, 22408 and 22409 for seed multiplication and regional testing (Table B).

Table A. Identification of *Cratylia argentea* accessions of agronomic interest. * *rainy/dry season value*

Group 1 (average/low yields, low regrowth, high dry season digestibility, high CP, high ADF):* CIAT 22382, 22390, 22392, 22393, 22394, 22396, 22399, 22411

Group 2 (high/low yields, low regrowth, high dry season digestibility, average CP, high ADF): CIAT 18675, 22380, 22383, 22384, 22386, 22387, 22391

Group 3 (average/low yields, low regrowth, average digestibility, lower CP than group 1, high ADF): CIAT 18672, 22376, 22378, 22381

Group 4 (very high/average yields, good regrowth, high digestibility, high CP, low ADF, low NDF): CIAT 22374, 22375, 22406

Group 5 (high/average yields, good regrowth, average digestibility, average CP, high ADF): CIAT 18676, 18957, 22373, 22400, 22410, 22412

Group 6 (very high/average yields, good regrowth, high digestibility, high CP, low AFD, higher NDF than group 4): CIAT 18516, 18667, 18668, 18671, 18674, 22379, 22404, 22407, 22408, 22409

Table B. Selected promising *Cratylia argentea* accessions and the two control accessions CIAT 18516/18668. Data of two evaluation cuts with 8 weeks of regrowth per season. IVDMD = *in vitro* dry matter digestibility, CP = crude protein.

Accession number	DM production (t/ha)[a]		IVDMD[b] (%)		CP[c] (%)		Seed production[d] (g/plant)
	Rainy	Dry	Rainy	Dry	Rainy	Dry	
18674	3.82	2.44	65	65	20	23	153
22375	3.12	2.41	65	66	21	23	255
22406	3.54	2.59	64	66	21	22	152
22408	3.25	2.45	69	67	21	22	153
22409	3.11	2.62	66	68	22	24	97
18516 (Control)	3.06	2.04	64	67	21	24	18
18668 (Control)	2.39	1.91	64	65	21	23	110

[a] Plant density 10 000 plants/ha
[b] Two-stage technique (Tilley & Terry 1963)
[c] Kjeldahl nitrogen x 6.25 (AOAC 2003)
[d] 15 months after sowing

In *F. macrophylla,* accessions evaluated differed in IVDMD, DM production, ECT, tannin extractability (ECT/total CT) and astringency (protein binding capacity) whereas CP and BCT showed only minor variability. IVDMD varied from 28 to 58% and crude protein content from 13 to 25%. Mean dry matter production was 2.08 t/ha in the wet and 1.18 t/ha in the dry season.

The chemical composition of 25 *F. macrophylla* accessions with contrasting digestibility varied greatly among accessions and in response to harvest season (Tables C and D).

Table C. *In vitro* digestibility, fiber and crude protein content of a representative subset of *F. macrophylla*. Data of one evaluation cut in the wet season and one in the dry season. n = no. of accessions evaluated, IVDMD = *in vitro* dry matter digestibility, CP = crude protein, NDF and ADF = neutral and acid detergent fiber, N-ADF = nitrogen bound to acid detergent fiber.

Forage quality[a]	IVDMD[b] (%)		CP[c] (%)		NDF[d] (%)		ADF[d] (%)		N-ADF[d] (%)	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
High (n=6)	52.8	48.0	22.4	21.6	33.0	33.8	20.3	23.7	11.1	11.2
Medium (n=12)	46.4	43.7	21.3	20.5	34.6	36.1	23.7	24.9	12.0	11.2
Low (n=7)	42.4	40.1	20.5	20.2	34.6	35.9	23.1	24.4	11.5	12.0
Minimum	39.9	36.8	17.0	17.6	29.5	31.2	17.0	21.5	9.1	6.6
Maximum	56.2	51.3	24.4	23.6	39.3	39.8	27.6	29.2	15.4	16.9
Mean	46.8	43.7	20.9	20.7	34.2	35.6	22.7	24.5	11.7	11.5

[a] high: average IVDMD ≥48%. intermediate: ≥43-47%. Low: <43%
[b] Two-stage technique (Tilley & Terry 1963)
[c] Kjeldahl nitrogen x 6.25 (AOAC 2003)
[d] van Soest et *al.* 1991, Robbins et *al.* 1987

Total condensed tannin content ranged from 1.5 to 16.7% in the rainy season and from 1.8 to 22.4% in the dry season. Astringency ranged from 1.7 to 6.8 (PBE) in the rainy season and from 2.4 to 7.9 in the dry season. The acetone-extractable CT among accessions ranged from 0 to 19.4%, whereas the content of acetone-bound CT ranged from 1.3 to 3.3%. The ECT represented 0% of total condensed tannins in CIAT 21090 but 95% in CIAT 20616. Positive correlations were found between ECT and astringency (r_{rainy} = 0.712, r_{dry} = 0.721, $P < 0.01$). IVDMD was negatively correlated with ECT (r_{rainy} = - 0.694, r_{dry} = - 0.576, $P < 0.01$) and astringency (r_{rainy} = - 0.632, r_{dry} = 0.548, $P < 0.01$).

Table D. Condensed tannin content and composition in 25 *F. macrophylla* accessions with contrasting digestibility. Data of one evaluation cut in the wet season and one in the dry season. ECT = acetone-extractable condensed tannins, BCT = acetone-bound condensed tannins, PBE = protein-binding entities, ND = not detectable. n.a. = not available.

		ECT[a] (%)		BCT[a] (%)		Extractability (ECT/TotalCT %)		Astringency[b] (PBE)	
		Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
18437	High Quality	4.2	7.6	2.33	2.65	64.54	74.05	4.21	4.57
18438		0.2	1.6	1.76	2.02	12.00	44.81	3.33	3.78
20065		7.7	9.7	1.30	2.24	85.47	81.26	4.84	n.a.
21083		0.1	0.0	1.36	1.81	7.48	0.00	1.65	2.39
21087		7.2	6.6	1.57	1.98	82.04	76.95	4.40	6.44
21090		ND	ND	1.57	1.99	0.00	0.00	2.03	2.53
17403	Intermediate Quality	4.3	9.6	2.12	2.43	67.08	79.83	4.52	4.99
20622		12.3	13.5	3.27	1.20	79.05	91.83	4.60	5.85
20744		11.5	13.2	1.91	1.75	85.77	88.29	4.39	5.79
20975		13.5	14.5	2.59	2.01	83.90	87.80	6.77	6.33
20976		13.4	16.7	1.85	1.33	87.90	92.62	6.09	5.33
21092		6.6	3.8	1.53	2.21	81.11	63.35	2.81	3.55
21249		9.0	10.7	2.18	2.59	80.55	80.51	4.44	5.70
21529		9.3	10.3	2.90	3.32	76.19	75.64	4.88	5.15
21982		8.0	12.5	1.87	2.90	81.13	81.16	6.03	7.70
21992		7.8	12.0	2.19	2.37	78.17	83.51	5.12	7.91
22082		0.3	0.3	1.86	1.96	11.85	11.31	2.73	2.88
J 001		7.1	15.8	2.55	1.73	73.63	90.15	4.11	5.58
17407	Low Quality	7.3	11.5	2.13	2.25	77.41	83.58	5.46	5.33
19457		8.5	11.6	2.76	2.65	75.51	81.46	5.80	6.74
20616		5.9	15.7	2.00	0.84	74.59	94.92	4.71	5.97
20621		14.2	17.1	2.54	0.96	84.78	94.67	5.42	5.88
21241		10.0	8.8	2.16	2.11	82.22	80.62	3.81	6.13
21580		9.4	5.7	1.79	2.07	84.06	73.22	5.49	5.07
21990		12.4	19.4	2.30	2.98	84.38	86.67	6.37	6.95
High		3.9	5.1	1.6	2.1	41.9	46.2	3.4	3.9
Intermediate		8.6	11.1	2.2	2.2	73.9	77.2	4.7	5.6
Low		9.7	12.8	2.2	2.0	80.4	85.0	5.3	6.0
Mean		7.9	10.3	2.1	2.1	68.0	71.9	4.6	5.4

[a] Butanol-HCl (Terrill et *al.* 1992, Barahona et *al.* 2003)
[b] Radial diffusion assay (Hagerman 1987, Lareo et *al.* 1990)

Monomer composition of the extractable CT fraction in 5 accessions *F. macrophylla* was quite variable due to accession but not due to season of the year (Fig. A). In four accessions (CIAT 20621, 20744, 20975 and 20976) prodelphinidin made up more than half of the proanthocyanidins (range from 49 to 79%). The second most important constituent

was propelargonidin, which ranged from 16 to 38%. Procyanidin was only present in small proportions (0 to maximum 16%). It was interesting to observe that in CIAT 21092 propelargonidin represented 82% of total proanthocyanidins in the rainy season and 95% in the dry season. Procyanidin was absent and prodelphinidin was less than 20%.

The five accessions for which we have reliable data on monomer composition of ECT are not representative of the entire *Flemingia* collection in terms of forage quality. However, four of them had very high ECT concentrations (13-17%) whereas CIAT 21092 presented relatively low ECT levels (7 and 4% in the rainy and dry season, respectively). The latter had an exceptionally high propelargonidin proportion but totally lacked cyanidin, which could indicate a relationship between monomer composition and forage quality.

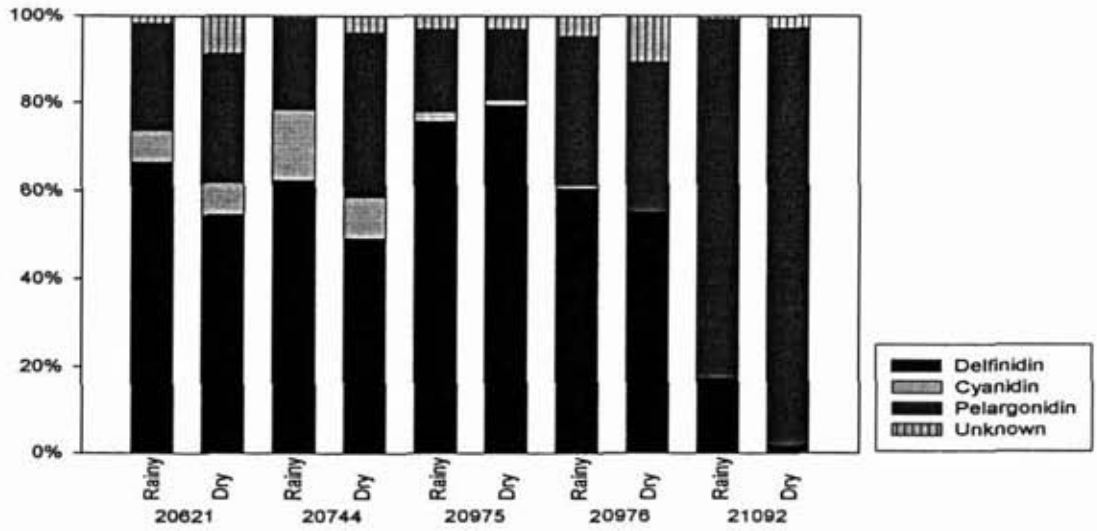

Fig. A. Monomer composition (procyanidin: prodelphinidin: propelargonidin ratios (C: D: P)) of the ECT fraction of five *F. macrophylla* accessions in rainy and dry season.

Analysis of a subset of 10 high-quality accessions (including control) showed that forage quality varied over time. Patterns were different in the rainy and dry season for both the averaged values of the 10 accessions and for individual accessions (Fig. B). Correlations found in this analysis confirmed the negative correlations between IVDMD and ECT and IVDMD and astringency.

Season had a large effect on IVDMD, DM production, plant height and diameter (higher in the rainy than in the dry season) and ADF, NDF, ECT and astringency (slightly higher in the dry season than in the rainy season). Extractability (percentage ECT of total CT) was relatively stable between harvest seasons (differences <10%). Only in six accessions (CIAT 17403, 18438, 20616, 20622, 21092 and J 001) differences up to 33% between

rainy and dry season were found. No genotype x season interactions were detected for DM production and regrowth.

The accessions with the highest average *in vitro* dry matter digestibility were CIAT 18437, 18438, 21083, 21090, 21092 and 21241. The most productive accessions were CIAT 7184, 21090, 21241, 21248, 21249, 21519, 21529, 21580 and CPI 104890 with a total DM production >3.5 t/ha in the rainy and >2 t/ha in the dry season.

Among the materials superior to CIAT 17403 (digestibility 36%, DM production 1.5 t/ha in the dry season) CIAT accessions 18437, 21083 and 21090 were identified for further testing as promising materials for dry season supplementation because they combined high digestibility with high productivity and low extractable condensed tannin content. These accessions had digestibility values > 48% and dry matter yields > 2 t/ha. Extractable condensed tannin content was 4.2 and 7.5% in the rainy and dry season for CIAT 18437 and nil in CIAT 21083 and 21090. However, their low seed production in the site where they were evaluated can limit their value.

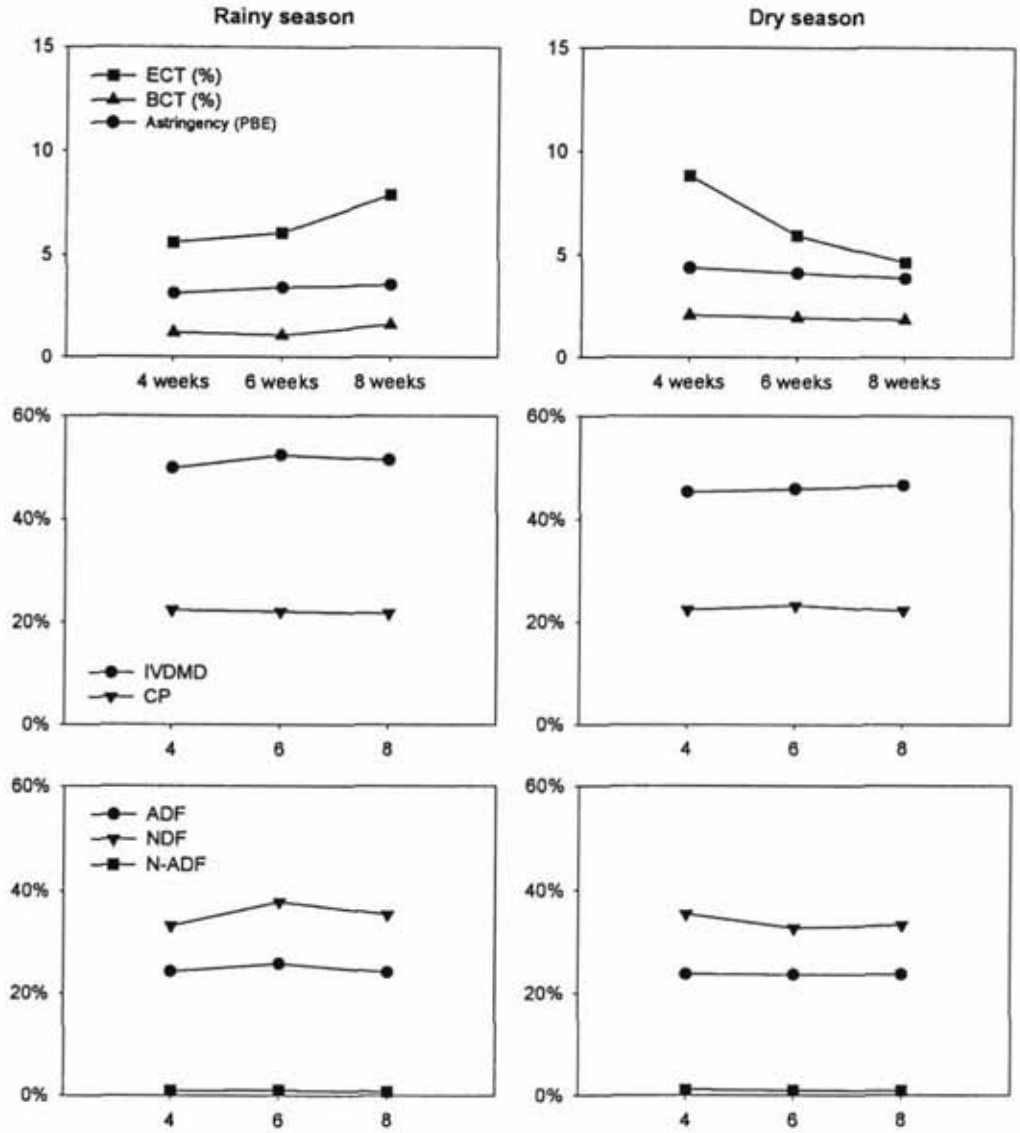

Fig. B. Variability in IVDMD, CP, fiber and tannin content of 10 *Flemingia macrophylla* accessions after 4, 6 and 8 weeks of regrowth in the rainy and dry season. ECT, BCT = acetone-extractable and bound condensed tannin, PBE = protein binding entities, IVDMD = *in vitro* dry matter digestibility, CP = crude protein, NDF and ADF = neutral and acid detergent fiber, N-ADF = nitrogen bound to ADF

The dendrogram (Ward's Method) was truncated at the 7-group level, explaining 72% of variation. The detailed agronomic characteristics of each group are listed in Table E. Group 4 was the one that had the most promising accessions from an agronomic point of view. It contained eight accessions (7 semierect, 1 'tobacco') with the highest digestibility values of the collection (51% in the rainy and 47% in the dry season) and high DM production (2.6 t/ha in the rainy and 1.2 t/ha in the dry season). The three selected promising accessions CIAT 18437, 21083 and 21090 were contained in this cluster.

Table E. Identification of *Flemingia macrophylla* accessions of agronomic interest. * *rainy/dry season value*

Group 1 (average/low digestibility, low yields, low regrowth, average CP, low vigor, low plant height):* CIAT 20973, 20977, 21080, 21086, 21994, 22053, 22087, 22090

Group 2 (average/low digestibility, average/low yields, high regrowth, average CP, low vigor, low plant height): CIAT 20979, 21079, 21990, 21993, 22285

Group 3 (high/low digestibility, low yields, average regrowth, high/average CP, low vigor, low plant height): CIAT 18048, 20972, 20976, 20978, 20980, 20982, 21982, 21991, 21992, 21995, 21996, 22327

Group 4 (high digestibility, high/average yields, good regrowth, high/average CP, high vigor, average plant height): CIAT 18437, 18438, 20975, 21083, 21087, 21090, 21092, 22082

Group 5 (average digestibility, high yields, average regrowth, high CP, high vigor, high plant height): CIAT 801, 7184, 20622, 20625, 20626, 20631, 20744, 21241, 21248, 21249, 21519, 21529, 21580, C104890, I15146, J001

Group 6 (like group 5, but lower yields, lower vigor and lower plant height): CIAT 19453, 19454, 19797, 19798, 19799, 20065

Group 7 (low digestibility, high/average yields, average regrowth, high CP, high vigor, average plant height): CIAT 17400, 17403, 17404, 17405, 17407, 17409, 17411, 17412, 17413, 18440, 19457, 19800, 19801, 19824, 20616, 20617, 20618, 20621, 20624

Genetic analysis by molecular markers (RAPDs): Out of 47 random primers tested, 9 were chosen that produced 171 RAPD bands ranging from 4 to 18 polymorphic bands per primer. Eight primers were selected for *Flemingia macrophylla* (D01, D04, D15, I07, J04, J06, J07, J12), and six for *Cratylia argentea* (D15, G12, I07, J06, J07, J12) (Table F).

Table F. Oligonucleotide primers employed in RAPD analysis, their sequence, number of bands obtained and percentage of polymorphic bands per species (% PBS).

Primer code	Sequence (5' to 3')	Number of bands		PBS (%)
		Polymorphic	Monomorphic	
Flemingia macrophylla				
D 01	ACCGCGAAGG	7	1	
D 04	TCTGGTGAGG	5	0	
D 15	CATCCGTGCT	18	1	
I 07	CAGCGACAAG	4	0	
J 04	CCGAACACGG	8	1	
J 06	TCGTTCCGCA	14	2	
J 07	CCTCTCGACA	13	0	
J 12	GTCCCGTGGT	6	2	
Total		*75*	*7*	*91.5*
Cratylia argentea				
D 15	CATCCGTGCT	10	4	
G 12	CAGCTCACGA	17	1	
I 07	CAGCGACAAG	17	4	
J 06	TCGTTCCGCA	13	1	
J 07	CCTCTCGACA	8	3	
J 12	GTCCCGTGGT	9	2	
Total		*74*	*15*	*83.1*

Clustering of 47 *Cratylia argentea* and 1 *C. mollis* (outgroup) accessions resulted in 5 groups (Fig. C), plus two genetically very distinct materials: "yacapani" (the only prostrate *C. argentea* accession) and *C. mollis* (data not shown here). Group 1 included 28 accessions and group 2 twelve. Group 3 comprised accessions 884 and CIAT 18668 and 22389. Group 4 was conformed of CIAT 22386 and 22387. Group 5 contained only CIAT 18674, one of the two agronomically most promising accessions. No correlation was found between the clustering according to RAPD polymorphisms and agronomic, morphological or geographical characteristics.

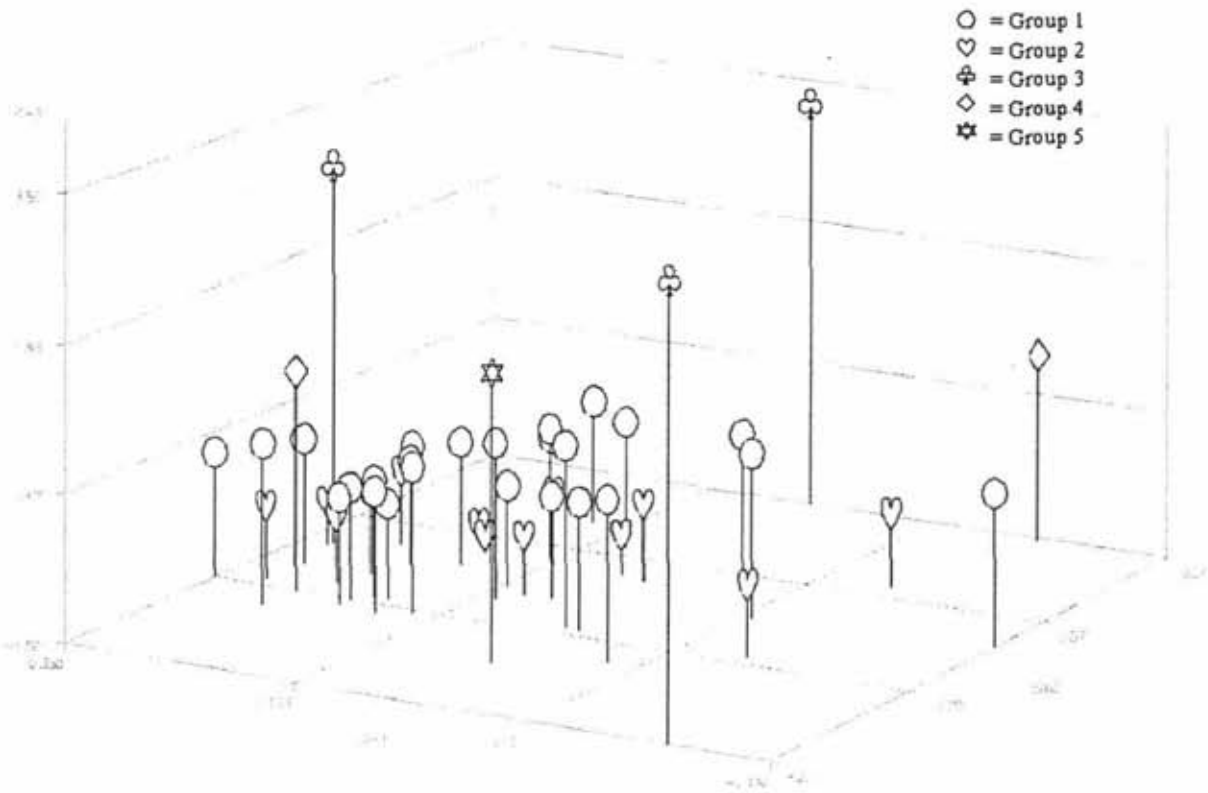

Fig. C. Tridimensional representation of five groups (without outgroup and prostrate accession "yacapani") resulting from clustering (UPGMA) of 47 *Cratylia argentea* and 1 *C. mollis* accessions according to molecular marker information (RAPDs).

Analysis of genetic diversity within accessions revealed high variability. Nei and Li similarity between groups often was as high or higher than within groups (Table G). This could indicate either seed contamination of accessions and/or outcrossing during multiplication in the field. Research on reproduction of *C. argentea* is urgently required to determine the rate and impact of outcrossing in this species.

Table G. Nei similarity within and between groups resulting from clustering (UPGMA) of 47 *Cratylia argentea* and 1 *C. mollis* accessions according to molecular marker information (RAPDs)

Group	N	1	2	3	4	5	6	7	Total
1	28	0.825	0.814	0.759	0.774	0.769	0.487	0.413	
2	12		0.839	0.721	0.764	0.720	0.515	0.404	
3	3			0.757	0.748	0.754	0.457	0.388	
4	2				0.717	0.757	0.479	0.400	
5	1					1.000	0.426	0.433	
6	1						1.000	0.444	
7	1							1.000	
Total	**48**								**0.776**

Clustering of 111 *Flemingia macrophylla* and 2 *F. paniculata* (outgroup) accessions resulted in six groups (Fig. D), distinguishing well among the different morphotypes of this species, which have been described in the morphological evaluation (Photo, see also CIAT Annual Report 2002).

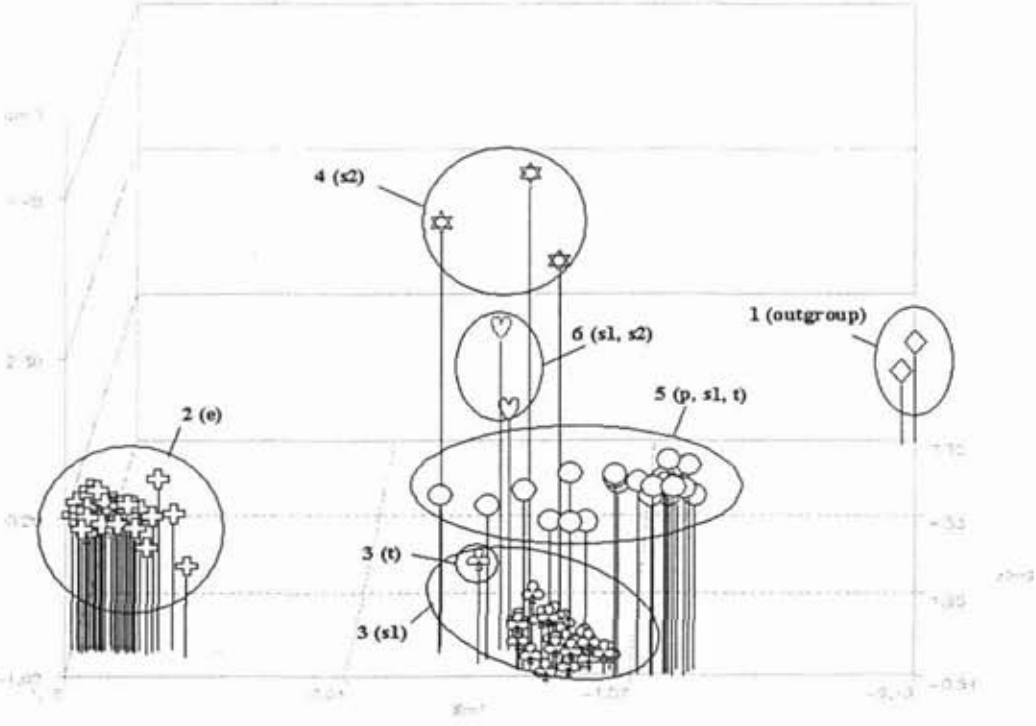

Fig. D. Tridimensional representation of the groups resulting from clustering (UPGMA) of 111 *Flemingia macrophylla* and 2 *F. paniculata* accessions according to molecular marker information (RAPDs). e = erect, s1 and s2 = semi-erect 1 and 2, p = prostrate, t = "tobacco" morphotype.

Group 1 included the two *F. paniculata* accessions. Group 2 was conformed by 55 of the 111 *F. macrophylla* accessions, which - with the exception of CIAT 20065 (prostrate) -

belonged to the erect growth type. Group 3 was composed of 23 semierect-1 and one "tobacco"-type accession and group 4 comprised 3 semierect-2 accessions. Group 5 included 8 prostrate, 14 semierect-1, 4 "tobacco" and 2 erect accessions and group 6 contained one semierect-2 and one semierect-1 accession.

No correlation was found between the clustering based on RAPD polymorphisms and agronomic or geographical characteristics. On the other hand, RAPD analysis proved to b useful for the identification/distinction of the different *F. macrophylla* morphotype. It is suggested that the employment of the more powerful AFLP markers would detect higher polymorphisms within the morphotypes of this species.

Photo . Four *F. macrophylla* morphotypes: 1=erect, 2=semi-erect 2, 3=prostrate, 4='tobacco'

Activity 1.2 Identification and mapping of useful genes and gene combinations

1.2.1 Development of a genome-wide anchored microsatellite map for common bean (*Phaseolus vulgaris* L.)

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Introduction

Microsatellites are polymerase chain reaction (PCR) based markers that have been developed for a wide range of plant species, including many commercial crops. Among he grain legumes, microsatellite markers are now available for soybeans, chickpea, cowpeas, peanuts and more recently common beans. Microsatellite markers have been developed for common beans from both non-coding (genomic) and coding (genic) sequences containing simple repeats. Our principal objective in this study was to map the both types of microsatellites in a single mapping population derived from the cross DOR364 x G19833 and to integrate this map with the genetic maps developed by Freyre et al. (1998) and Vallejos et al. (1992). Given the different sources of the microsatellites we compared the polymorphism rates of markers derived from genes versus those derived from random genomic sequences.

Materials and Methods

Populations and DNA extraction. Two populations of recombinant inbred lines (RILs) were used for this study: the first population was based on the cross DOR364 x G19833 (and heretofore will be referred to as the DG population). A total of 87 RILs were developed for this cross by a modified single seed descent from the F2 to the F9 generation. The plants within the F9 progeny row were bulked and used for subsequent genetic analysis. The second population was based on 91 RILs from the cross BAT93 x JaloEEP558 whose development and origins are described by Freyre et al. (1998). Total genomic DNA for each of the recombinant inbred lines in both populations was isolated from bulked leaf tissues of eight greenhouse-grown plants per line, using a CTAB extraction method.

Source and development of markers. We used three sets of markers in this study: 1) genomic microsatellites developed in this laboratory by Gaitán-Solís et al. (2002); 2) gene-coding microsatellites developed by Yu et al. (1999, 2000) and 3) additional gene-coding and non-coding microsatellites from searches for SSR containing Phaseolus sequences deposited in the Genbank database before July 15, 2001. SSRs were found using the SSR identification tool (SSRIT) that screens for all possible dimeric, trimeric and terameric repeats. Only sequences containing a minimum of three tetra-nucleotide, four tri-

nucleotide or five di-nucleotide motif repeats were used for primer design. Primers were designed using Primer 3.0 software to produce PCR amplification fragments that were on average 150 bp long, and PCR primers with consistent melting temperatures of 55°C or above and an average length of 20 nucleotides. Primer pairs were checked to make sure that they had similar melting temperatures and did not suffer from palindromes or end pairing.

Microsatellite analysis. Polymorphisms between the mapping parents were determined on parental survey gels. Standard microsatellite PCR conditions were used throughout and the PCR reaction was carried out in 20 mL final volumes. Gel staining and image capture are as described in more detail by Gaitan et al. (2002). The sizes of the parental alleles were estimated based on 10 bp and 25 bp molecular weight ladders. To determine the genotypes of the progenies, alleles were scored based on the parental bands that were amplified as controls along with the RIL individuals.

Data Analysis. Segregation distortion was measured with a Chi-square test for an expected 1:1 ratio and segregation data was used to place the microsatellites on the established genetic maps for the DG and BJ populations (Beebe et al. 1998; Freyre et al. 1998). The DG map included 240 RFLP, RAPD, SCAR and AFLP markers described in Beebe et al. (1998) while the BJ map contained 141 markers as described in Freyre et al. (1998). The two maps were linked by common RFLP markers with the map described by Vallejos et al. (1992). Linkage analysis was conducted with the software application Mapmaker 2.0 using a minimum LOD of 4.0.

Results and Discussion

A total of 150 common bean microsatellites were used in this study. Of these, 81 were anonymous genomic or non-coding microsatellites and 69 were gene-derived microsatellites. In the Genbank searches, SSRs were found in a range of coding and non-coding sequences. The highest number of repeats detected in the simple sequence repeats was nine, while the average number of repeats was 5.5 among all the microsatellites identified. Genomic microsatellites had a significantly higher (P=0.02) average number of repeats than gene microsatellites (6.2 versus 5.3) in unpaired t-test. In this study, di and tri-nucleotide motif containing microsatellites did not have significantly different average number of repeats. All the microsatellite markers were screened for amplification products and polymorphism in the parents of the DG and BJ populations and no difference in band intensity between the cDNA and genomic derived microsatellites was observed. A majority of the microsatellites produced single bands for the parents. Polymorphism rates for the DG and BJ populations were 65.4 and 63.2% for the genomic microsatellites, and 46.3 and 46.2% for the genic microsatellites, respectively. Overall the percentage of polymorphism between the parents of both populations was very similar: a total of 84 out of the 150 microsatellites tested for the parents of the DG population were polymorphic (56.0%), while a total of 68 out of the 122 microsatellites tested for the parents of the BJ population were polymorphic (55.7%).

A total of 100 new microsatellite loci were placed on the two genetic maps (78 on the DG population and 22 on the BJ population) during this study. Microsatellite loci were found on each of the eleven chromosomes of the species and each chromosome was tagged with at least five or more microsatellite. Two chromosomes, B02D and B04B had a relatively greater number of microsatellites placed on them, with 17 and 13 markers respectively; while the average number of microsatellite loci per chromosome was 10. The total cumulative map length for the DG population was 1720 cM with an average chromosome length of 156.4 cM. The average distance between microsatellite loci in this map was 19.5 cM; however the distribution of loci was variable and several large gaps between microsatellites remained on the map. The largest gaps between microsatellite markers remained on chromosomes b01, b07, b08 and b11. Among the markers that presented multiple bands, duplicate loci could be mapped for two markers. The gene-based microsatellites were better distributed than the genomic microsatellites and several clusters of genomic microsatellites were found on almost every chromosome except b06 and b08 which were the linkage groups with the fewest microsatellites.

This study brings to a total of 115, the microsatellite loci located on the bean genetic map and provides coverage for every chromosome in the genome with from five to twenty markers each. Although the genomic distribution of microsatellite markers in this study tended to be random, some large gaps between microsatellites occurred and certain chromosomes contained more microsatellite loci than others. As single-locus markers, the microsatellites in this study were specific to a given place in the genome and this allowed them to be used for comparative mapping across both the DG and BJ populations. This comparative mapping showed the consistency of microsatellite location on both populations: with all the microsatellites mapping to the same individual chromosome and equivalent map locations in each of the populations. Mapping in two populations was useful for placing microsatellites that were monomorphic in one or the other of the populations. Comparative mapping allowed us to determine the identity and orientation of each linkage group and to obtain a more accurate position for each of the microsatellites. The synteny between maps as reflected by the map order of jointly mapped markers was conserved providing further evidence that no major rearrangements have occurred in the common bean genome.

The current set of microsatellite markers described in this study provides the basis for anchoring and aligning genetic maps one to each other based solely on PCR-based markers, something that previously was done with single-copy RFLP markers or by tentatively associating RAPD bands found in different populations. Therefore, the microsatellites make ideal second-generation markers for the whole genome analysis important for gene tagging and quantitative trait loci studies. The microsatellites mapped during the course of this research will also be invaluable for marker assisted selection because they are simple to analyze, specific for single genes of interest and diagnostic in most crosses due to their high level of polymorphism. The mapped microsatellites can also provide a good set from which to chose markers for studies of genetic diversity in common bean.

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1.2.2 Analysis of iron reductase as a mechanism for enhanced iron uptake in common beans

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Introduction

Nutritional genomics is being used as part of the Biofortification Challenge Program to discover the basic mechanisms for mineral uptake and accumulation. As part of this program CIAT is collaborating with the Grusak lab at the USDA-Baylor College of Medicine to determine the genes in common beans that determine iron uptake and utilization. As part of the overall genomics approach, information from other well studied species such *Medicago truncatula*, peas and soybeans, as well as other model species such as *Arabidopsis thaliana* which have extensive genetic and molecular resources are being used for gene discovery and functional analysis. The underlying concepts of this work are to take advantage of metabolic unity among plants to characterize gene function and to apply bioinformatics and molecular cloning approaches to identify potential orthologous genes.

As a first example of this approach the Grusak lab is trying to dissect the importance of iron reductase in the accumulation of iron in beans by assaying iron reductase activity in roots and by cloning an ortholog of the gene from common bean based on similarity to the same gene already isolated from *Medicago truncatula* and from *Pisum sativum* (Pea). Iron reductase is a member of the protein super-family of flavocytochromes and functions to convert iron from an unavailable form (ferric, Fe3+) to an available form (ferrous, Fe2+) that can be readily absorbed by plants. The iron reductase protein (FRO) is located in roots and straddles the root cell membrane where it is active for iron reduction and was first

isolated from *A. thaliana* and *P. sativum*, both of which are fairly efficient at extracting iron from the soil and serve as model species for enzyme activity.

Methodology

SCAR marker. the Grusak lab selected conserved primers for RT-PCR based on the Pisum iron reductase gene (PsFRO1). At CIAT we tried using these primers for mapping of the gene as a SCAR marker.

Reductase Assay. In the Grusak lab, seeds are germinated for 3-4 days then planted in a hydroponic system for 12 days of growth in various levels of iron concentration (eg. 2, 5 , 10 and 20 uM Fe). Iron reductase assay is conducted at the end of this period by removing the entire root systems of four plants and staining for reduced iron measured as umol Fe reduced/g FW/hr.

Results and Discussion

The SCAR primers produced multiple banding patterns that were not of the expected size range, suggesting that the conservation between common bean and peas for this gene is low, and that it will be difficult to clone via a direct PCR approach. Given this the Grusak lab has been screening a common bean leaf cDNA library which we made at CIAT and will begin next year to screen a set of two root leaf cDNA libraries, where the mRNA for iron reductase is more likely to be expressed.

The iron reductase assay is producing interesting results that suggest that there are differences between parents of several mapping populations for their ability to reduce iron (Table 1). These differences are evident more at low Fe concentration than at high iron concentration and seem to be somewhat correlated with the seed iron status of the parents, for example in the cross of G21242 x G21078, the high iron parent G21242 has higher reductase activity than the low iron parent G21078. Many of the wild accession also had low reductase activity as well, except for the Colombian accession G24404, which in contrast had high reductase activity and therefore will be investigated further in future experiments.

It was notable that significant difference exist between the parents of the DOR364 x G19833 mapping population at a range of hydroponic Fe concentrations (Figure 1) and that these results were consistent with the results observed in the first trial with multiple parents at 2 uM and 15 uM Fe concentrations (Table 1). More information on this trait will be reported next year when a set of recombinant inbred lines have been fully tested and the QTLs for this trait localized.

Future Steps

- Evaluate Fe reductase activity in a greater number of parents of other populations and a range of Fe concentrations for each parent.

- Evaluate the full set of recombinant inbred lines from the DOR364 x G19833 and G21078 x G21242 crosses to determine inheritance and location of QTLs for Fe reductase activity.

- Develop a DNA marker for Fe reductase activity either based on QTL mapping or cloning and mapping of orthologs of the Fe reductase gene.

Table 1. Common bean genotypes tested for root reductase activity at low (2 uM) and high (15 uM) Fe concentration.

Genotype	Status	Origin	Seed Fe content	Root Fe(III) Reductase Activity umol Fe reduced/g FW/hr	
				2 uM Fe	15 uM Fe
G11350	Cult.	Meso	High	0.835	0.350
G11360	Cult.	Meso	Low	0.454	0.165
G19227A	Cult.	Meso	ND	0.879	0.047
G19833	Cult.	Andean	High	1.054	0.123
G19839	Cult.	Andean	High	1.083	0.200
G19842	Wild	Andean	ND	0.227	**0.248**
G21078	Cult.	Andean	Low	**0.049**	**0.307**
G21212	Cult.	Meso	ND	1.089	**0.482**
G21242	Cult.	Andean	High	0.713	0.528
G21657	Cult.	Andean	ND	0.639	0.231
G24390	Wild	Meso	ND	0.187	0.668
G23585	Wild	Andean	ND	0.048	0.036
G24404	Wild	Meso	ND	1.039	0.705
G24423	Wild	Andean	ND	0.180	0.342

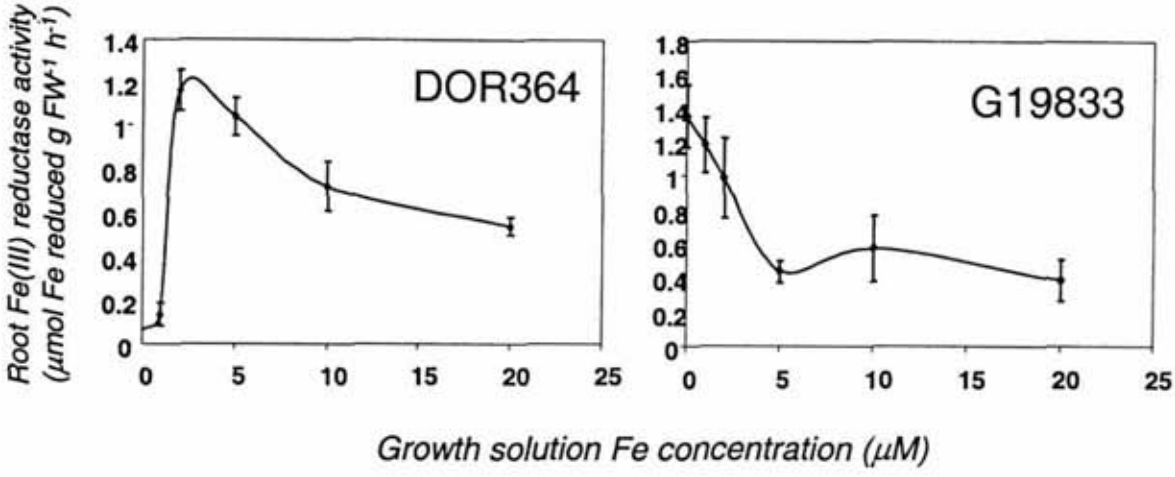

Figure 1. Parental genotypes tested for root reductase activity over a range of Fe concentrations.

1.2.3 Tannin studies on parents and progeny of the DOR364 x G19833 population

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[1]SB-2, CIAT [2]IP-1, CIAT,[3]Forages, CIAT

Introduction

Part of the effort to increase the nutritional quality of common bean has concentrated on increasing iron bioavailability, where bioavailability is the proportion of the consumed nutrient that is digested, absorbed and utilized by human beings. Bioavailability is determined by both food composition and the nutrient status of the consumer as well as a mix of promoters (such as sulfur amino acids: methionine and cysteine, vitamin A or C and lipid content) and anti-nutritional factors (including fiber, lectins, phytates, polyphenolics and tannins, as well as Calcium and Manganese). Among the anti-nutrients, tannins are important because of their ability to interact with proteins and to chelate minerals which results in reductions in protein digestibility and mineral bioavailability. Tannins are derived from phenolic compounds and contribute to the coloring found in common bean seed coats. They can be divided into hydrolizable / soluble tannins (derived from Gallic acid) and condensed tannins / proanthocyanadins (derived from polymerized flavonoids), which are measurable by different techniques.

Previous studies looking at overall tannin content in bean seed coat, using a water/methanol extraction found variability in the content of tannins in seed coats of different varieties of common beans. In this study our objective was to identify the genetic

variability for tannin in a segregating population and to collect preliminary evidence on the inheritance of soluble and insoluble tannin content in the seed coats of recombinant inbred lines from the cross DOR364 x G19833. In this study we applied a more accurate extraction technique and began to look at the inheritance of various fractions of tannin content.

Methodology

Tannin extraction. Total tannin extraction and analysis followed the methods of Jones et al. (1976) and Terrill et al. (1992). Extraction involved the use of a mix of acetone/water/diethyl ether. Colorimetric tannin analysis was realized with a Butanol-HCl method which allows total condensed tannins to be measured. A butanol-water (5%) mix was used as a blank. This method had been standardized for forage tannin analysis and has been used successfully for the analysis of tannins in sorghum grain.

Plant Material. Tannins were extracted from seed coats that had been peeled from common bean seed, dried at 60 C for one hour and ground into a fine powder to use in the analysis. An n-heptane treatment was used to facilitate seed peeling and consisted in 12 hours immersion in the n-heptane solution, followed by seed drying and hand-peeling. Different amounts of ground seed coat were used for the parents (30 g) than for the individual recombinant inbred lines (15 mg). This was done to obtain enough purified tannin from the parents to construct the concentration calibration curves used in the analysis of the progeny. Three replicates were used per seed coat sample for the analysis.

Results and Discussion

Soluble and insoluble tannins were successfully purified from DOR364 and G19833, the parents of the population, to use for the determination of a calibration curve for absorbance vs. concentration to use in estimating the amount of tannins in the progeny. The color of the dried tannin extract was darker and more reddish for the DOR364 tannins than for the G19833 tannins as would expected from their respective seed coat colors. When the progeny and parents were tested against the calibration curve, a range of seed coat tannin concentrations (expressed in percent) were observed (Figure 1). Soluble tannin was found to range from 13 to 41%, while insoluble tannin ranged from 1 to 8 %, depending on the progeny lines tested in the population. The parents had similar amounts of soluble tannins (both around 26%) while DOR364 contained more insoluble tannin (5.3 %) than did G19833 (2.9 %). The normal distribution of both soluble and insoluble tannins in the population suggests that both are inherited as quantitative traits.

Conclusions and future plans

We will use a QTL analysis approach to determine the genes involved in tannin content in common bean seed coats and use this information to devise a strategy for reducing specific fractions of tannins with the hope of increasing bioavailability of iron in beans. The

strength with which we pursue the strategy of reducing tannins to increase iron bioavailability, must be counterbalanced by the evidence that some tannins have been shown to have beneficial aspects as anti-oxidants and anti-carcinogens.

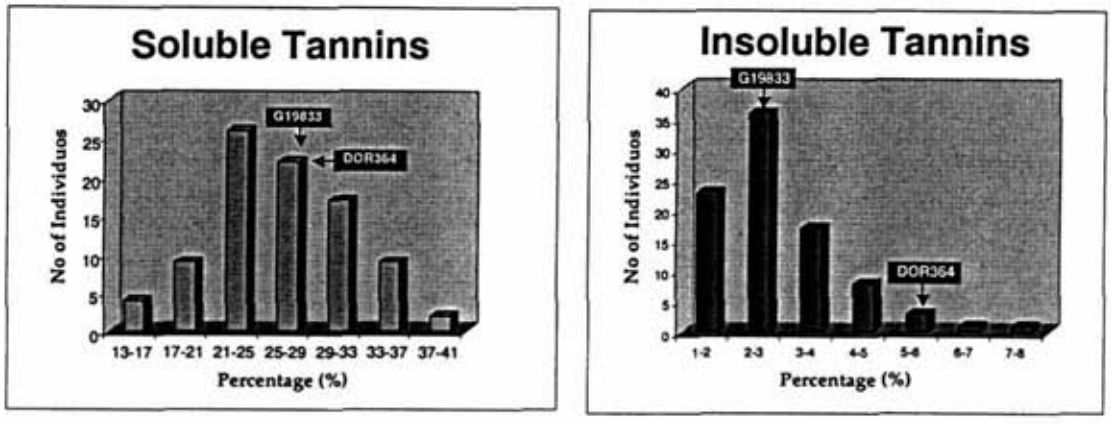

Figure 1. Histogram showing the population distribution for soluble and insoluble tannin content among the recombinant inbred lines of the cross DOR364 x G19833.

1.2.4 Identification of QTLs for resistance to *Thrips palmi* in common bean

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Introduction

Thrips palmi is a damaging insect pest of common bean and other dicotyledenous crops that was introduced from Asia (Java, Indonesia) into the Americas during the last decade. Starting in the Caribbean, (Cuba, Dominican Republic, Haiti and Puerto Rico) the species spread rapidly into the United States and northern South America (Brazil, Colombia, Ecuador and Venezuela). The greatest damage inflicted to common bean production in Colombia is seen in climbing bean varieties that are grown for the fresh market (including snap beans and Cargamanto dry beans). Sequential plantings, common in the production of snap beans is very conducive to heavy infestations of thrips and whiteflies, which are synergistic in the damage that they inflict. Misuse of insecticides also can lead to resurgence in thrips populations. Host plant resistance on the other hand is a promising component in an integrated cropping system to reduce damage by *Thrips palmi* Karny. Therefore the objective of this research was to identify the stability of Thrips resistance across environments and the genes and QTLs controlling this resistance.

Methodology

The BAT881 x G21212 population, consisting in 139 F5:7 generation RILs, was evaluated over three seasons at a field site in Pradera, Valle, Colombia. The details on phenotypic data collection are reported in CIAT- AR (2000). A genetic map was constructed based on the screening of 151 RAPD markers and 107 microsatellites as reported in CIAT-AR (2002). A linkage map was constructed in two phases; first with the RAPD markers only and then with both RAPD and microsatellite markers. The software package MAPMAKER3.0 (Lander et al., 1987), was used to generate the genetic map, whereby genetic mapping was done by: first, grouping markers at LOD > 5.0 and then, ordering them at LOD > 3.0 using three point analysis with a maximum inter-marker distance of 37.2 cM. Broad sense heritabilities (h2) on an entry-mean basis in seasons and across seasons were calculated for both resistance traits based on mean square ANOVA results, genotypic variance (σ2g), phenotypic variance (σ2p), genotype x season interactions (σ2gs), and error variance (σ2e). Arbitrary linear (orthogonal) contrasts were conducted, using the Scheffe's F-test, to compare among (1) the five most resistant RILs, (2) the five most susceptible RILs, (3) the BAT881 parent, (4) the G21212 parent and (5) the susceptible PVA773 check (Statistix, 1998). Quantitative trait loci (QTL) were identified through single-point regression analysis (SPA) and interval mapping analysis (IM) with the software packages Qgene, and QTLCartographer V1.21, respectively. In the SPA analysis, probability thresholds of 0.05, 0.01, and 0.001 were used. In the IM analysis a LOD threshold of 2.5, a window size of 10 cM and a 2 cM walking step were used to determine the presence and location of QTLs and whether there was evidence for more than one QTL on linkage groups with multiple LOD peaks.which had been run on the progeny.

Results and Discussion

The cross BAT881 x G21212 was found to produce progeny showing transgressive segregation for thrips resistance. Correlations between damage and reproductive adaptation scores were significant in seasons, and significant correlations existed between seasons. Broad sense heritabilities were moderate ranging from 32.4 to 63.4% depending on the parameter and the season (Table 1). The genetic map constructed for the cross had 11 linkage groups, eight of which could be identified as homologous to the chromosomes of the integrated linkage map of common beans, three of which remained unidentified. The most important thrips resistance QTL was located on chromosome b06, linked to two microsatellites and one RAPD marker, explaining up to 27.7% of variance in SPA. This QTL was located at the same region as the bc-3 and Ur-4 resistance genes. Other minor QTLs for thrips resistance were found on chromosomes b02, b03, b08, and b09, some of which were located in regions of genes encoding for disease resistance. The identification and mapping of thrips resistance genes is one of the first studies on insect resistance QTLs in common beans and is expected to facilitate the development of resistant bean cultivars by using molecular marker assisted selection.

Future Studies

Continue mapping with additional microsatellites to achieve complete map coverage in the BAT881 x G21212 population

Table 1. Estimates of variance components and heritabilities among RILs grown in two seasons in Pradera, Colombia (1999B, 2000B) for *Thrips palmi* damage and reproductive adaptation (RA) scores (both measured on 1-9 scales).

Parameters	Damage 1999B	RA 1999B	Damage 2000B	RA 2000B
Within seasons				
Variance components				
genotypic variance (σ^2_g)	0.36	0.14	0.51	0.32
phenotypic variance (σ^2_p),	0.58	0.44	0.97	0.67
Heritability (h^2)	0.619	0.324	0.526	0.478
Across seasons	Damage		RA	
Variance components				
genotypic variance (σ^2_g)	0.32		0.135	
phenotypic variance (σ^2_p),	0.50		0.31	
genotype x season interactions (σ^2_{gs})	0.03		0.02	
error variance (σ^2)	1.01		0.98	
Heritability (h^2)	0.634		0.436	

1.2.5 Development of SCAR and microsatellite markers for Apion resistance

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Introduction

This year we continued a project begun in 2001 to tag resistance to the bean pod weevil (*Apion goodmani* Wagner) which damages beans grown in Mexico and Central America. Resistance is controlled by two possible mechanisms – either antibiosis involving a hypersensitive response that encapsulates the oviposition site – or antixenosis that affects the preference of oviposition sites. Epistasis between two independent genes, *Agr* and *Agm*, has been suggested to control the hypersensitive response. The fact that a few genes control resistance may explain why it has been relatively easy to transfer resistance from Mexican landraces where it is found to new breeding lines with Central American grain types. The objectives of this research were to identify additional markers linked to the genes controlling resistance in the recombinant inbred line (RIL) population derived from

Jamapa x J117 and to try to identify the chromosomal position of the resistance QTLs identified so far.

Methodology

Parental Survey and Genetic Mapping. Genetic material consisted in a total of 104 F5 derived recombinant inbred lines (RILs) from the cross Jamapa x J117, where Jamapa is a susceptible cultivar released in Mexico and J117 is a resistant breeding line. Screening of susceptible and resistant bulks (of 4 lines each) has continued from last year with a total of over 150 microsatellite markers. A genetic map was constructed with the new dataset of 104 lines and all the polymorphic RAPD markers using the program Mapmaker.

RAPD cloning and SCAR primer design. Two RAPD bands (W9-1300S; Z4-800R) that were polymorphic from last year's survey and which were significantly associated with the resistance phenotype were selected for cloning. The RAPD bands were purified in 4% polyacrylamide gels which were used directly for a second round PCR amplification. Upon confirming that a single band had been amplified this DNA was purified from a 1% low melting point agarose gel using a Wizard PCR prep purification system (Promega). The purified insert DNA was cloned into the PGEM-T easy vector system for further analysis. Several clones were picked per ligation reaction and their inserts sequenced using standard techniques, T7 and Sp6 primers and an ABI377 DNA sequencer. Specific primers were designed for each unique sequence using Primer 3.0 software and these were tested for their ability to amplify SCAR products. Any monomorphic SCAR products were digested with frequent cutting restriction enzymes (*Alu*I, *Hae*III, *Rsa*I and *Sau*3AI).

Results and Discussion

As in last year's results, most of the markers were linked to each other in five tight linkage groups representing chromosome b01, B05, B07, B08 and B11, each with four or more markers per linkage group. The most significant markers occurred on chromosome b01 including the two RAPDs targeted for this SCAR development, W9-1300S and Z4-800R. We will continue to refine the genetic map with new markers for the other four significant linkage groups.

BLAST searches identified homologies for the two cloned RAPD bands. Several similar clones from each RAPD, showed that W9-1300S was derived from a retrotransposon, while Z4-800R was derived from an unknown Soybean gene.

A total of 5 primer sets were designed for the two RAPD band sequences and these were tested on the population parents and on the bulks. All the primer sets showed monomorphism as SCARs. When the PCR products were digested with the frequent cutting restriction enzymes, polymorphism was revealed for the fragment W9-1300-15. This potential CAPS (Cleaved amplified product) marker will be tested across the full population during the upcoming year.

Future Plans

- Test the new CAPS marker in the entire population and use the same procedure to determine if other SCARs developed from the cloned RAPD fragments can be converted into polymorphic markers.

- QTL analysis will be carried out when phenotypic data is available for the entire set of recombinant inbred lines which is expected for later in 2003.

1.2.6 Marker-assisted selection for BGYMV resistance in small-seeded beans

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Introduction

Bean golden yellow mosaic virus (BGYMV) is a devastating disease of common beans (*Phaseolus vulgaris* L.) in Latin America. It was first observed in Brazil in the early 60s, and a decade later it became the main biotic constraint to bean production in Brazil, demonstrating its considerable epidemiological potential for this country, Central America, the Caribbean and Mexico (Morales, 1994). Although the disease is not found in Colombia, indirect selection through molecular markers can be accomplished successfully (Beebe et al., 2002). Since 1999, a marker-assisted selection (MAS) breeding scheme has been applied using the SCAR marker DOR21 linked to the *bgm-1* gene. Breeding for BGYMV has been facilitated and accelerated with this strategy, which is beginning to be used in other countries such as Cuba (Rodríguez et al., 2002). Last year efforts were made to use a second marker for a QTL (quantitative trait locus), identified in collaboration with USDA-Puerto Rico and the University of Puerto Rico several years ago. This marker is also a SCAR (named W12) and was planned to be used as a large-scale tool, such as DOR21, in the identification of plants carrying the resistance genes for BGYMV.

Materials and methods

Alkaline DNA extraction and PCR for the *bgm-1* marker were performed for MAS purposes as described previously (Quintero et al., 2002). Visualization of amplified products was carried out as usual, except that samples were loaded three times per comb instead of two as done previously.

Given that the PCR conditions for W12 SCAR used last year yielded either unamplified products or spurious bands that made the screening difficult and time consuming, changes in annealing temperatures of the oligonucleotide primers and different PCR profiles were

assayed first in a set of 27 bean varieties, and those that gave best results were applied for screening the F_7 red- and black-seeded families. Once PCR conditions were set, a multiplex assay including both markers was performed as follows: each reaction contained 5 µl of the alkaline DNA diluted 1:1 in sterile water or 20 ng of pure DNA; 0.2 mM each dNTPs, 0.2 µM each forward and reverse W12 primers, 0.1µM each forward and reverse DOR21 primers, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase for a total volume of 15µl. PCR products were resolved in a 0.5X TBE agarose gel with ethidium bromide at a final concentration of 0.02 µg/ml. Presence or absence of both SCAR markers was scored.

In addition, new primer sets were designed from the sequence of the amplified fragment provided by P. Miklas (USDA-ARS Prosser, WA). These new primer sets were also assayed with bean varieties known for the presence/absence of DOR21 and/or W12 SCARs. The assays were first performed with DNA extracted using the protocol of Afanador et al. (1993) and then with the alkaline method routinely used for MAS.

Results and discussion

MAS for introducing BGYMV resistance in small-seeded beans continued as reported last year, and then F_1 plants were screened for the presence of the *bgm-1* marker. Twenty-four segregant populations (BGMV code 548 to 607), corresponding to F_1 multiple crosses for drought stress and high iron content, were evaluated. Of 1794 individual plants, 722 had the *bgm-1* marker in homogeneous state and 122 in heterogeneous state. These plants were selected and will be evaluated under drought stress next season.

In addition, a set of F_6-F_7 derived families under drought stress was screened for the presence of DOR21 and W12 SCAR markers. The marker for *bgm-1* was detected in either the heterogeneous or homogenous state in 644 out of 1350 F_7 families tested. The W12 marker was detected in 585 of these families (Table 1). Both markers occurred at a similar frequency (*bgm-1*, 43% and W12, 48%). Also, the number of red- and black-seeded families having both markers was comparable: 200 (20%) and 74 (22%), respectively.

Table 1. Families expressing or lacking two SCAR markers for resistance to BGYMV in F_6-F_7 derived families.

Marker	Color Group	Heterogeneous[§]	Present	Absent	Total
bgm-1	Red	55	407	556	1018
	Black	14	168	150	332
Subtotal		69	575	706	
W12	Red	-	478	540	1018
	Black	-	107	225	332
Subtotal			585	765	
TOTAL					**1350**

[§]The *bgm-1* marker is co-dominant for the resistant and susceptible allele, which permits defining heterogeneous class, while the W12 marker is dominant and only presence/absence classes are scored.

A multiplex assay using DOR21 and the original W12 primers was conducted in four bean varieties and pure extracted DNA. Identical PCR products were obtained when comparing individual amplification of the SCARs and the multiplex (Fig. 1).

Figure 1. Multiplex using W12 and DOR21 markers for BGYMV resistance. Lane 1, W12 check; lane 2, DOR21 check showing susceptibility allele (upper) and resistance allele (lower); lane 3, lanes 4-6, Tío Canela 75, SAM1, DOR 364, EMP496, multiplex; lanes 7-10, W12 on the same set of varieties; lanes 11-14, DOR21 amplified on the same set of varieties.

Using a multiplex assay would speed up the MAS process, but a more specific W12 marker would be needed. Among six sets of designed forward and reverse primers, two sets were selected. The first yielded even more spurious bands than the original W12 oligonucleotides, but the second was more specific and produced a single band at 614 bp (Fig. 2).

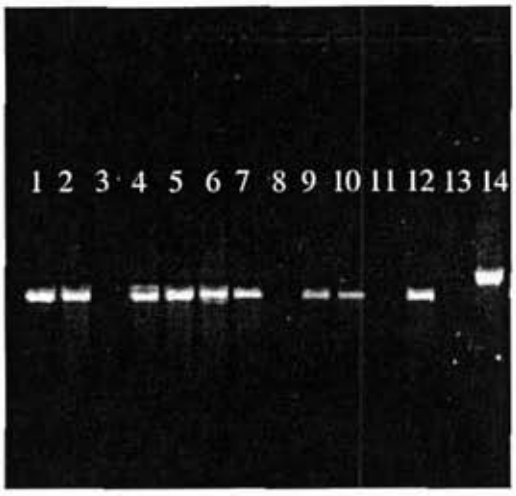

Figure 2.PCR amplification of shorter region W12 marker (614 bp). Lane 1-8, bean varieties whose DNA was extracted using the alkane method; Lanes 9-13, pure DNA checks, Tío Canela 75, DOR 364, G19833, SAM1 and EMP496. Lane 14, original W12 fragment (732 bp).

These new W12 oligonucleotides were more specific, at least in the small set of varieties selected for this study. Since no amplification was obtained in G19833 in contrast with the original oligonucleotides, it would be useful to run this new set of primers in the mapping population DOR 364 x G19833, prior to applying it for large-scale screening.

Conclusions and ongoing activities

The *bgm-1* gene is being introduced through MAS on small-seeded families also tolerant to the main biotic and abiotic constraints to bean production.

Attempts were made to bring W12 SCAR to a high-throughput screening strategy, comparable to that of DOR21. Changes in PCR profiles for the amplification of the original fragment yielded more confident results than last year assays and were started to be used.

A multiplex of both SCARs available for BGYMV resistance was successfully run in a set of bean varieties with known performance for both SCARs. Troubles with spurious bands or no amplification seemed to be overcome as a more specific set of forward and reverse primers for W12 fragment was designed.

New W12 primers should be tested in the mapping population DOR 364 x G19833.

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Acknowledgments

We would like to thank Eliana Gaitán, BRU, CIAT for providing assistance in primer designing.

1.2.7 Marker assisted selection of Arcelin-derived bruchid resistance

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Introduction

Last year we described the testing on a total of 63 genotypes (including 7 wild accessions of common bean that were the sources of the seven variants of arcelin known to exist; 28 advanced breeding lines from the bruchid resistance program (either RAZ or GG designations); and 28 bruchid-susceptible parents used in crosses with Arc1 or Arc 5 containing lines) of seven microsatellite markers that were linked to the Arcelin resistance gene which is the most effective resistance factor for the most common storage pests of common bean, namely the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman). This year, our objective was to replace the serological / protein based selection of arcelin in our Red Mottled common bean breeding program with a genetic assay using two of the microsatellites that were most closely linked to arcelin. We analyze the two markers for their ability to distinguish susceptible and resistant genotypes and individual alleles of the Arcelin gene and confirm these results with both protein assays, insect feeding tests and

replicated trials for the most resistant lines. The long-term objective of this work is to increase the efficiency of breeding for multiple constraint resistance and facilitate the pyramiding of bruchid resistance with other biotic and abiotic stress resistances. The conversion of the protein based selection of arcelin to a usable DNA marker obviates the need for arcelin-specific antibodies and protein electrophoresis and streamlines arcelin selection with the widespread use of other SCAR markers that we have also embarked on in our breeding program. In addition to its compatibility with other types of DNA based markers, the advantages of using the microsatellites over the time-consuming protein based selection was they were amenable to high-throughput and fast analysis.

Methodology

Genetic materials and Phenotyping. A set of 796 F_4 and F_5 derived advanced lines from multiple crosses between RAZ lines and susceptible parents were used in the marker assisted selection experiments. Of these, 772 were Andean genotypes from the breeding program for Red Mottled beans and 24 were Mesoamerican genotypes from the breeding program for Small Red beans. All 796 lines were tested for bruchid resistance using a single replicate of 30 seeds infested with 6 pairs of *Zabrotes subfasciatus* (Boheman) in a small mesh covered clear plastic vial whose walls were covered with sandpaper (No. 150, rough side of sandpaper facing inwards) to avoid egg-laying on the plastic surface rather than the bean seed coat. Data was collected on number of eggs, number of emerged adults and percentage emergence. Genotypes with 0 to 15% adult emergence were classified as highly resistant (HR), from 15 to 30% as resistant (R), from 30 to 50% as intermediate (I) and from 50 to 100% as susceptible (S). A replicated, confirmation test was conducted with the 60 best, highly resistant advanced lines as well as the 2 worst, susceptible lines and the check varieties, RAZ44 (resistant) and ICA Pijao (susceptible). Days to emergence and percentage of damaged seed were measured along with the above parameters using the same protocol. A total of 4 replicates were used in these trials.

DNA extraction. Two DNA extraction techniques were attempted. One was a rapid, high-throughput "microprep" method based on alkaline lysis. The other was an ammonium acetate based "miniprep". Tissue was harvested in the greenhouse as leaf disks cut with a hole-puncher for the microprep or newly emerging trifoliates for the miniprep. The DNA from the miniprep was found to be more adequate for microsatellite amplifications than the DNA from the microprep so subsequent analysis were done with this techniques.

Microsatellite markers. Two microsatellite markers were used for the marker assisted selection scheme: Pv-AG004 (X04660) and Pv-ATCT001 (M68913). Microsatellite amplifications were conducted according to standard PCR protocols. Microsatellite markers were run at 1800 volts, 120 Wats and temperature of 45C for one to two hours on 4% polyacrlamide gels and silver-stained with a recirculating tank system. Alleles were identified as reported in last year's annual report for the 63 parental materials used in multiple crosses to generate the advanced lines tested this year.

Additional Tests. In addition to the marker assisted selection screening of the 796 lines, two other tests were conducted: 1) an additional microsatellite evaluation on a blind sample of 102 genotypes for which known arcelin reactions and alleles had already been determined but for which marker genotype was unknown and 2) a protein assay was conducted to confirm the presence or absence of arcelin in the 64 genotypes selected in for the replicated confirmation test described above.

Protein extraction and arcelin determination. 0.75g of bean flour was dissolved in 250 ul of extraction buffer, vortexed and centrifuged 14,000rpm for 15 minutes. The supernatant was transferred and mixed with 50ul of cracking buffer, which was vortexed, boiled for 5 minutes, allowed to cool and centrifuged before loading 5 ul onto a stacking polyacrylamide gel. Samples were run at a constant 150 volts until the sample passed into the running gel where a constant 25 mA was maintained. Protein gels were stained for 4 to 5 hours in 120 ml of 0.25% Coomassie Blue R-250, then transferred to Destaining solutions I and II for approximately 4 to 5 hours.

Results and Discussion

The phenotyping results are reported in another section of the CIAT annual report, therefore here we will concentrate on the results of the genotyping that was carried out. Preliminary to the process of marker assisted selection we carried out a parental survey, in which we found the number of alleles and level of polymorphism present for each of the seven microsatellites evaluated. In this parental survey, two microsatellites were found to be more tightly associated with the Arcelin gene: of these the microsatellite Pv-AG004 (X04660) was more polymorphic than Pv-ATCT001 (M68913) presenting 6 alleles versus 2 alleles, respectively. For both markers, unique alleles were found for the parent that provided Arcelin 1 (G12882) giving a diagnostic test for this widely used allele of the Arcelin gene. Although this marker produced multiple bands, the pattern of bands produced was diagnostic for the G12882 allele and for resistant genotypes derived from this source, producing bands that were 184, 195 and 207 bp long, while susceptible genotypes had bands that were 207 and 245 or 207 and 285 bp long. Furthermore for Pv-AG004 (X04660) there was also a unique allele for the parent that provided Arcelin 5 (G02771) that was different from the Arcelin 1 associated allele. This banding pattern consisted of fragments that were 184, 203 and 207 bp long. Meanwhile, the other marker, Pv-ATCT001 (M68913), could not distinguish between susceptible genotypes and Arcelin 5 containing genotypes. This marker produced single amplification products were the resistant allele of Arcelin 5 was associated with the 190 bp band while the susceptible allele of Arcelin 5 was associated with the 195 and/or 200 bp bands. It was decided that both markers would be used to screen the full set of advanced lines.

In the marker assisted selection tests with Pv-AG004 and Pv-ATCT001, the percentage of correctly identified susceptible genotypes was higher (95.4 and 94.9%, respectively) than the percentage of correctly identified highly resistant or resistant genotypes (60.2 and 62.0, respectively). Both markers were more accurate in eliminating susceptibles than in

distinguishing within genotypes that were likely to contain the Arcelin 1 allele, whether they would be highly resistant, resistant or intermediate. Chi-square tests confirmed the linkage between the markers and the Arcelin resistance gene.

The blind test using the Pv-AG004 marker showed the capacity to distinguish whether a genotype had Arcelin or not, but also what type of Arcelin it had as well (Table 1). The accuracy level was 100% for the absence of Arcelin and 81.4% for the presence of Arcelin. As mentioned above this marker was able to distinguish those materials containing Arcelin 1 allele versus those containing Arcelin 5 allele. These results confirm that linkage disequilibrium exists between this Phytohemaglutinnin gene microsatellite and the Arcelin locus.

As marker assisted screening proceeded, improvements were made in the experimental technique resulting in a successful analysis of 92.8% of the DNA samples analyzed for Pv-AG004 (833 genotypes) and 85.6% for Pv-ATCT001 (769 genotypes). The remaining DNA samples did not amplify well and were therefore not scored. It was noteworthy that higher quality DNA was needed for the microsatellite amplification and this slowed down the process compared to SCAR screening which proceeds normally with alkaline extraction microprep DNA. The miniprep extraction technique produced on average 200 ul of 60ng/ul DNA which was more than sufficient for the PCR reactions realized and did have the advantage of being storable for a long period of time allowing screening to proceed over a period of four months, given the need for DNA extraction, PCR amplification and gel electrophoresis of the large number of samples.

The confirmation test using arcelin protein analysis with 60 highly resistant and 2 susceptible advanced line genotypes plus controls also showed a high level of accuracy. Resistance was associated in its entirety with the presence of the heavy 35Kda band that represents arcelin 1 in the seed protein extract. In the replicated insect resistance trial some variability was observed for the arcelin protein containing genotypes (where a few were re-classified from HR to only R).

Future Plans

- Improve the efficiency of the screening technique, adapting the microsatellite amplification to the alkaline extraction technique.
- Test the remaining two microsatellite markers in the region of the arcelin locus.
- Determine the level of linkage disequilibrium between the markers and the arcelin locus in breeding populations.
- Use the markers to select for greater recombination around the arcelin locus and break the linkage drag associated with this locus which has a negative affect on plant vigor of arcelin-derived lines.

Table 1. Results of the blind test with 102 common bean genotypes evaluated for the markers Pv-AG004 and Pv-ATCT001.

Hypothesis tested	Pv-AG004	Pv-ATCT001
Correct in absence of Arcelin	100	100
Correct in presence of Arcelin	81.5	80.7
Incorrect in presence of Arcelin	18.5	19.3
Correct in presence of Arc 1	82.6	86.36
Incorrect in presence of Arc 1	17.4	13.6
Correct in presence of Arc 5	100	0
Incorrect in presence of Arc 5	0	100

1.2.8 Generation means analysis of climbing ability in common bean crosses

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Introduction

Climbing beans are an important component of traditional and modern agriculture in highland areas of Central and South America. In Colombia, where production is limited to areas above 2000 masl, highly productive trellis systems are used that can produce yields of over 4 tons/hectare. New varieties are in demand as production of climbing beans is increasing in the Andes. One of the basic characteristics needed for the breeding of new varieties of climbing beans is their ability to accumulate biomass and height quickly over the season, which significantly affects yielding ability of climbing beans. In this study, our objectives were to identify the genetic components of climbing ability, plant height and internode length, through a generation means analysis of crosses between indeterminate bush and climbing beans and to evaluate the correlation of these traits with yielding ability and yield components.

Materials and Methods

Genetic Material. The F1, BC1P1, BC1P2 and F2 generations were obtained by standard hybridization techniques for three crosses between parents with contrasting growth habits and different genepools: G2333 x G19839, Tio Canela x G2333 and BRB32 x BRB1448, where G2333 is a Mesoamerican type IVa climbing bean, BRB1448 is an Andean type IVa climbing beans, Tio Canela is a Mesoamerican type IIa bush bean, BRB32 is a type IIb

Andean bush bean and G19839 is type IIIa Andean bush bean. Note that only indeterminate growth habits were used for all the crosses.

Field trials. Field experiments were conducted in Darién, Valle (1500 masl, 19°C, 1200 mm average rainfall) in the 2003A season. For each cross a Randomized Complete Block Design was used with six treatments consisting in parent (P1 and P2) and offspring (F1, F2, BC1P1 and BC1P2) generations and 3 repetitions for each cross. The P1 and P2 treatments consisted in 20 plants each, while the BC1P1 and BC1P2 treatments had 30 plants each. The F2 treatments had a total of 80 plants per repetition. The three crosses were planted in three separate but adjoining experiments. The variables that were evaluated on a plant-by-plant basis were Plant Height (PH) and internode length (IL). This was done at 40 and 70 days after planting (dap) for all three populations. For the Mesoamerican x Mesoamerican and Andean x Andean crosses an additional reading of plant height was realized at 90 dap. At harvest, yield component data (raceme length, number of pods per raceme, number of seed per pod, and 100 seed weight) were taken.

Table 1. Crosses made for the Generation Means analysis of plant height and internode length in climbing beans

Cross	Growth Habits	Genepools
G2333 x G19839	IVa x IIIa	Mesoamerican x Andean
Tiocanela x G2333	IIa x IVa	Mesoamerican x Mesoamerican
BRB32 x SEL1448	IIa x IVa	Andean x Andean

Agronomic management. The trials were protected with two preventative fungicide treatments at planting and again at flowering, and one insecticide treatment to control thrips and whitefly. The cropping systems consisted in a bamboo and wire trellis, with strings tied to individual plants within the plot. The trellis had a height of 2 m.

Data Analysis. Results were analyzed using the Generation Means model of Mather and Jinks (1971). Sums of squares and determination coefficients were estimated for additive and additive x dominance models for generation means analysis of plant height and internode length at 40, 70 and 90 days after planting in the three populations. Genetic parameters were based on variances (s^2) for each generation estimated with the formulae: $s^2E = (s^2P1 + s^2P2 + 2s^2F2)/4$; $s^2G(F2) = s^2F2 - s^2E$; $s^2A(F2) = 2s^2F2 - (s^2$ BC1P1 + s^2 BC1P2) where s^2E was environmental variance, s^2G was genotypic variance and s^2A was additive variance, while other variances corresponded to the indicated generations (F1, F2, BC1P1 and BC1P2). Broad sense and narrow-sense heritabilities (h^2) were estimated based on genetic variance values using the formulae: h^2(broad-sense) = 100 x $s^2G(F2)$ /s^2F2 and h^2(narrow-sense) =100 x $s^2A(F2)$ /s^2F2. Data obtained from the single plant scoring of F2 plants was used for evaluating partial correlations between yield components and climbing ability.

Results and Discussion

In the G2333 x G19839 cross, generation means analysis for plant height in the first reading at 40 dap showed a better fit to an additive model of inheritance (R^2=95.8%), while for the data collected at 70 dap for this cross, the inheritance of plant height had a better fit to an additive-dominant model (R^2=99.5%) over just an additive model (R^2=88.7%). These results suggest a) that additive effects on plant height are more important than dominance effects in this cross and b) that the dominance effects are expressed later in the season than the additive effects (goodness of fit test, Table 2). The results for internode length were very similar, with an additive model evident in the first reading at 40 dap and an additive-dominant model at the second reading at 70 dap. This was not surprising given that the two traits have been seen to be associated in our previous studies (CIAT, 2002). Likewise in the Tio Canela x G2333 cross, the same pattern of inheritance held true for both traits with additive-dominant models for both second and third readings, while in the BRB32 X SEL1448 cross, dominance effects were evident in all three readings, although additive effects continued to be more important explanations for total variance.

Narrow-sense heritability values for plant height at the three growth intervals and in the three populations varied from 52.53 to 79.63% (Table 3). Similar narrow sense heritability values were observed for internode length except in the case of the second reading for the population BRB32 X SEL1448. These results suggest that both characteristics are not very influenced by the spatial environment within a single site and probably are controlled by a few genes. Interactions of these two traits with soil fertility, photoperiod and temperature would of course be interesting to test but are difficult when analyzing an experiment with hybrid seed such as this generation means analysis.

Partial correlations were significant between height and internode length in all three populations suggesting that genepool source of climbing ability does not affect this association (Table 4). In two populations, G2333 x G19839 (Meso x Andean) and Tio Canela x G2333 (Meso x Meso), raceme length was significantly correlated with plant height. Therefore it may be postulated that longer racemes are an inherited character from G2333. No such correlation was found in the BRB32 x SEL1448 (Andean x Andean) population where less variability existed for raceme length. The number of vines was also significantly correlated with plant height in the G2333 x G19839 population however the correlation value was low (r=0.172) and the results were not consistent in the other two populations.

Several correlations were observed between yield components and between yield components and climbing bean ability. The number of pods per raceme was positively associated with the length of the raceme in all three populations (r=0.156 to 0.618). Number of seed per pod was also positively correlated with pod length in all three populations (r=0.361 to 0.713). However larger number of seeds per pod was associated with smaller seed in two populations, G2333 x G19839 and Tiocanela x G2333 (r = -0.500 and –0.440 respectively) although this was not observed in the Andean x Andean

population, BRB32 x SEL1448, possibly because the parents were less contrasting in seed weight than the parents of the other two populations.

Plant height presented significant correlation with 100 seed weight in the Andean x Andean (BRB32 x SEL1448) and Meso x Meso (Tio Canela x G2333) populations but not in the Meso x Andean (G2333 x G19839) population. However correlation coefficients were generally low (r=0.175 and 0.222, respectively) suggesting that within genepools there is only a slight tendency for higher, more aggressive plants to produce larger seeds. On the other hand, that when genepools are combined this tendency is overshadowed by the innately larger seed of the Andean genepool and the separate inheritance of this quantitative trait. Finally, plant height was highly correlated with pod length and number of seeds per pod in the Andean x Andean (BRB32 x SEL1448) population but not in the other populations possibly due to the large differences between parents in this population only. The inferences made from these populations will need to be proven in additional populations to make conclusions about the association between these traits across the multiple races and genepools found in cultivated common bean.

Conclusions and Future Plans

This is one of the first studies to investigate the inheritance of climbing ability in common bean through generation means analysis. Apart from the determinacy and photoperiod response genes, no other loci, which affect plant architecture in climbing beans, have been studied. This study is attempting to determine the inheritance of climbing ability and to better understand the consistency of additive and dominance effects across several populations.

Table 2. Sums of squares and determination coefficients for generation means analysis of plant height and internode length at 40, 70 and 90 days after planting in three crosses of common bean a) G2333 x G19839, b) Tio Canela x G2333 and c) BRB32 x SEL1448

a)

Model	PH (40)		PH (70)		IL (40)		IL (70)	
	SS	R^2	SS	R^2	SS	R^2	SS	R^2
M	6315.7		9995.4		8119.8		13927.0	
m [add.]	9376.3	95.8	12796.7	88.7	10010.6	96.1	15420.3	86.9
m[add.] [dom.]	9433.7	99.3	13136.1	99.5	10071.6	99.2	15624.6	98.8
Total SS	9509.5		13153.2		10087.4		15645.2	
Goodness of fit [dom.] – F value	2.27^{ns}		59.98^{**}		7.02^{ns}		29.91^{**}	

b)

Model	PH (40)		PH (70)		PH (90)		IL (40)		IL (70)	
	SS	R^2	SS	R^2	SS	R^2	SS	R^2	SS	R^2
M	3355.9		7453.0		8196.3		8359.8		8834.1	
m [add.]	6742.2	97.6	11148.3	93.7	12370.2	93.8	11937.2	91.9	11028.4	93.4
m[add.] [dom.]	6753.3	99.7	11362.2	99.4	12623.2	99.6	12173.8	97.9	11170.4	99.5
Total SS	6820.1		11397.9		12643.9		12254.1		11182.3	
GF [dom.] – F value	0.50^{ns}		17.95^{*}		36.72^{**}		8.33^{ns}		35.57^{**}	

c)

Model	PH (40)		PH (70)		PH (90)		IL (40)		IL (70)	
	SS	R^2	SS	R^2	SS	R^2	SS	R^2	SS	R^2
M	6330.1		7766.8		11074.0		7014.9		9693.6	
m [add.]	7735.7	97.7	8842.2	94.7	11405.2	82.4	7990.0	99.3	10999.6	98.6
m[add.] [dom.]	7764.1	99.7	8900.7	99.9	11474.6	99.7	7991.0	99.4	11003.2	98.8
Total SS	7768.7		8902.2		11475.8		7997.0		11018.6	
GF [dom.] – F value	18.18^{*}		121.95^{**}		182.6^{**}		0.46^{ns}		0.70^{ns}	

*, ** and *** represent significance levels of P=0.05, 0.01 and 0.001, respectively.

Table 3. Genetic parameters and estimates of broad and narrow-sense heritabilities for three F2 populations.

Parameter	Population	PH (40)	PH (70)	PH (90)	IL (40)	IL (70)
Genotypic Variance	G2333 x 19839	0.174	0.386		16.98	15.60
	Tiocanela x G2333	0.085	0.334	0.360	10.83	13.00
	BRB32x SEL1448	0.170	0.519	0.440	36.58	13.73
Additive Variance	G2333 x G19839	0.143	0.252		13.65	14.71
	Tiocanela x G2333	0.071	0.330	0.340	9.33	12.70
	BRB32 x SEL1448	0.142	0.480	0.360	36.17	4.02
Broad-sense Heritability	G2333 x G19839	81.45	85.60		76.80	79.80
	Tiocanela x G2333	62.32	80.64	83.31	68.72	71.24
	BRB32 x SEL1448	78.30	81.30	73.36	83.56	66.54
Narrow-sense Heritability	G2333 x G19839	66.95	56.03		61.71	75.26
	Tiocanela x G2333	52.53	79.63	80.66	59.19	69.62
	BRB32 x SEL1448	65.42	75.27	60.07	82.63	66.54

Table 4. Partial correlations between climbing ability and yield and yield components in the F2 populations from the crosses a) G2333 x G19839, b) Tio Canela x G2333 and c) BRB32 x SEL1448.

a)

Trait	PH	IL	RL	VN	P/R	PL	S/P	100S
Plant Height (PH)	1	0.556**	0.287**	0.172**	0.126	0.099	-0.083	-0.086
Internode length (IL)		1	0.002	-0.016	0.118	-0.004	0.052	-0.003
Raceme length (RL)			1	0.136*	0.156*	0.003	0.025	0.135*
Vine no. (VN)				1	0.079	-0.051	0.118	0.049
Pods/raceme (P/R)					1	-0.030	0.0216	0.001
Pod length (PL)						1	0.713**	0.499**
Seed/pod (S/P)							1	-0.505**
100s weight								1

b)

Trait	PH	IL	RL	VN	P/R	PL	S/P	100S
Plant Height (PH)	1	0.738**	0.400**	0.029	-0.010	0.151	0.154	0.222**
Internode length (IL)		1	-0.178*	0.027	0.023	-0.072	0.005	-0.055
Raceme length (RL)			1	-0.026	0.618**	0.048	0.020	-0.143
Vine no. (VN)				1	0.247**	-0.018	0.085	0.070
Pods/raceme (P/R)					1	0.039	-0.013	-0.0349
Pod length (PL)						1	0.505**	0.409**
Seed/pod (S/P)							1	-0.445**
100s weight								1

c)

Trait	PH	IL	RL	VN	P/R	PL	S/P	100S
Plant Height (PH)	1	0.476**	0.041	0.126	0.260**	0.139*	0.235**	0.175**
Internode length (IL)		1	0.119	-0.064	0.081	0.114	0.009	0.028
Raceme length (RL)			1	0.050	0.347**	-0.020	0.074	0.119
Vine no. (VN)				1	-0.050	-0.089	0.213**	0.187**
Pods/raceme (P/R)					1	-0.019	-0.041	-0.040
Pod length (PL)						1	0.361**	-0.018
Seed/pod (S/P)							1	-0.060
100s weight								1

*, ** and *** represent significance levels of P=0.05, 0.01 and 0.001, respectively.

1.2.9 Adaptation and use of SCAR markers for the BCMV resistance gene *bc*-3 in the Andean bean breeding program

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Introduction

Although virus resistance can be screened phenotypically, the frequency of escape, the complex interaction of multiple genes and the recessive nature of most of these makes marker assisted selection (MAS) the best option for rapidly breeding resistant varieties. This is especially true in the case of climbing beans which are longer season and more expensive to produce than bush beans and therefore can proportionally benefit from MAS derived savings in the time and expense of producing a fully tested variety. Climbing beans are grown in both intensive (trellised/staked monoculture) and extensive (inter-cropping with corn) farming systems. In either system the need to protect the crop from diseases is great, especially seed borne and easily transmitted viral diseases such as bean common mosaic virus (BCMV or BCMNV). A number of BCMNV resistance genes have been tagged including the dominant *I* gene, as well as the recessive *bc3*, *bc2* and *bc1-2* genes.

The genes can be distinguished by inoculation with different viral isolates. BCMNV resistance is very important in Africa were necrotic strains are prevalent: Therefore, it is important to incorporate recessive resistance based on the *bc-3* gene into varieties for most parts of the continent. Although most BCMV and BCNMV resistance genes have been tagged with SCAR markers very little work has been done to validate and utilize the markers in applied breeding programs. Therefore the objective of this research was to validate a SCAR marker for the most important BCNMV resistance gene, *bc-3*, and use the marker to introgress this gene into new bush bean and climbing bean germplasm being produced at CIAT. One target of the breeding effort are the MAC lines that we have recently developed and distributed to East Africa. These are mid-altitude climbing bean (MAC) lines which are more heat tolerant and higher yielding than many traditional climbing bean varieties and have great promise for the region.

Materials and Methods

Triple crosses and backcrosses were made between BCMV resistant, *bc3*-containing, bush-type small red or red mottled beans and a series of mid-altitude climbing (MAC) bean advanced lines bred recently at CIAT as well as the landraces G2333 and G685. The MAC climbing beans included red and cream mottled selections, namely SEL1445, 1446, 1447, 1448, 1452, 1453 and 1454. The BCMNV sources included BRB29, BRB 32 and BRB191. Selection were made based on:

*Viral inoculations.*were carried out on the F1:2 bulk seed in the greenhouse using a necrotic strain of BCMNV to evaluate viral resistance or susceptibility reactions. After approximately 10 days, the plants were scored for necrotic hypersensitivity I gene resistance (N), susceptible mosaic symptoms (M) or immune resistance response indicating presence of the bc3 gene (0).

Genotyping. was carried out on young bean plants in the field before flowering. Vegetative tissue was collected for alkaline DNA extraction from four leaflets from four individual plants per line using a hole-puncher. The leaf disks were placed directly into 96 well plates that were kept on ice while in the field. Tissue was kept frozen at –20 °C until use. Alkaline extraction involved adding 60 µl of 0.25M NaOH to the samples, heating the 96 well plates to 100 °C for 2 minutes, neutralizing with 60 µl 0.25M HCl, and 30 µl of 0.5M pH 8.0 Tris- HCl buffer and heating again to 100 °C for 2 minutes. This extract was transferred to a clean 96 well plate and diluted 1:1 with sterile water. A total of 5 ul of this dilution was used for PCR amplification of two sequence characterized amplified region (SCAR) markers: ROC11 used to detect the *bc*-3 resistance gene (Johnson et al. (1997) and SW13 used to detect the dominant *I* gene (Melotto et al., 1996). PCR products were visualized on 1.5% agarose gels run in 0.5X TBE buffer. Up to three loadings were used per 42 well comb and products were run for 45 minutes at 150 volts. Gels were photographed for scoring. The presence or absence of PCR products was evaluated for each genotype to determine if the genotype was likely to contain the resistance allele or the susceptible allele.

Results and Discussion

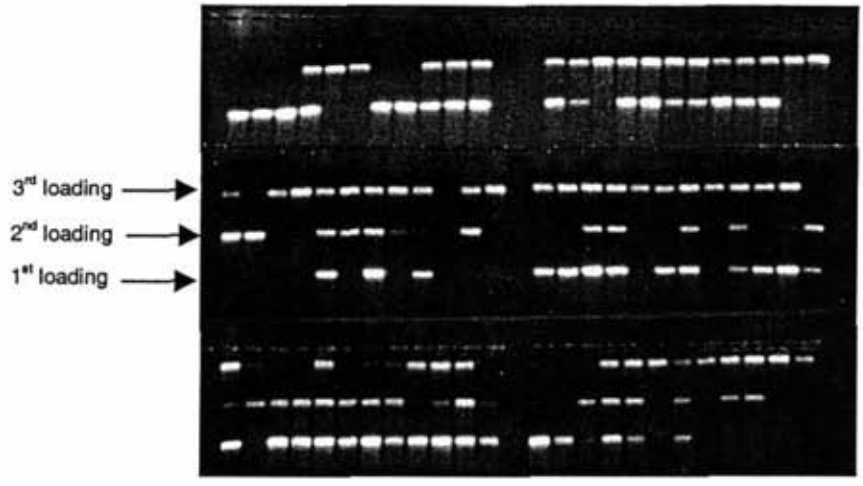
Breeding methods. The bush bean resistance sources were crossed with most of the climbing beans and the F1 hybrids were either backcrossed or crossed to a second climbing bean as indicated in Table 1. The pedigrees segregating for BCMV resistance were single plant harvested and their progeny screened for resistance in the greenhouse. The F3 families were planted and mass selection was practiced for the best individual plants of each family.

Phenotypic screening. Among the crosses segregating for BCMNV resistance, a total of 97 selection were made in the greenhouse from the 228 seed samples that had been selected from within the F1:2 bulks. These samples were separated based on seed color, since some of the bulks were mixed. Any families that were positive or had good seed type were planted in the field as F3 families in 2002A. BCMV was prevalent in the field that semester and resistance observed in the field in the F3 generation agreed well with the results for the F2 families tested in the greenhouse. The natural epidemic also allowed us to screen for resistance in the field.

Genotyping. ROC11 marker is dominant (a band is either present or absent) and in repulsion for the *bc*-3 gene. Furthermore ROC11 is a single-copy SCAR marker which

does not produce extra bands. Therefore in advanced generations, it is easy to score the resistant and susceptible genotypes based on presence/absence of the PCR product / band as shown in Figure 1. The marker and the gene are tightly linked, although some cross-over events have been observed.

Figure 1. The SCAR marker ROC11 used to evaluate the *bc-3* resistance gene in .



Efficiency of marker assisted selection was increased by multiplex loading of agarose gels (first with two and then three loadings), increasing numbers of wells per comb (first 30 well and then 42 well combs were used), use of 384 well PCR plates and multipipettor loading of gels. The resulting savings decreased the time to PCR amplify and load a gel by approximately 50% and increased the number of genotypes run per gel by 225%.

Parental survey: Parental surveys were conducted to determine the genotype and expected genotype for the SCAR markers. A total of 74 parents involved in the crosses described above were evaluated with both viral inoculations and the SCAR markers to determine if they contained the recessive *bc*-3 or dominant *I* resistance genes and the expected marker alleles (Figure 2, Tables 2 and 3). Polymorphism was easily observable in the Andean genepool for the *bc*-3 gene, since the majority of susceptible Andean genotypes produce a band for the ROC11 SCAR, while the majority of *bc*-3 resistant Andean genotypes do not produce a band for ROC11. Likewise, the band for the SW13 SCAR for the dominant *I* gene is mainly present in resistant parents (Figure 3, Table 3). Therefore in Andean breeding populations segregation for presence or absence of the SCARs was expected to predict well the presence or absence of the resistance alleles.

Figure 2. Parents evaluated in double loading for the ROC11 SCAR marker for *bc-3* resistance

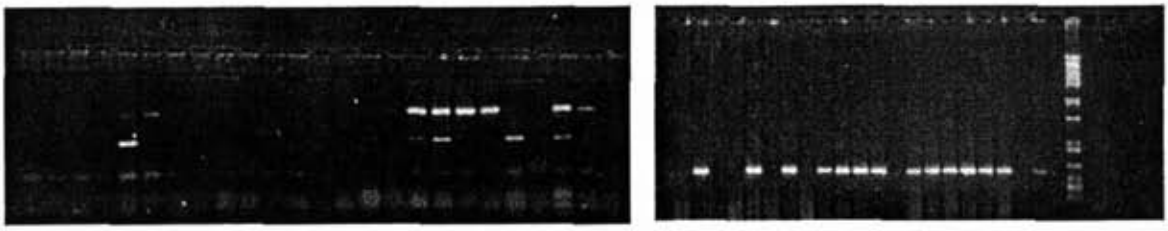


Table 2. Parents evaluated for the BCMNV virus resistance in the greenhouse and for the SCAR marker for the *bc-3* resistance gene.

Parents	SCAR ROC 11	bc-3 Gene	Virus response (Greenhouse)			Parents	SCAR ROC 11	bc-3 Gene	Virus response (Greenhouse)		
			N = necrosis	M = mosaic symptoms	O = bc-3 resistance				N = necrosis	M = mosaic symptoms	O = bc-3 resistance
AFR298	A	+	-	-	15	G12582	A	+	-	11	-
BRB029	A	+	-	-	15	G12727	A	+	-	12	-
BRB032	A	+	1	-	14	G19833	P	-	-	15	-
BRB151	A	+	-	-	14	G2333	A	+	-	14	-
BRB181	P	-	-	-	15	G23604	A	+	-	14	-
BRB183	P	-	-	-	14	G23614	A	+	-	11	-
BRB189	A	+	-	-	15	KABOON	A	+	-	10	-
BRB197	A	+	-	-	14	MAM49	A	+	17	-	-
BRB203	A	+	-	-	15	MDRK	A	+	-	14	-
BRB204	A	+	-	-	15	MICHELITE	A	+	-	14	-
BRB211	A	+	-	-	15	PVA773	P	-	-	15	-
BRB217	A	+	-	-	15	S31465	A	+	-	15	-
CAL096	A	+	-	12	-	SEL1445	P	-	-	15	-
CAL143	P	-	-	12	-	SEL1446	P	-	-	15	-
CALIMA	P	-	-	12	-	SEL1447	A	+	7	6	-
COS16	P	-	14	-	-	SEL1448	P	-	12	-	-
DOR476	P	-	15	-	-	SEL1449	P	-	-	14	-
DOR482	P	-	10	-	-	SEL1450	A	+	-	14	-
EMP122	P	-	-	14	-	SEL1451	P	-	-	15	-
EMP250	P	-	14	-	-	SEL1452	A	+	-	15	-
EMP320	A	+	-	15	-	SEL1453	A	+	-	11	-
EMP364	P	-	14	-	-	SEL1454	A	+	-	15	-
EMP496	P	-	14	-	-	SEQ1033	A	+	-	15	-
G685	A	+	-	14	-	SEQ1040	A	+	15	-	-
G11785	A	+	-	6	-	TO	A	+	15	-	-
G12572	A	+	-	13	-	UPR9745226	A	+	15	-	-

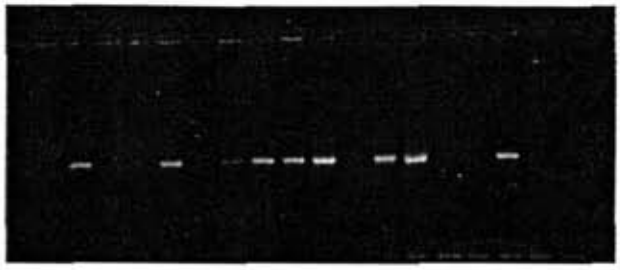


Figure 3. Parents evaluated for SCAR SW13 for the dominant *I* gene for BCMV resistance

Table 3. Additional parents evaluated for the *I* and *bc-3* resistance genes and ROC11 and SW13 SCAR markers.

Padre	Origin	*bc-3* reaction	*I* gene reaction	Marker ROC11	Marker SW13
G10909	Mesoamerican	+	-	A	A
G855	Mesoamerican	-	-	P	A
G22036	Mesoamerican	+	-	A	P
G22041	Mesoamerican	+	-	A	A
BRB191	Andean	-	-	P	A
BRB29	Andean	+	-	A	P
CAL143	Andean	-	-	P	A
MAM38	Mesoamerican	+	+	A	P
POA12	Andean	-	+	P	P
SUG131	Andean	-	+	P	P
SUG137	Andean	-	(+)	P	P
PVA1441	Andean	-	+	P	A
AFR619	Andean	+	-	A	P
SEL1448	Andean	-	-	P	P
CALIMA DAR	Andean	-	???	P	A
SEL1449	Andean	-	-	P	A
SEL1445	Andean	-	-	P	P
S31465	Andean	-	-	P	A
SEL1446	Andean	-	-	P	A
BRB32	Andean	+	-	A	-
BRB198	Andean	+	-	(T)	-
G2337	Mesoamerican	-	-	-	-
MAM49	Mesoamerican	???	(+)	-	-
SEA15	Mesoamerican	???	(+)	-	-

Marker assisted selection of breeding lines. 2002B: In this season we worked out the technical practicalities of applying the ROC11 marker in the breeding program to gear up for the following semester. We also analyzed which populations with which pedigrees segregated for ROC11 as expected. A total of 180 F4 lines were evaluated for the ROC11 marker at CIAT headquarters (Table 4). Of this total, 139 lines were climbing beans and 41 lines were bush beans. A total of 90 *bc*-3 positives were diagnosed with the SCAR marker.

2003A: In this season we applied the SCAR marker on a mass scale at two locations and tested the efficiency of marker assisted selection for the *bc*-3 gene. A total of 1923 F4 lines were evaluated for the ROC11 marker both at CIAT headquarters (Location 1) and at an off-station breeding site (Location 2) used by our breeding program (Table 5). Of this total and across both locations, 1481 lines were climbing beans (246 from simple crosses and 1235 from triple crosses), while 442 lines were bush beans. A total of 350 of these same lines were also evaluated for a second marker (data not shown) showing the potential for multiple screening for pyramiding resistance genes.

Table 4. Climbing bean advanced lines evaluated for *bc-3* marker in semester B 2002.

Location	Type of cross	ROC11 evaluation		Total Climbers	ROC11 evaluation		Total Bush
		+	-		+	-	
Location 1 (D)	Triple crosses with BRB32			86			
	Triple crosses with BRB29			43			
Total lines evaluated for *bc*-3		82	50	139	8	33	41

Table 5. Climbing and Bush bean advanced lines evaluated for *bc-3* marker in semester A 2003.

Location	Type of cross	Climbers		Total	Bush		Total	Total		Total lines
		+	-	Climbers	+	-	Bush	+	-	evaluated
Location 1 (D)	Simple Crosses	142	104	246	----	----	----	142	104	246
	Triple Crosses	386	275	661	90	2	92	476	277	753
	Total Lines / location			907			92			999
Location 2 (P)	Simple Crosses	----	----		----	----		0	0	0
	Triple Crosses	100	474	574	159	191	350	259	665	924
	Total Lines / location			574			350			924
Grand total										1923

Conclusions and Future Plans

Gamete selection was applied to the F1 plants from these triple crosses based on the marker results, resulting in a savings of 50% of the individual selections that would be

made if the plants had been saved for phenotypic evaluation. The populations were then advanced by mass and pedigree selection to the F4 and F5 generation when single plant selections were made and evaluated for the resistance gene. In the meantime, an additional group of populations made from the simple crosses between virus resistant bush bean parents and susceptible climbing bean parents is being advanced by mass selection through the F3 and F4 generations for good climbing bean genotypes. When we increase seed of the F5 lines and make individual selections from the new F4 populations in the upcoming year we will validate the fidelity of marker assisted selection by testing whether the selections have the virus resistance gene or not. The rapid increase in efficiency obtained during the application of the ROC11 marker shows the advantages of testing new markers in practicing breeding programs.

1.2.10 Identification of an important QTL for symbiotic nitrogen fixation (SNF) in Mexican environments

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Funding by BADC through the VLIR program, in collaboration with the Catholic University of Leuven, Belgium.

Introduction

Genetic maps combined with phenotypic data can serve to identify markers for economic traits to be used for Marker Assisted Selection. Maps based on locus-specific markers such as microsatellites are superior since they permit comparative mapping of traits, are typically more stable and repeatable than other markers such as RAPDs, and can serve as a framework within which to map other markers such as RAPDs and AFLPs. Last year we reported on the evaluation of a number of microsatellites on the parents to determine polymorphism, and the mapping of nineteen microsatellites on the map of DOR 364 x BAT 477.

Methods

A partial map of BAT 477 x DOR 364 based on RAPD has existed for several years. In the course of the year we continued with the effort to improve the map, amplifying another 29 microsatellites on the mapping population. To develop the linkage map of DOR 364 x BAT 477 the data were first analyzed with the program MAPMAKER 3.0. Linkage groups were established using as a referente mapped microsatellite markers that had been mapped on the populations BAT93 x Jalo EEP558 and DOR364 x G19833. Each marker was assigned to a group with a minimum LOD score (probability that a marker is linked to a specific position) of 3.0, which is to say, a probability of 1 in 1000.

A subset of the 94 Recominant Inbred Lines (RILs) were selected and planted in multiple locations, to verify if QTL that were expressed in the greenhouse in previous years did in fact express under field conditions. Thirty-six lines and checks were planted in Mexico again in 2002 (as in 2001) and in Darién, Colombia with stress from low P and without stress. Yield data served as phenotypic data for the QTL analysis. Identification of QTLs was carried out using simple linear regresión, or Single Point Análisis (SPA) con the program Q-Gene, which permits determining the level of significance between the molecular markers and the quantititative characters. At the same time a second analysis was implemented by composite interval mapping (CIM) with the program QTL Cartographer 2.0 to determine the location and effect of QTLs. The probability of the presence of a QTL was expressed as LOD and the threshold value for recognizing a QTL was fixed at 2.8.

Results

Map development. At present 190 microsatellite primers have been evaluated on the parental genotypes DOR 364 and BAT 477 of which approximately 25% (48 primers) have presented polymorphism. These forty-eight microsatellites have been amplified and read on the 132 RILs, these corresponding to 30 co-dominant and 4 dominant markers. The linkage map of DOR 364 x BAT 477 contains at the moment 185 markers, of which 137 are RAPDs, and the 48 remaining are microsatellites. Of these markers 130 (96 RAPDs and 34 micros) are already mapped and distributed in 10 linkage groups covering a total distance of 515 cM.

Identification of QTLs. A total of eighty-two phenotypic characters were analyzed with the programs Q-Gene and QTL Cartographer to determine their level of association with the 130 mapped markers. With the QTL Cartographer program it was possible to identify 22 markers for 17 characters on six linkage groups. The SPA analysis identified 26 characters associated with 29 markers in three linkage groups, which explain more than 15% of the phenotypic variation. Other QTL were identified with a smaller effect, but these were examined for their consistency across sites, especially those associated with yield and SNF characters.

Some of these QTL had been identified in previous studies of SNF under different phosphorus regimes, in a search for genes for SNF that represented tolerance to low P. The present analysis was repeated with yield data taken in the field in Mexico, under different moisture regimes, and in Colombia under both moisture and low P stress. The intention was to find QTL that were apparently associated with low P tolerance of SNF and that impact on yield. Several QTL identified in the greenhouse had an effect in the field and of these, one of the QTL appears to be especially interesting, for reasons specified below:

1) QTL on chromosome B02, in the vicinity of RAPD V1701 and microsatellite CLON1598: This QTL was associated with parameters of phosphorus use efficiency (PUE) for SNF as well as biomass accumulation in the greenhouse trial. It is also

expressed in several (not all) yield trials, both in Mexico and in Colombia. It is normal that QTL do not express in all sites, rather it is encouraging that a QTL associated with SNF in the greenhouse is in fact carrying over to the field. In the field this QTL gave a yield advantage of 15-49 kg/ha grain yield, with an average of 33 kg/ha (Table 1). This is a rather modest absolute increase but considering that this increase occurred in some heavily stressed trials with low yields (600 kg/ha or less), it represents an average of 5% and as much as 8% of yield, which is quite respectable for a single SNF gene. It is noteworthy that this yield level is in fact quite common and is representative of much of bean production in Mexico. If this yield advantage were extended to some 200,000 ha, which is the area occupied by improved varieties in the Mexican highlands, it could represent an additional 8,000,000 kg of grain, or about $2,000,000 per year. This is the most interesting QTL and we will want to look at RILs with and without this QTL to see possible interactions with the bacterium.

2) QTL on chromosome B03: This QTL also was associated with some greenhouse traits associated with P use efficiency, and with yield in the field, but sometimes it gave a positive effect on yield and sometimes a negative effect! This region also appears to be associated with longer growth cycle, and in spite of its effect on P use efficiency in the greenhouse, its yield effect may be associated with days to maturity. In other words, longer growth cycle results in better yield, *except* in drought trials where long growth cycle leads to lower yield. Hence its positive effect in some environments, and negative effect in others. Nevertheless, given its effect on PUE in the greenhouse, we have identified some RILs that possess this QTL for further study

3) QTL on chromosome B09: This QTL had a very marked effect in the greenhouse on PUE and biomass accumulation, but in the field *only* gives negative associations with yield. It is also associated with growth cycle but its pattern is not as consistent as the case of 2) above. It is also included for further study, for contrast with the other QTL mentioned above.

Conclusion

In the past year we have made significant progress toward the tagging of genes for SNF that are associated with tolerance of low P and that contribute to yield in field conditions. The improved SNF potential associated with this QTL will be introduced to commercial cultivars, with the expectation that this can increase yields by at least 5% under stress conditions. Gains that have been observed reflect SNF with native strains, and our hope is that more dramatic gains can be realized with improved strains. RILs with the CLON1598 QTL are being delivered to colleagues for studies of the interaction with modified strains.

Table 1. Effect of the CLON1598 yield QTL in several environments in Colombia and Mexico.

	Yield increasing effect (kg / ha)	Trial average (kg / ha)
Darién, Colombia (high P)	27	3435
Darién, Colombia (low P)	47	714
Darién, Colombia (low P)	18	630
Palmira, Colombia (drought)	27	560
Celaya, Mexico (unstressed)	38	1050
Cotaxtla, Mexico	32	818
Isla, Mexico (acid soil stress)	15	422
Zacatecas, Mexico (drought)	49	600
Sandovales, Mexico (drought)	47	558

1.2.11 Molecular Marker-Assisted Breeding for Resistance to the Cassava Mosaic Disease in Latin American Cassava Gene pools

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Introduction

Molecular marker-assisted selection (MAS) for CMD resistance at CIAT is both a pre-emptive measure, should in case the disease is accidentally introduced in Latin America, and a dynamic measure, to enable a true evaluation of the value of CIAT improved germplasm in India and Africa. MAS using the single dominant gene CMD2 as source has completed the first year at CIAT. A total of 2315 seeds harvested from more than 3000 controlled crosses between CMD resistant parents introduced from IITA and elite parents of the 5 cassava gene pools by agro-ecology or backcross derivatives of M. esculenta sub spp flabellifolia resistant to the green mite. More than 1,100 genotypes were germinated as embryo axes and multiplied for molecular analysis with the SSR marker NS158 closely linked to CMD2. Establishment of breeding populations in vitro is to aid shipment to collaborators in Africa and India. CMD resistant genotypes, as revealed by MAS, will be sent to the green house for hardening and also shipped to partners in India and Africa. We describe here results of the first year of MAS at CIAT.

Methodology

Sexual seeds of crosses between CMD resistant parents and elite parents of cassava gene pools were germinated from embryo axes (see Activity 22) and molecular analysis performed on the plantlets. DNA isolation was using a rapid mini prep method developed

for rice (Nobuyuki et al 2000) and 2 young leaves from the in vitro plantlets. The leaves were placed in an 1.5 ml eppendorf tube and 200ul of TE buffer (10 mM tris-Cl, 1 mM EDTA; pH: 8.0) added. The leaves were then squashed in the buffer using a small pestle and incubated in a water bath at 100 °C for 15 minutes. Next, 800ul of TE buffer was added and the tube inverted gently several times to mix the content followed by centrifugation in a table-top centrifuge at 14,000rpm for 10 minutes. The supernatant contains about 10 to 20ng/ul and was used directly in the PCR amplification reaction. The supernatant was transferred to eppendorf 96-well plates (Costar) using 8 tip multi-channel pipette for easy dispensation into 96-well PCR plates and for long term storage. DNA obtained is sufficient for 100 reactions and can be held in the Costar plates for 2 months at –20°C without any degradation. PCR and PAGE gel analysis of the PCR product is as described by Mba et al. (2001). Gel image from the SSR analysis is entered into an excel sheet containing other information, such as pedigree, phenotypic evaluation, number of plants available and where.

Results

More than 1,100 plants representing the first breeding population for CMD resistance at CIAT were analyzed using the NS158 marker associated with CMD resistance. Results of the molecular analysis are shown in Figure 1. An information management system to handle the MAS data and to make it easily accessible was developed in Micro soft Excel. The versatility of Excel spreadsheets make it the appropriate software to handle the diverse information generated by MAS. We also reviewed the process of MAS as conducted by CIAT´s cassava molecular lab with respect to time, labor and costs. The time required to pick leaves from in vitro plantlets, extract DNA and completely fill a 96-well plate is approximately 9 hours. To set up a 96-well PCR reaction and complete the temperature cycling, in this case for SSR marker NS158 associated with CMD2, is 4 hours. Running the amplification product on a 6% acrylamide gel and silver stain also requires 4 hours. In total it takes 17 hours or 2 working days for a single person to complete DNA isolation and marker analysis for 96 genotypes. The cassava molecular marker lab currently has two persons working on MAS for CMD and together they can process 192 genotypes in 2 days or 480 genotypes per week or over 24,000 samples in a year. We are working on improving this by doing the grinding and DNA isolation in 96-well plates. Current costs of a single SSR marker data point analysis for cassava at CIAT is US$0.30, processing 24,000 samples in a year requires a budget of US$7,200.

Conclusions

MAS for CMD resistance breeding has been initiated at CIAT. More than 1,100 genotypes have been processed this year and it is expected that three times that number will be processed that year as the entire system from crosses to embryo rescue to molecular analysis is made more efficient. Future perspectives include development of a 96-well method for grinding leaf tissue and DNA isolation to eliminate the need for time-consuming transfers from eppendorf tubes to 96-well plates.

MATERIALES CR					In Vitro		MAS		Green house plants
Number	Code	Mother	Father	TC Media	Jars	Tubes	PCR	Score	
1	CR52A-30	C-243	SM1219-9	4E	2**	0	SI	S	
2	CR52A-31	C-243	SM1219-9	4E	2**	0	SI	R	
3	CR52A-32	C-243	SM1219-9	4E	2**	0	NO	-	
4	CR52A-33	C-243	SM1219-9	4E	2**	0	SI	R	
5	CR52A-34	C-243	SM1219-9	4E	2**	0	SI	S	
6	CR52A-35	C-243	SM1219-9	4E	2**	0	SI	R	
7	CR52A-36	C-243	SM1219-9	4E	2**	0	SI	R	
8	CR52A-37	C-243	SM1219-9	4E	2**	0	SI	R	
9	CR52A-40	C-243	SM1219-9	4E	2**	0	SI	R	
10	CR42-1	C-18	MCOL 2206	4E	2**	0	SI	R	
11	CR42-2	C-18	MCOL 2206	4E	2**	0	SI	R	
12	CR42-3	C-18	MCOL 2206	4E	2**	0	SI	R	
13	CR42-4	C-18	MCOL 2206	4E	2**	0	SI	R	
14	CR42-5	C-18	MCOL 2206	4E	2**	0	SI	R	
15	CR42-6	C-18	MCOL 2206	4E	2**	0	SI	S	
16	CR42-7	C-18	MCOL 2206	4E	2**	0	SI	R	
17	CR42-9	C-18	MCOL 2206	4E	2**	0	SI	R	
18	CR9A-153	C-4	MTAI 8	4E	2**	0	SI	S	
19	CR9A-154	C-4	MTAI 8	4E	2**	0	SI	R	
20	CR9A-155	C-4	MTAI 8	4E	2**	0	SI	S	
21	CR9A-156	C-4	MTAI 8	4E	2**	0	SI	S	
22	CR20A-1	CM3306-4	C-33	4E	2**	0	SI	R	
23	CR20A-2	CM3306-4	C-33	4E	2**	0	SI	R	
24	CR20A-3	CM3306-4	C-33	4E	2**	0	NO	-	
25	CR20A-4	CM3306-4	C-33	4E	2**	0	SI	R	
26	CR20A-5	CM3306-4	C-33	4E	2**	0	SI	R	
27	CR20A-6	CM3306-4	C-33	4E	2**	0	SI	R	
28	CR14B-1	C-4	CM523-7	4E	2**	0	SI	R	
29	CR14B-3	C-4	CM523-7	4E	2**	0	SI	R	
30	CR14B-4	C-4	CM523-7	4E	2**	0	SI	R	
31	CR14B-5	C-4	CM523-7	4E	2**	0	SI	R	
32	CR14B-6	C-4	CM523-7	4E	2**	0	SI	R	
33	CR14B-7	C-4	CM523-7	4E	2**	0	NO	-	
34	CR14B-8	C-4	CM523-7	4E	2**	0	SI	R	
35	CR14B-9	C-4	CM523-7	4E	2**	0	SI	R	
36	CR14B-10	C-4	CM523-7	4E	2**	0	SI	R	
37	CR14B-11	C-4	CM523-7	4E	2**	0	NO	-	
38	CR14B-12	C-4	CM523-7	4E	2**	0	SI	R	
39	CR14B-13	C-4	CM523-7	4E	2**	0	SI	R	
40	CR14B-14	C-4	CM523-7	4E	2**	0	SI	R	
41	CR14B-16	C-4	CM523-7	4E	2**	0	SI	R	
42	CR52A-41	C-243	SM1219-9	4E	2**	0	SI	R	
43	CR52A-42	C-243	SM1219-9	4E	2**	0	NO	-	
44	CR52A-43	C-243	SM1219-9	4E	2**	0	SI	R	
45	CR9B-5	MTAI 8	C-4	4E	2**	0	SI	S	
46	CR25-2	CM7951-5	C-33	4E	2**	0	NO	-	
47	CR23-1	CM7951-5	C-4	4E	2**	0	SI	R	
48	CR23-2	CM7951-5	C-4	4E	2**	0	SI	S	
49	CR23-3	CM7951-5	C-4	4E	2**	0	SI	R	
50	CR23-4	CM7951-5	C-4	4E	2**	0	SI	S	
51	CR23-5	CM7951-5	C-4	4E	2**	0	SI	S	
52	CR23-6	CM7951-5	C-4	4E	2**	0	SI	R	
53	CR23-7	CM7951-5	C-4	4E	2**	0	SI	R	

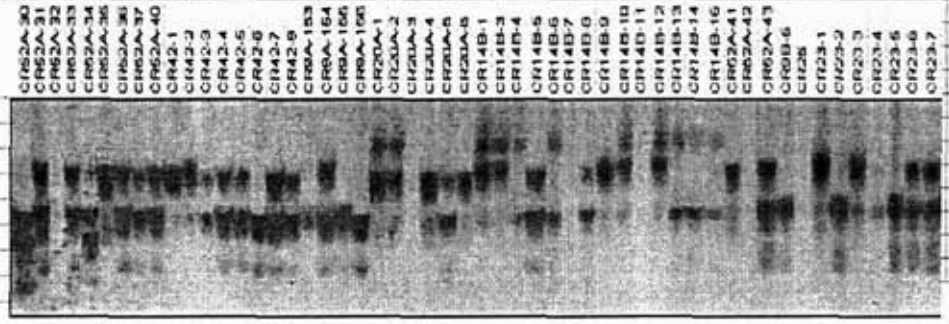

Fig1. The cassava MAS data management system in Microsoft excel, the spreadsheet shows part of a 96-well plate molecular marker (NS158) analysis. The allele associated with CMD resistance is the middle one.

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1.2.12 Molecular Marker-Assisted and Farmer Participatory Improvement of Cassava Germplasm for Farmer/Market Preferred Traits in Tanzania

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Introduction

Tanzania is the fourth largest producer of cassava in Africa with average yields of about 8 t/ha (FAO, 2001). This is well below the continent's average of 10tons/ha and the average yield of 14 tons/ha of Africa's (and the world's) largest producer, Nigeria. The low yield is caused by many factors including susceptibility of commonly grown varieties to major diseases and pests such as cassava mosaic diseases, caused principally by the East African Cassava Mosaic Virus (EACMV), its Ugandan variant (UgV), and the African Cassava Mosaic virus (ACMV), cassava brown steak disease (CBSD), cassava bacterial blight (CBB), cassava green mite (CGM), cassava mealy bug (CM) and nematodes. Previous research at Kibaha and Naliendele reported as high as 55% loss in the local cultivar "Albert" (Mtunda 2003 pers comm). A recent survey in Tanga has revealed crop losses of up to 74% (Muhanna and Mtunda, 2002). In severely affected areas, entire fields may be destroyed.

A farmer participatory, molecular marker-assisted, decentralized, breeding scheme has recently been approved for funding by the Rockefeller Foundation to speed up the process of improving local cassava germplasm for resistance to pests and diseases in Tanzania. The proposed breeding project will take farmer preferred germplasm by agro-ecology and cross them to improved introductions that have resistance to Cassava Mosaic Disease (CMD), Cassava Green Mite (CGM), and Cassava Bacterial Blight (CBB). Given the fairly large number of parents that will be used, molecular markers associated with pest and disease resistance will be employed to reduce, in a logical manner, the number of progeny to a manageable number. The progeny selected by MAS will be evaluated in a single season in the corresponding agro-ecology and then evaluated over two cycles in collaboration with

end-users (rural communities and cassava processors). The project will be carried out in a total of six years divided into 2 three-year phases. A principal objective of the project is the development of capacity for participatory plant breeding and marker-assisted breeding. This would be achieved by training 2 national program breeders at the MSc. and PhD level, and through 2 training workshops on participatory plant breeding and marker-assisted breeding.

Methodology

Activities of the first year of the RF Tanzanian project includes the collection and evaluation of local germplasm and the introduction of improved progenitors for use as parents in the breeding project. Improved progenitors were designed to have resistance to CMD, CBB, and CGM as well as molecular markers associated with these genes. The improved progenitors were developed as described below. The RF funded project "Molecular Mapping of Genes Conferring Resistance to the Cassava Mosaic Disease (CMD) in African Cassava Germplasm" has led to the identification of 3 SSR and 2 RAPD markers tightly linked to a novel source of CMD resistance controlled by a single dominant gene designated CMD2 (Akano et. al. 2002; Moreno and Fregene unpublished data). The CMD2 source shows high levels of resistance against a wide spectrum of strains of the virus in sub Saharan Africa, including the aggressive Ugandan variant (UgV), ACMV and EACMV (Akano 2002, CIAT 2001). Excellent resistance to CGM have also been observed in 4 F_1 inter-specific hybrid families obtained by crossing the cassava clones CG487-2, CG501-16, MCol2215, and CM2766-5 to a genotype of M. esculenta sub spp flabellifolia. (Belloti and Fregene 2002, unpublished data; CIAT 2002). Bulk segregant analysis (BSA) was quickly used to identify several SSR markers associated with CGM resistance in the MCol2215 cross (CIAT 2002). The inter-specific hybrids of *M. esculenta* sub spp *flabellifolia* that carry the novel CGM resistance were crossed extensively to elite parents of cassava genepools from the 5 agro-ecologies, a number of these parents have the SG107-35 source of CBB resistance. These first back cross derivatives were then crossed to CMD 2 donor parents to obtain more than 600 BC_2 progenies.

Progeny from the above cross were established from embryo axes of mature sexual seeds, multiplied and kept in vitro. Two *in vitro* plants were used for marker evaluation to identify progeny CMD, CBB and CGM resistance. Another 5 in vitro plants were sent to the green house for phenotypic evaluation of yield and yield components. About 200 genotypes that combine resistance to CMD, CBB and CGM and high productivity are being prepared for shipment to Tanzania, at least 20 plants per genotype will be shipped. An import permit has been issued by the Tanzanian phyto-quarantine authorities for the import of this germplasm.

Results

Progenies of inter-specific hybrids crossed to parents of cassava gene pools adapted to the sub-humid lowland, acid savannah, mid-altitude and semi-arid agro-ecologies, with good

resistance to CBB were crossed to CMD2 donor parents to obtain more than 600 BC_2 progenies. Table 1 summarizes the BC_2 families obtained and the number of plants per genotype in vitro. In addition, parents of the above progenitors were indexed for the frog skin disease (FSD) and other commonly found diseases in South America as well as checked for pests. Embryo rescue and multiplication of the BC_2 families to obtain 10 plants per genotype was done as described earlier (CIAT 2002). About 5 in vitro plants per genotype was used in marker analysis using markers associated with CMD and CGM according to methods described earlier (CIAT 2002). A special format in microsoft excel was developed to display results of the molecular marker-assisted evaluation of the BC_2progenies as described in the MAS for CMD at CIAT activity. Plants selected by molecular marker analysis were further multiplied to obtain more than 20 plants per genotype and will be shipped to Tanzania once the import permit have been issued and received at CIAT, at least 20 plants per genotypes will be shipped. On arrival in Tanzania, 18 plants of all genotypes will be hardened in the screen house and 6 plants each sent to the 3 different target agro-ecologies for field establishment and evaluation for use as improved parents. The remainder 2 plants per genotype will be multiplied and kept in vitro as back-up. At least 30 genotypes of the 200 improved introductions will be selected for crosses to the20 selected local varieties. Selection parameters will include harvest index, pest and disease resistance and root quality. The parents will be crossed in all possible combinations using a polycross design. The crossing blocks, with 40 plants each per local variety and 20 plants each of the improved introductions, will be set up at two sites, namely SRI-Kibaha, Eastern zone, and Maruku, in the lake zone, where cassava flowers profusely, to maximize the possibilities of getting seeds from all genotypes. Hand pollination will also be carried out to ensure certain combinations are obtained. The local land races and introductions will be used as female parents to achieve a wide base of cytoplasm, therefore open pollinated and controlled pollinated sexual seeds will be harvested from both. Between 10,000 – 20,000 seeds are expected from crosses for each agro-ecology.

Table 1. ***List of BC_2 families developed for the introgression of resistance to CGM from wild progenitors of cassava into CMD resistant genotypes. Crosses were made to cassava parents adapted to the semi-arid low land tropics (ZO1) and Acid savannahs (ZO2).***

Family	Mother	Father	No. of Genotypes	Zone
CW 74- 1	CM 2177- 2	CW 65- 77	1	Z02
CW 75- 1	CM 3306- 4	CW 66- 60	7	Z01
CW 76- 1	CM 3306- 4	CW 68- 3	9	Z01
CW 77- 1	CM 7951- 5	CW 65- 77	5	Z02
CW 78- 1	CM 7951- 5	CW 66- 19	4	Z02
CW 79- 1	CM 7951- 5	CW 66- 62	1	Z02
CW 80- 1	CM 7951- 5	CW 67- 42	5	Z02
CW 81- 1	CM 7951- 5	CW 67- 98	3	Z02
CW 213- 1	SM 805- 15	CW 67- 39	1	Z01
CW 214- 1	SM 805- 15	CW 67- 87	11	Z01
CW 215- 1	SM 909- 25	CW 66- 60	8	Z02
CW 217- 1	SM 1219- 9	CW 65- 77	17	Z02
CW 218- 1	SM 1219- 9	CW 66- 73	12	Z02
CW 220- 9	SM 1219- 9	CW 67- 123	5	Z02
CW 223- 1	SM 1460- 1	CW 66- 19	11	Z02
CW 224- 1	SM 1460- 1	CW 66- 60	16	Z02
CW 225- 1	SM 1460- 1	CW 66- 62	30	Z02
CW 226- 1	SM 1460- 1	CW 66- 73	15	Z02
CW 227- 1	SM 1460- 1	CW 68- 3	3	Z02
CW 229- 1	SM 1511- 6	CW 67- 87	26	Z01
CW 230- 1	SM 1565- 15	CW 66- 19	4	Z02
CW 231- 1	SM 1565- 15	CW 66- 60	24	Z02
CW 232- 1	SM 1665- 2	CW 66- 19	23	Z01
CW 233- 1	SM 1665- 2	CW 66- 60	32	Z01
CW 235- 1	SM 1665- 2	CW 67- 87	117	Z01
CW 236- 1	SM 1669- 5	CW 66- 19	33	Z01
CW 237- 1	SM 1669- 5	CW 66- 60	8	Z01
CW 238- 1	SM 1669- 5	CW 66- 62	1	Z01
CW 239- 1	SM 1669- 5	CW 66- 73	5	Z01
CW 240- 1	SM 1669- 5	CW 66- 74	28	Z01
CW 241- 1	SM 1669- 5	CW 67- 123	4	Z01
CW 242- 1	SM 1669- 7	CW 67- 87	9	Z01
CW 243- 1	SM 1741- 1	CW 66- 19	6	Z02
CW 244- 1	SM 1741- 1	CW 66- 60	15	Z02
CW 245- 1	SM 1741- 1	CW 66- 62	1	Z02
CW 246- 1	SM 1741- 1	CW 67- 91	9	Z02
CW 247- 1	SM 1778- 45	CW 66- 19	3	Z02
CW 248- 1	SM 1778- 45	CW 67- 45	2	Z02
CW 257- 1	MTAI 8	CW 65- 77	25	Z01
CW 258- 1	MTAI 8	CW 66- 60	26	Z01
CW 259- 1	MTAI 8	CW 66- 73	46	Z01
CW 260- 1	MTAI 8	CW 66- 74	6	Z01
CW 261- 1	MTAI 8	CW 67- 123	4	Z01
Total			621	

Conclusions

A molecular marker-assisted breeding project to develop improved germplasm for small cassava farmers have been initiated. The first year will be spent in the development of parents and generating broad-based breeding populations for subsequent selection by molecular markers and in collaboration with farmers. The project is a first and experience gained is expected to guide the application of molecular markers in cassava breeding.

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1.2.13 Molecular Marker-Assisted Selection (MAS) for Breeding Early Root Bulking in Cassava

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CIAT

Introduction

Early root yield is an important trait of cassava (Nweke et al. 1994), critical to the crop's role as food security crop in sub-Saharan Africa. Furthermore, as globalization picks up speed, new opportunities have arisen for cassava as a source of industrial raw material which requires a great deal of flexibility of harvest, that is varieties that attain close to maximum yield at 8-10 months after planting. Genetic mapping of early root bulking in a full-sib cassava genetic map population was extended to a S_1 family of 268 genotypes from a progeny, K150, of the same map population. Several QTLs for foliage weight and harvest index, 2 traits that strongly influence early root bulking, were found in both studies. They include a major QTL for foliage weight, explaining 31% of phenotypic variance, and 3 QTLs for harvest index, explaining between 15 and 22% of phenotypic variance, the QTL for foliage showed a dominance gene action while those for harvest index were additive (Okogbenin et al. 2003). The mapping of major QTLs is an important step towards the development of molecular markers for breeding of early yield, which like yield is a complex trait. To identify parents for the development of breeding populations for early root bulking, 25 of the progenies from the S_1 population that had the highest yield at 7 months after planting in the QTL experiments last year were evaluated for a second

year in larger plot sizes and more replications. The best 5 genotypes will be used as parents for making crosses to elite cassava parents for the development of breeding populations for molecular marker-assisted selection (MAS).

Methodology

An S_1 family was developed from K150, a progeny of the cassava map population having a large number of positive QTLs for foliage weight, harvest index and number of roots, was developed in 1998 and evaluated 2001 in a replicated trial (Okogbenin 2003). A second year evaluation was conducted in 2002 with the difference that harvest was conducted at 7 months after planting. Twenty five of the highest yielding genotypes with harvest index more than 0.50 and strong canopy, foliage weight per plant greater than 1.5kg, were selected and re-established for a third year evaluation at the CIAT station in Santander de Quilichao a high stress environment. Previous work has shown than selection for early root bulking in a low stress environment may not be replicated in a high stress environment (Kawano et al, 2001). Experimental design and evaluation of the above early bulking genotypes was as described for the 2002 evaluation (Okogbenin 2003). Ten months after planting, the genotypes were evaluated for root yield, foliage weight, harvest index, and dry matter content. The best five genotypes from this experiment will be used as parents in the development of populations for a molecular breeding scheme for improved early bulking.

Results

Evaluation of the best 25 genotypes from last year's evaluation for early yield confirmed earlier observation that a strong canopy and high harvest index are essential for early root yield (Okogbenin and Fregene 2003). The 3 genotypes with the highest foliage weight are also those with the highest root yield (Table 1). Another observation is the very low standard deviation for harvest index in the 25 genotypes, all but one genotype have harvest index of more than 0.6, again revealing the strong correlation between harvest index and early yield, selection of these genotypes last year was based only on early yield. The genotypes selected as parents from this evaluation are AM150-241, AM150-332, AM150-185, AM150-150, and AM150-309. Stakes from these materials have planted in the crossing block for next year.

Conclusions

A scheme to improve early bulking via marker-assisted selection has been initiated with the selection of parents for the development of breeding populations. Future perspectives include evaluation of breeding populations obtained next year with markers associated with QTLs for early bulking in the S1 family from K150.

Table 1 Some statistics yield and yield components of the 25 most early yielding genotype from the S_1 family from K150

Genotype	Mother	Foilage/plant (g)	Yield/plant (g)	HI	% Dry matter	Yield (t/ha)
AM150-10	CM 7857- 150	1187.50	3850.00	0.76	34.11	38.50
AM150-34	CM 7857- 150	325.00	1150.00	0.78	27.19	11.50
AM150-43	CM 7857- 150	650.00	1650.00	0.72	33.16	16.50
AM150-56	CM 7857- 150	1550.00	2625.00	0.63	31.53	26.25
AM150-70	CM 7857- 150	1937.50	2750.00	0.59	32.08	27.50
AM150-83	CM 7857- 150	687.50	2600.00	0.79	30.24	26.00
AM150-96	CM 7857- 150	850.00	2750.00	0.76	31.78	27.50
AM150-100	CM 7857- 150	1375.00	5500.00	0.80	32.90	55.00
AM150-104	CM 7857- 150	775.00	1887.50	0.71	29.94	18.90
AM150-111	CM 7857- 150	1191.67	1600.00	0.57	32.40	16.00
AM150-114	CM 7857- 150	950.00	3950.00	0.81	33.72	39.50
AM150-119	CM 7857- 150	1125.00	3750.00	0.77	36.27	37.50
AM150-124	CM 7857- 150	518.75	1856.25	0.78	34.63	18.60
AM150-137	CM 7857- 150	1125.00	2956.25	0.72	34.44	29.60
AM150-143	CM 7857- 150	1237.50	3750.00	0.75	32.84	37.50
AM150-154	CM 7857- 150	1700.00	1000.00	0.37	28.03	10.00
AM150-165	CM 7857- 150	641.67	1875.00	0.75	36.76	18.80
AM150-179	CM 7857- 150	508.33	2483.33	0.83	32.20	24.80
AM150-182	CM 7857- 150	387.50	2087.50	0.84	32.02	20.90
AM150-185	CM 7857- 150	3800.00	6737.50	0.64	36.89	67.40
AM150-206	CM 7857- 150	625.00	1550.00	0.71	30.37	15.50
AM150-241	CM 7857- 150	2350.00	8725.00	0.79	32.55	87.30
AM150-290	CM 7857- 150	825.00	1440.00	0.64	31.35	14.40
AM150-309	CM 7857- 150	1375.00	4275.00	0.76	33.95	42.80
AM150-332	CM 7857- 150	4012.50	9550.00	0.70	36.00	95.50
	Maximum	4012.50	9550.00	0.84	36.89	95.50
	Minimum	325.00	1000.00	0.37	27.19	10.00
	Average	1268.42	3293.93	0.72	32.69	32.95
	Std. Deviation	934.26	2228.53	0.10	2.46	22.29

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1.2.14 Genetic Mapping of Beta-Carotene Content

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CIAT

Introduction

A project to fortify cassava varieties grown by rural communities with higher levels of beta-carotene has been initiated as a way of combating deficiency of this key micronutrient in areas where cassava is a major staple. The experimental approach on increasing cassava beta-carotene content includes conventional breeding and genetic transformation. The discovery of a wide segregation pattern of root color in two S_1 families from the Colombian land race MCol72 (AM273) and the Thai variety MTAI8 (AM320), led to the commencement of molecular genetic analysis of beta-carotene content in cassava. The cost-effectiveness of breeding for high beta-carotene content in cassava can be considerably enhanced if the mode of inheritance and the number of genes involved are known. Regions of the genome associated with beta-carotene content can also be examined for the presence of known genes involved in the biosynthesis of beta-carotene content can. This information can be used for functional diversity analysis of natural variation of beta carotene content for a more rational exploitation of naturally occurring variability. We describe here results of bulk segregant analysis and the identification of two regions of the cassava genome associated with beta-carotene content.

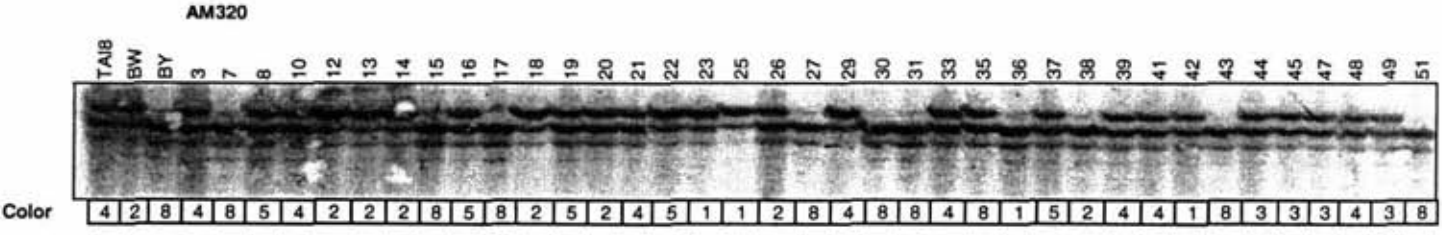
Methodology

Ten white colored and 10 orange/pink colored genotypes were selected from each of the S_1 family to serve as individuals for the high and low beta-carotene bulks. For DNA isolation, 1-2g of young leaves was harvested from genotypes of both bulks for each family and from all 38 genotypes of family AM273 and 102 genotypes from family AM320. The leaves were dried for 24h in an oven at 48°C and then ground into a fine powder using a power drill and washed sand. DNA was isolated from 200mg using a miniprep version of the Dellaporta (1983) protocol. The bulks of white and orange/pink roots were then created per family to give a total of 4 bulks. DNA from the bulks and the parents were then genotyped with the 650 available cassava SSR markers according to methods described by Mba et al (2001). Markers polymorphic in the bulks were employed to analyze individuals of the bulks and, where the polymorphism remained consistent in the individuals as with the bulks, the markers were analyzed in the entire family. Association with orange/pink color was determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the Microsoft Excel. The amount of phenotypic variance explained by each marker l was obtained from the R^2 value.

Results

A total of 6 markers, namely NS189, NS980, SSRY240, SSRY251, SSRY9, and SSRY63 were polymorphic in the bulks from the S_1 family AM320 derived from MTAI8. Six markers, NS980, SSRY240, SSRY9, SSRY251, common to both families, and 2 additional markers, SSRY192 and SSRY54 unique to AM273, were found polymorphic in the bulks. On analyzing individuals of the bulks, polymorphism was consistent in the individuals for markers SSRY251, NS980, SSRY240 in both families while the remaining markers did not show a clear-cut pattern of polymorphism between the individuals of the bulks. All the three markers are located on linkage group D of the molecular genetic map of cassava. The 3 polymorphic markers were analyzed in all individuals of both S_1 families and results reveal NS251 has the strongest association with beta-carotene content in both families (Fig1). This marker explains 30%

Figure 1. Silver-stained polyacrylamide gel of PCR amplification of individuals from family AM320 with SSR marker NS251. The color code is as follows: 8= orange/pink roots, 1-2= white roots, 4-5= cream roots



of phenotypic variance for beta-carotene. From the segregation of SSR marker NS251 and scorings of color, known to be highly correlated with beta-carotene content ($r>0.8$), of some individuals in the family AM320 in figure 1, it can be observed that the most intense color, a score of 8, is associated with homozygosity of the smaller sized allele of NS251 in this family. The same was pattern was also observed in family AM273.

Conclusions

An SSR marker, NS251 that explains 30% of phenotypic variation for beta-carotene content has been identified in cassava. A search for markers more tightly associated with beta-carotene in the linkage group D region of the genetic map of cassava that may have been missed in the screening of the bulks due to a lack of polymorphisms is ongoing. A modified BSA method using recombinants found with marker SSRY251 and several marker systems, including RAPDs, AFLPs, and known biosynthetic genes of beta-carotene is ongoing.

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1.2.15 Genetic Mapping of Dry Matter Content (DMC)

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CIAT

Introduction

Few key traits in cassava hold potential for increasing cost-effectiveness via molecular marker assisted selection (MAS) compared to root dry matter content (DMC). It is measured at the end of the growth cycle and affected by the time of evaluation, DMC is generally high before the onset of the rains but drops after the rains begin as the plant mobilizes starch from the roots for re-growth of leaves (Byrne 1984). A tool to evaluate early and accurately DMC can increase cost-effectiveness of breeding from DMC, via an elimination of a large number of genotypes leaving the breeder more time to concentrate on the evaluation of a reduced number of plants. Every plant with low DMC transplanted to the field uses up space, resources and time (at evaluation) that considerably lowers breeding efficiency.

The entry point for developing markers associated with DMC was three diallel experiments that were began in 2000. The diallels, made up of 90 families, is an ideal experiment to identify genes controlling DMC that are useful in many genetic backgrounds. In May 2002, a final evaluation of the three diallel experiments was done. Estimates of general combining ability (GCA) and specific combining ability (SCA) for many traits of agronomic interest was calculated, an emphasis was placed on DMC. Based on general combining ability (GCA) estimates, parents were selected to generate larger sized progenies for DMC mapping. Sizes of families in the original diallel experiment were about 30 progenies, a rather small size for genetic mapping. Parallel to the development of mapping populations was the search for markers associated with DMC using 2 F_1 families, GM312 and GM313 selected from the diallel experiment having parents with high GCA for DMC.

Methodology

A crossing block of high and low GCA parents for DMC (Table 1) was established in CIAT, Palmira in October 2002, with the aim of generating larger sized progenies for QTL mapping of DMC. Crossing is ongoing for all genotypes except for SM1741-1 x MECU) and SM 1411-5 x CM 4574-7), which have either few plants or have poor flowering. Bulk segregation analysis (Michelmore et al., 1991) was conducted for 2 F_1 crosses, GM 312 and GM 313, each with MECU 72 (high dry matter and good GCA) as a parent. DNA samples were extracted from 3g of fresh leaves according to Dellaporta et al., 1983. Between 500µg to 1000µg of high quality DNA was obtained from each extraction and quantified using flourometer. The samples where then diluted to 10ng/µl for PCR amplification. DNA from genotypes with percent dry matter of 34.3 - 37.7 and 24.5 - 31.5 were bulked to form high and low bulks respectively. The Bulks and parents were screened with polymerase chain (PCR) reaction using 650 SSR primers developed in CIAT. PCR product was denatured and electrophoresed on 6% polyacrylamide gels according to Mba et al., 2001.

Table 1: Status of the crosses for QTL mapping.

Cross	Number of crosses		Target area
	Direct	Reciprocal	
SM 1741-1 x MPER 183	269	7	Mid altitude
SM 1741-1 x SM 1219-9	104	114	Mid altitude
SM 1741-1 x MECU 72	0	0	Mid altitude
SM 1411-5 x MTAI 8	36	97	North coast
CM 8027-3 x CM 6757-8	224	154	North coast
SM 1665-2 x SM 805-15	544	570	North coast
SM 1411-5 x CM 4574-7	2	-	"out cross"
SM 1219-9 x SM 1565-15	188	0	Acid savannah soils
CM 4574-7 x SM 1565-15	124	163	Acid savannah soils

Results

A summary of crosses made so far for the development of QTL mapping populations for DMC can be seen in table 1.

Thirty-one primers were found to be polymorphic between the bulks of high and low dry matter content and in individuals of the bulks from family GM 313. These were tested on the remaining genotypes of the cross. Of these eleven NS 701, NS 717, NS781, NS80, NS 9, NS 909, NS 917, NS 955, SSRY 150, SSRY 160 SSRY 88 and NS 371 remained polymorphic. A simple regression of dry matter content on the genotypic classes of the SSR markers was highly significant for the 2 markers, SSRY160 and SSRY150, with

regression coefficient (R^2) of 29.3 and 18.1 respectively. The phenotypic variance explained by these two markers, based on their regression coefficient, is enough to consider them as markers for marker-assisted selection (MAS). All other markers had R^2 of between 0.05 and 0.1. The other family GM 312, yielded no markers polymorphic in the individuals of the bulks in the BSA. The markers SSRY 160 and SSRY150 associated with DMC in the GM 313 cross are being tested on about 700 genotypes from 23 crosses of the diallel experiment. The 23 crosses were selected from crosses with a high standard deviation for dry matter content and from parents with good general combining abilities. Theses crosses had earlier been planted in the field in Quilichao for further evaluation of DMC in a completely randomized block design of 3 blocks (replicates) and up to10 plant per block. At harvest data was collected on fresh foliage yield, fresh root yield, number of tubers, harvest index, number of tubers per plant, yield, percentage dry matter and dry matter yield. Results are summarized in tables 2. Low standard deviation values were estimated for dry matter content compared to other yield related parameters suggesting that it is a relatively stable trait. All the parameters were highly correlated (Table 3) confirming their importance to yield. Dry matter and number of tubers per plant were not significantly correlated to the number of plants harvested suggesting that they are not easily influenced by environment, which is important to note since there was non-uniform establishment. There was an incidence of frog skin disease (FSD) in this trial and it was highly and negatively significant to all yield related traits, and it is likely to influence results of the study. The affected plants were therefore eliminated from the final results. Cross GM 311 had the highest standard deviation for dry matter (8.58) followed by CM 9901 (6.47) while GM 228 had the lowest of 2.40 (Table 4).

Table 2:Some statistics of yield related parameters for the crosses evaluated in Quilichao, during the 2002-2003 season.

Cross	No of genotypes	Harvest Index (0-1)		% Dry matter		Yield (t/ha)		Dry yield (t/ha)	
		Mean	St. Dev	Mean	St. Dev	Mean	St.Dev	Mean	St. Dev
CM 9642	27	0.48	0.08	33.46	3.41	20.74	21.30	7.02	7.45
CM 9733	25	0.52	0.13	28.20	4.34	25.94	19.28	7.68	6.30
CM 9901	31	0.47	0.18	28.53	6.47	14.17	12.69	4.53	4.32
GM 228	6	0.46	0.17	35.39	2.40	19.85	14.58	7.29	5.65
GM 257	33	0.55	0.15	29.66	4.80	23.06	18.13	7.13	5.84
GM 260	31	0.65	0.10	30.54	4.02	20.33	13.02	6.29	4.55
GM 265	34	0.55	0.12	29.03	4.03	19.60	12.25	5.95	4.08
GM 267	8	0.45	0.17	31.33	5.39	21.01	26.41	10.30	9.14
GM 268	29	0.52	0.15	33.93	3.97	22.49	19.12	7.96	7.03
GM 269	25	0.52	0.13	33.79	3.47	25.74	17.98	8.91	6.60
GM 283	29	0.59	0.17	30.46	5.37	18.86	16.86	6.13	5.63
GM 284	42	0.54	0.18	31.82	5.39	17.76	12.93	5.77	4.45
GM 285	13	0.51	0.19	29.45	4.27	22.74	16.49	7.02	5.33
GM 286	28	0.48	0.13	31.43	2.85	28.09	17.26	8.94	5.77
GM 293	27	0.50	0.14	32.04	3.33	22.31	17.54	7.08	5.47
GM 294	26	0.54	0.12	31.74	3.18	25.15	18.93	8.01	6.23
GM 306	27	0.40	0.11	29.52	4.44	20.40	15.02	6.12	4.62
GM 309	36	0.49	0.19	29.26	5.35	21.38	16.01	6.68	5.42
GM 310	30	0.57	0.13	33.98	4.08	28.20	21.44	9.94	7.99
GM 311	34	0.45	0.19	30.23	8.58	19.32	14.86	5.86	4.81
GM 312	19	0.46	0.11	32.49	4.83	27.32	15.54	9.17	5.70
GM 313	30	0.45	017	32.21	5.13	22.14	16.05	7.02	5.22
GM 314	19	0.47	0.12	31.33	3.77	20.59	13.53	6.68	4.96
Minimum	6	0.40	0.08	28.20	2.40	14.17	12.25	4.53	4.08
Max	42	0.59	0.19	33.46	8.58	28.20	26.41	10.30	9.14

Table 3: Correlation table of frog skin disease incidence and yield related parameters

	Plants[a]	FSD[b]	ComRt[c]	TbNo[d]	FolWt[e]	HI[f]	Yield[g]	DM[h]
Plants								
FSD	-0.06NS[i]							
ComRt	0.57***[j]	-0.23***						
TbNo	0.05NS	-0.21***	0.56***					
FolWt	0.57***	-0.02NS	0.58***	0.28***				
HI	0.02NS	-0.24***	0.36***	0.35***	-0.29***			
Yield	0.58***	-0.20***	0.88***	0.52***	0.63***	0.39***		
DM	0.06NS	-0.33***	0.29***	0.22***	0.07NS	0.26***	0.27***	
Dyield	0.54***	-0.22***	0.87***	0.52***	0.61***	0.39***	0.98***	0.38***

[a]=plants harvested per plot; [b]=frog skin disease; [c]=number of commercial roots per plot; [d]=tubers per plant; [e]=foliage weight; [f]=harvest index; [g]=fresh yield (t/ha); [h]=dry matter content(0-1)

Conclusion

A study of genes controlling dry matter content in many full-sib families of cassava has been initiated towards the development of molecular markers for increasing the cost-effectiveness of breeding for dry matter content. Initial marker analysis has led to the discovery of 2 markers, SSRY160 and SSRY150 that explain about 30% and 18% respectively of phenotypic variance for DMC. These markers are being analyzed in 23 crosses with high standard deviation and derived from parents with high GCA for DMC o confirm their utility across genetic backgrounds. Parallel to this, larger families are being developed from selected parent for QTL mapping of DMC.

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1.2.16 Genetic Mapping of Cyanogenic Potential (CNP)

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Introduction

The project on genetic mapping of cyanogenic potential in cassava, a collaborative project between the Swedish Agricultural University (SLU), Uppsala, the Medical Biotechnology Laboratories (MBL), Kampala, and CIAT, is in its second year. The project is being conducted as a Ph.D. thesis by Elizabeth Kizito at SLU. A review of the project was conducted at a meeting in Kampala April this year, where several changes in the project were decided. The principal change was to discontinue the development of F_2 populations derived from the cross between the bitter Ugandan variety, Tongolo, and the CMD resistant land race, TME4, due to the time limit for the completion of Elizabeth's Ph.D. research. It was decided that the S_1 family AM320 derived from the bitter variety MTAI8 available at CIAT should be used instead. This family is currently being genotyped with more than 800 DArT makers, at CAMBIA, Australia, and 200 SSR markers, at CIAT, for gene tagging of beta-carotene content, dry matter and harvest index in cassava.

A new initiative was also embarked during the year on the genetic mapping of the two cytochrome P450 genes, CYP79D1 and D2 that catalyze the rate-limiting step of the biosynthesis of the cyanogenic glucosides, linamarin in the S_1 family AM320 and a search for association with QTLs for CNP. This project is collaboration with the Royal Veterinary and Agricultural University Copenhagen, Denmark (Prof Birger Moller), where the genes were cloned. The discovery of molecular markers for CNP will provide a tool to efficiently select for low cyanogenic potential in cassava breeding.

Methodology

The 102 individuals of the family AM320 in the field were transferred in vitro following methods routinely used in the cassava tissue culture facility (Activity 23 of this report). Another 32 plants were obtained from embryo rescue of residual sexual seeds of AM320. Ten copies per genotype were shipped to SLU, Uppsala for evaluation of root and leaf cyanogenic potential under uniform nutrient, temperature, light and humidity conditions in a phytotron. A field evaluation of CNP in the roots will also be conducted at CIAT. For genetic mapping of SSR markers and the CYPD1 and D2 genes, DNA was isolated from green house plants of all AM320 genotypes using a modified Dellaporta et al. (1983) DNA isolation protocol. DNA from MTAI8, parental genotype of family AM320, and 5 S_1 progenies was screened with 650 SSR markers to identify polymorphic markers for genetic mapping. To map the cytochrome P-450 genes, primers were designed from thes sequence of CYPD1 and D2 using the "Primer3" primer picking software found at http://waldo.wi.mit.edu/cgi-bin/primer/primer3 (Whitehead Institute for Biomedical Research). The genes were then amplified in 50ul volume reactions containing 50-100ng of genomic DNA from MTAI8, 0.2 µM of each forward and reverse primers, 10mM Tris-HCL (pH 7.2), 50mM KCL, 1.5 or 1mM $MgCl_2$, 200mM of each dNTP, and about 1U of Taq DNA polymerase. Temperature cycling profile was: an initial denaturation step for 5min at 94°C, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 55°C (D1) or 60°C (D2) for 2 min and primer extension at 72°C for 2 min. A final extension cycle of 5 min at 72°C was added. Between 4 and 5 µl of the PCR reaction was electrophoresed on 5% ethidium bromide stained metaphor agarose gels and visualized under UV light. The fragments are to be mapped either as cleaved amplicon polymorphism (CAPs), RFLPs or SNPs according to standard methods (Cortes et al. 2003; Fregene et al. 1997).

Results

A total of 130 genotypes from the S1 family AM320 have been shipped to SLU, Uppsala for phenotypic evaluation of CNP in the leaves and roots. Evaluation of the same family will be conducted on field grown plants by October at CIAT. Seventy three polymorphic SSR markers, that segregate in the expected fashion has been found out of 320 SSR markers surveyed till date.

Primers designed for the CYP79 D1 and D2 genes are shown below. They were used in amplifying a part of the genes from parent MTAI8 and some of its S_1 progenies (Fig1).

CYP79 D1
Forward primer AAAGAGTGCTGCTAACAAGG
Reverse primer CCATTGTTGAATCCTTTCAT

CYP79 D2
Forward primer GGTACAGACCGACGTTTCGT
Reverse primer AATGGCTTGCCATCTGAATC

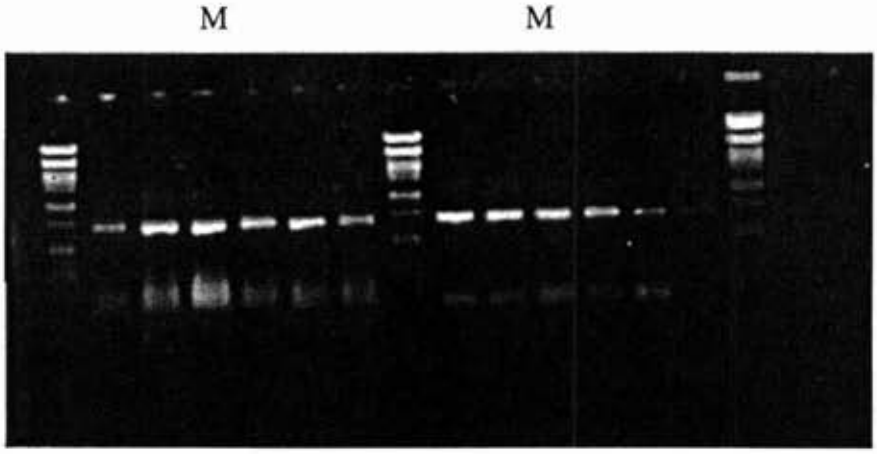

Fig 1. Ethidium bromide stained gel of PCR amplification product of MTAI 8 and 5 S_1 progenies amplified with CYP79 D1 primers (first panel of six lanes), and CYP79 D2 primers (second panel of six lanes). Lanes labelled M are molecular weight markers (Lambda DNA digested with Pst I restriction enzymes).

The search for single dose fragments as CAPs, RFLPs, or SNPs in genes CYPD1 and D2 are ongoing using the genotype in MTAI8. Once these markers have been identified they will be mapped in the S_1 family AM320.

Conclusions

The genetic mapping of CNP has continued with the S_1 family AM320 derived from MTAI8. Phenotypic evaluation of root and leaf CNP is being conducted at SLU, Uppsala, Sweden under uniform conditions in a phytotron. The same family is being genotyped with SSR (at CIAT) and DArT markers (at CAMBIA, Australia), root CNP will also be evaluated in the field at CIAT. Parallel to this effort is the mapping of the CYP D1 and D2 gene in the AM320 family. It is expected that markers associated with CNP will be identified at the end of the study.

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1.2.17 Genetic Mapping of Leaf Retention

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Introduction

Leaf retention or the ability of some cassava genotypes to retain their leaves at 5, 6 and up to 7 months after planting, is a trait that has been shown to be associated with dry matter yield. An experiment conducted in the northern coast of Colombia (sub-humid agro-ecology) to measure the effect of leaf retention revealed that clones that retained leaves at 5-6 months had a 26.4%, 7%, and 32% increase at harvest in fresh root yield, dry matter content and dry matter yield respectively (Lenis et al 2003). Leaf retention in these trials was also associated with higher root dry matter content and harvest index (7% increase). This experiment was repeated during a second season with similar results. Similarly it has been shown that leaf retention and dry matter content are negatively correlated making it possible to simultaneously improve both traits. Preliminary genetic analysis suggests that the trait maybe simply inherited. Development of markers for carrying out pre-selection for leaf retention of breeding populations at CIAT before they are sent to the target environment should therefore reduce the load of evaluation and increase cost-effectiveness of breeding.

Methodology

More than 100 full-sib and half-sib families were evaluated for leaf retention in a clonal field trial in Santo Tomas, the Colombian north coast. Leaf retention was measured visually, on a score of 1 to 9 at 17, 19, 21, and 23 weeks after planting. Two full-sib families, and 1 half-sib family were selected based on the wide segregation for leaf retention. Ten good leaf retention and 10 poor leaf retention genotypes were selected from each of the families to serve as bulks for bulk segregant analysis (BSA) for the discovery of markers associated with leaf retention. For DNA isolation, 1-2g of young leaves was harvested from genotypes of all the 6 bulks and from all other genotype in each family. The leaves were dried for 24h in an oven at 48°C and then ground into a fine powder using a power drill and washed sand. DNA was isolated from 200mg using a miniprep version of the Dellaporta (1983) protocol. The bulks of high and low leaf retention were then created per family to give a total of 6 bulks. DNA from the bulks and the parents will be genotyped with the 650 available cassava SSR markers according to methods described by

Mba et al (2001), and is just beginning. Markers polymorphic in the bulks were employed to analyze individuals of the bulks and, where the polymorphism remained consistent in the individuals as with the bulks, the markers were analyzed in the entire family.

Results

Evaluation of leaf retention in the North coast of Colombia in more than 100 families led to the selection of three families for molecular analysis. Table 1 describes some statistics of leaf retention in the 3 families. It can be observed that leaf retention evaluated at 23 weeks after planting has the widest variability within families and is the most appropriate measurement for use in molecular analysis of the trait. The choice of 3 families for marker analysis is to identify markers that are useful across genetic backgrounds that can be used in a breeding program regardless of the parental genotypes.

Table 1. Descriptive statistics of 3 families evaluated for leaf retention in the Colombian North Coast at 17 (RF1), 19 (RF2), 21 (RF3) and 23 (RF4) weeks after planting.

Clon	Madre	Padre		RF1	RF2	RF3	RF4
CM 9775- 12	CM 7514- 7	MNGA 19	MAX	9.00	9.00	9.00	9.00
			MIN	8.00	6.00	4.00	2.00
			AVERAGE	8.87	8.25	7.53	6.51
			STD DEV	0.33	0.92	1.91	2.49
SM 2783- 45	SM 1511- 6		MAX	9.00	9.00	9.00	9.00
			MIN	8.00	6.00	4.00	3.00
			AVERAGE	8.74	7.61	6.55	5.71
			STD DEV	0.44	1.04	1.81	2.17
SM 2615- 70	CM 4365- 3		MAX	9.00	9.00	9.00	8.00
			MIN	7.00	7.00	4.00	3.00
			AVERAGE	8.76	8.06	6.85	5.21
			STD DEV	0.55	0.76	1.57	1.81

Conclusions

Genetic mapping of genes controlling leaf retention has been initiated using 2 full-sib and one self family and BSA. Markers identified to be associated with leaf retention will serve as a means to screen populations for the trait at CIAT before they are sent to the field.

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1.2.18 Mining the Primary Gene Pool of Cassava: Introgression of High Root Protein from Accessions of *Manihot esculenta* sub spp *Fabellifolia* and *Manihot Tristis* into Cassava

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Introduction

As a major staple food crop across the tropics, cassava can serve as a cheap means of deploying adequate protein requirement amongst the poor and for feeding animals. A major effort has therefore been embarked upon to increase protein content of cassava roots. An advanced back cross QTL (ABC-QTL) to introgress high protein content from wild relatives into cassava is in its third year at CIAT. The first year saw the evaluation of wild relatives of cassava for high protein content and in the second year, inter-specific hybridization between selected high protein lines and some improved elite parents, including some yellow varieties. Wild by wild crosses were also carried out to investigate if protein content can be further increased by combining favorable alleles from different populations or species of the wild accessions. The inter-specific hybrids were evaluated in a seedling trial this year.

Other activities this year include an amino acid profile of root flour from 4 wild and inter-specific accessions and standardization of the SDS-PAGE methodology for the determination of size and isolation of root protein from high protein accessions, towards a proper characterization of the protein. During this year also, cassava varieties that were found to be high in protein from an evaluation conducted in 2001 were re-established in the field from tissue culture plants for another round of evaluations. If the previous results are confirmed, genetic crosses will be made with elite parents of the cassava gene pools for breeding high protein content and QTL mapping studies.

Methodology

Sexual seeds of inter-specific hybrids obtained from last year crosses were evaluated in a seedling trial this year. Total protein was measured in root flour, from 3 root per plant, using the Kjedahl method, in selected individuals of about half the total number of families. The decision to evaluate a fraction of the F_1 lines was due to cost considerations and size of roots, only genotypes with fairly large sized roots were used for protein measurement. In collaboration with the starch company AVEBE, amino acid profile was obtained from root flour from 1 genotype each of M.esculenta sub spp flabellifolia and M. tristis high in protein, as well as from 2 inter-specific hybrids high in protein. Pooled leaf flour sample from 10 cassava varieties with high protein was also analyzed.

Several protein extraction protocols were evaluated for SDS-PAGE analysis of root protein in cassava in order to select the most suitable one (Table 1). About100mg of root flour

was used in each case, suspended in a 500µl volume of sample buffer. The samples were centrifuged in an eppendorf tube for 5 min at 14000 rpm and the supernatant was transferred to a new eppendorf tube and, in the case of some protocols, mixed in a 1:1 (v:v) ratio with SDS-PAGE disruption buffer (Laemmli, 1970). Proteins were completely dissociated by immersing the samples in a boiling water bath for 5 min, then briefly centrifuging at 14000 rpm to pellet cellular debris. The resulting supernatants (total protein extracts) were stored at –20°C until SDS-PAGE analysis .

One-dimensional SDS-PAGE was run with 4.5% acrylamide (w/v) loading gel and 10%, 12%, 13.8%, 15% acrylamide (w/v) separation gel concentrations were tested (Table 2) using a Bio-Rad gel electrophoresis apparatus. The 4.5% acrylamide loading gel was prepared by mixing 9.0ml water, 2.25 ml acrylamide (30% solution), 3.75 ml Tris 1.5M pH: 6.8, 150µl SDS (10% solution), 150µl ammonium persulfate (10% solution) and 20µl TEMED and the casting (Laemmli,1970). Ten microliters of each sample was loaded per lane. Constant voltage of 50V was applied for 1 h at 10 °C and increased to 150V for the remaining duration of the run, until the tracking dye reached the gel mold. A Phaseolus vulgaris protein sample (Phaseolin type T) was used as a check in every protocol assayed. After electrophoresis, gels were fixed in 12% tri chloro acetic acid, then stained in a solution containing 2% phosphoric acid, 10% ammonium sulphate, 0.15% Coomassie Blue G, and 20% methanol for 12 hours. Gels were then destained in 20% methanol and scanned.

Table 1. Different extraction buffers for isolation of proteins in cassava root

Buffer	Reference
1M Tris-HCl pH: 7.5, 0.5M EDTA, 1% Ascorbic acid, 0.1M PMS	Gutierrez 2003,Personal communication
1a. 1M Tris-HCl pH: 7.5, 0.5M EDTA, 1% Ascorbic acid, 0.1M PMS and boiling for 5 min.	
0.005M Sodium phosphate, sucrose 5% 0.05% β-mercapthoethanol, pH 7.0	Suiter, 1988
0.1M KCl, 20 mM cystein, pH 7.3	Bourdon, 198
0.0625 M Tris-HCl, 2% SDS, 10% Glycerol, 5% (β-mercapthoethanol, 0.001 bromophenol	Laemmli, 1970
5.50mM Sodium phosphate	Shewry, 1992
5a.50mM Sodium Phosphate, 10mM PMSF, 10% PVP	Shewry, 1992
6. 0.5M NaCl, pH 3.2, 2mM EDTA, 2% SDS, 40% Sucrose, 1% (β-mercapthoethanol, 0.01% Bromophenol blue in 0.0625M Tris-HCl pH 6.8	CIAT, Gutierrez 2003, Personal communication

Table 2. Conditions of SDS-PAGE for the visualization of root protein in cassava

1.5M Tris^; 30% Acrylamide*	3.5M Tris^; 30% Acrylamide*		3.5M Tris^; 22.8% Acrylamide*	
	10%	15%	12%	13.6%
Water	37.5ml	23ml	33ml	11.88ml
Acrylamide*	30ml	50ml	40ml	54.72ml
Tris pH: 8.8	22.5ml	25ml	25ml	22.5ml
SDS (10% solution)	0.4ml	0.5ml	0.5ml	0.4ml
APS (10%solution)	0.4ml	0.5ml	0.5ml	0.2ml
TEMED	0.05ml	0.04ml	0.04ml	0.02ml

37:1 = w/w ratio of acrylamide to N,N'-methylene bis-acrylamide

Thirty one cassava varieties that were found to have between 6 and 8% of crude protein in their roots in an evaluation conducted in 2001 (CIAT 2002) were re-established again, from *in vitro* culture for a second year evaluation.

Results

A total of 4,271 sexual seeds organized into 58 families were obtained from inter-specific crosses between high protein accessions of M. esculenta sub spp flabellifolia, M. tristis and elite parents of the cassava gene pool. An evaluation of root protein content was made of 579 genotyopes , based on root size, and a summary of the results are shown in Table 3. Results reveal that some wild genotypes such as OW 231-3, 280-2, OW132-2, and OW284-1 have good general combing ability for root protein content. It was also observed that crosses between 2 wild parents, both high in protein (WW), had more uniform high root protein progenies, compared to wild by cultivated crosses, suggesting that a number of genes for protein content might be recessive or additive. The inter-specific hybrids are being evaluated for a second year in a single row trial (SRT) experiment. Amino acid profile revealed very high amounts of arginine, about half the total amount of amino acids, and low levels of methionine and lysine in the roots, but high levels in leaves.

Table 3. Descriptive statistics of % Protein, based on dried root basis, in 31 inter-specific families . Genotypes OW230, 231, 180, 181, 189, are accessions of M. esculenta sub spp flabellifolia, while Genotype OW280, 284, 132, 131, 146 are accessions of M. tristis

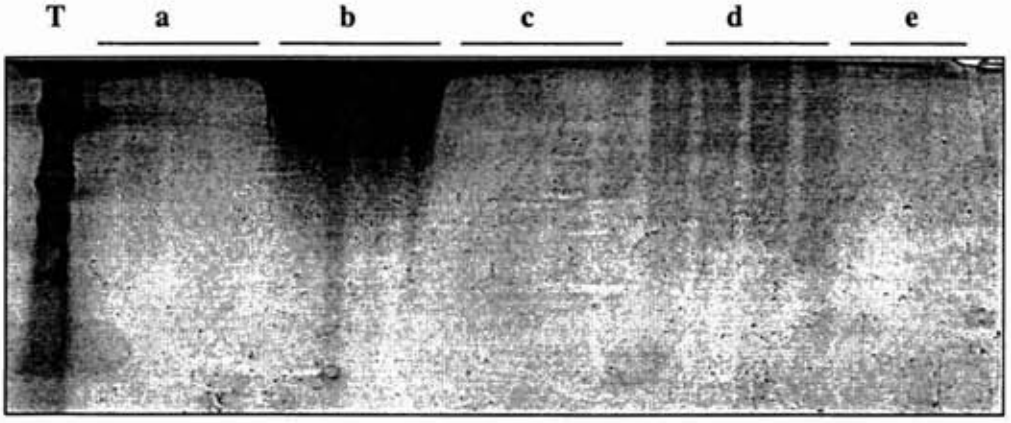
Family	Mother	Father	Size	Maximum	Minimum	Average	Std. Deviation
CW 205	OW 231-3	MTAI 8	8	10.96	5.37	8.11	2.80
WW 14- 51	OW 181- 2	OW 280- 2	58	10.49	4.39	6.42	1.47
WW 40- 1	OW 284- 1	OW 280- 2	76	10.46	4.04	6.28	1.26
WW 22- 3	OW 231- 3	OW 240- 8	82	9.72	3.19	5.37	1.21
WW 41- 9	OW 284- 1	OW 146- 1	41	9.70	3.26	6.21	1.51
CW 177-1	OW 132-2	CM 1585- 13	63	8.52	3.75	5.71	1.04
CW 161	CW 56- 5	OW 189- 1	3	8.03	5.37	6.64	1.29
CW 160- 1	CW 56- 5	OW 181- 2	3	7.86	4.63	6.06	1.64
CW 179-1	OW 132-2	MTAI 8	17	7.86	3.80	5.65	1.17
WW 24- 2	OW 231- 3	OW 280- 2	35	7.80	3.42	5.63	1.09
CW 99- 26	CW 30- 29	OW 280- 1	30	7.66	3.85	5.27	0.87
CW 185	OW 180- 1	MTAI 8	6	7.58	3.10	5.13	1.66
WW 3-1	OW 132-2	OW 240-6	6	7.32	4.05	5.77	1.43
WW 39- 3	OW 280- 1	OW 280- 2	23	7.27	3.54	5.46	1.06
CW 256- 1	MCOL 1734	OW 280- 1	9	7.20	2.87	4.86	1.63
CW 200- 1	OW 230- 3	CW 47- 3	5	7.10	4.00	5.04	1.09
CW 201- 2	OW 230- 3	CW 56- 5	5	7.10	4.00	5.60	1.15
CW 73- 2	CM 1585- 13	OW 284- 1	12	6.84	4.27	4.97	0.86
CW 198	OW 230- 3	CW 30- 65	31	6.77	3.97	5.30	0.73
CW 212- 1	OW 284- 1	MCOL 1734	7	6.53	4.40	5.25	0.66
CW 251- 2	MCOL 1734	OW 189- 1	5	6.46	5.22	5.89	0.55
CW 203- 1	OW 230- 4	CW 48- 1	3	6.37	5.29	5.76	0.55
CW 184- 2	OW 180- 1	MCOL 1734	5	6.27	3.01	4.73	1.33
CW 204- 1	OW 231- 3	AM 244- 31	5	6.25	4.46	5.04	0.75
CW 183	OW 180- 1	CW 48- 1	2	6.05	4.66	5.36	0.98
WW 21- 10	OW 231- 3	OW 240- 6	14	5.86	3.11	4.74	1.00
WW 20- 2	OW 231- 3	OW 146- 1	8	5.74	3.30	4.98	0.90
CW 202- 2	OW 230- 4	CW 30- 73	4	5.66	4.02	4.61	0.72
WW 9- 1	OW 180- 1	OW 234- 2	2	5.58	5.39	5.48	0.13
CW 186- 1	OW 181- 2	CM 1585- 13	4	5.27	3.72	4.69	0.67
WW 19- 2	OW 230- 3	OW 240- 8	7	4.80	3.01	3.72	0.96
Total			579				

Table 4. Total protein and Amino acid profile of root flour from 2 high protein inter-specific hybrids, 2 high protein *M. escuenta* sub spp *flabellifolia* accessions used as parent and a pooled leaf sample of 10 high leaf protein cassava varieties

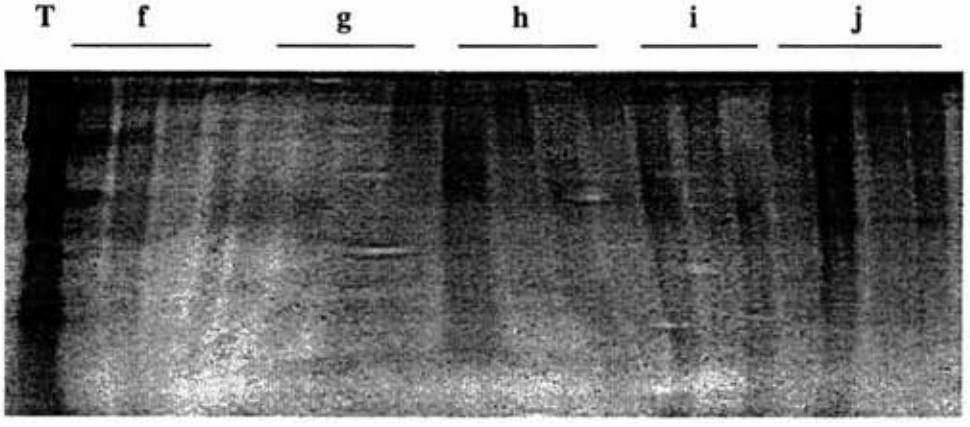
Variety:	OW235-3 g/kg	CW66-18 g/kg	CW66-48 g/kg	OW132-2 g/kg	Leaf (pooled) g/kg
N	15.7	13.8	14.9	18.7	35.5
CP=N*6.25	98	86	93	117	222
Cys	0.3	0.6	0.3	0.4	2.9
Met	0.6	0.2	0.5	0.5	3.5
Asp	2.1	1.3	1.6	2.7	22.2
Thr	1.1	0.5	0.8	1.1	9.4
Ser	1	0.6	0.8	1.2	10.8
Glu	7.7	4.7	8.6	9.7	31
Pro	0.9	0.6	1	1.1	10.3
Gly	0.9	0.7	0.8	1.1	10.7
ala	1.5	0.9	1.4	2.2	12.1
val	1.3	0.6	1	1.2	12.2
ile	0.8	0.5	0.7	0.8	9.5
leu	1	0.8	1	1.4	17.2
tyr	0.5	0.4	0.5	0.1	8.1
phe	0.7	0.5	0.7	0.9	11.4
gaba	0.9	0.7	0.8	1.5	2
his	1.1	0.9	1	1.5	4.7
ornt	1.3	0.6	3.8	1.8	0.1
lys	1.5	1.5	1.5	1.8	12.3
arg	27	28.5	23.8	27	11.1
trp	0.6	0.3	0.4	0.4	4.4
sum ex gaba, ornt	50.6	44.1	46.4	55.1	203.8
sumAA/CP	0.52	0.51	0.50	0.47	0.92
sumAA/N	3.23	3.20	3.12	2.94	5.74

To get a better understanding of these unusual amino acid profile preliminary SDS-PAGE analysis of the protein was conducted on the 4 high protein samples. Extraction buffers number 3, and 5 gave the best quality protein extract as demonstrated by the visualization of some bands (Fig.1). Gel concentrations of 13,8 and 12% were more suitable in resolving bands. The bands obtained are still quite faint and need to be improved before proper band sizing and protein isolation can be conducted. The success of SDS-PAGE relies upon selection of the buffer which in turns depends of the characteristics of the

tissue. The ratio of buffer to tissue needs to be optimized in materials with low content of proteins like cassava root flour. Efforts continue to standardize the SDS-PAGE protocol for root proteins in cassava.



T: Phaseolus (control)
a: samples with buffer 1
b: samples with buffer 1a
c: samples with buffer 2
d: samples with buffer 3
e: samples with buffer 4



T: Phaseolus (control)
f: samples with buffer 5
g: samples with buffer 5a
h: samples with buffer 6
i: samples with buffer 6a
j: samples with buffer 5b

Fig. 1. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total proteins from root of four cassava cultivars. In order to standardize the protocol in cassava different extraction buffers were evaluated.

A group of 33 genotypes, 5 plants each, that showed high root protein in an analysis conducted in 2001 were requested from the genetic resource unit and sent to the green house for hardening (Table 5).

Table 5. List of cassava varieties with high protein content in an evaluation conducted in 2001 that are to be re-evaluated again this year.

Clone and protein (%)		Clone and protein (%)		Clone and protein (%)	
CM 5620-3	8.31	MCOL 2436	6.25	MBRA 101	5.94
SM 1406-1	8.13	MBRA 26	6.25	MCOL 219	5.94
MCOL 689B	7.75	MCR 136	6.13	MGUA 33	5.94
MCOL 1563	7.38	MGUA 9	6.13	CM 7310-1	5.88
MGUA 76	6.94	MGUA 91	6.06	MCOL 678	5.88
MCR 142	6.63	MMEX108	6.06	MMEX 95	5.81
CM 696-1	6.44	SM 629-6	6.00	MGUA 79	5.81
CM 3199-1	6.44	SM 673-1	6.00	MBRA 300	5.75
SM 734-5	6.44	MCOL 2532	6.00	MCOL 2459	5.75
MCR 38	6.31	MGUA 19	6.00	MBRA 1384	5.75
MGUA 86	6.31	CM 3236-3	5.94	MCOL 2694	5.75

Conclusions

This year, inter-specific hybrids from wild relatives with high root protein and cassava were evaluated and results reveal the consistency of the trait, amino acid profile and preliminary SDS-PAGE analysis was also conducted with the root proteins. Future perspectives include genetic back crosses, to cassava, of inter-specific hybrids with high protein and evaluation of putative high root protein cassava varieties

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1.2.19 Mining the Primary Gene Pool: Green Mites (CGM) Resistance Genes from *Manihot esculenta* sub spp *Fabellifolia*

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Introduction

Following a very dry spell in January 2002 at CIAT Palmira and a subsequent heavy incidence of the green mites, 4 inter-specific hybrid families, CW68, CW65, CW67, and CW66, from the wild *Manihot* accession MFLA 437- 007 showed a very high level of resistance to the green mites with an almost equal number of susceptible(score of 3-4) and resistant (score of 1-2) genotypes. Bulk segregant analysis (BSA) was used to identify 4 SSR markers NS74; NS217; NS260; SSRY330 polymorphic in the bulks and individuals of the inter-specific families resistant to green mites (CIAT 2002). At the same time, an attempt was made to transfer the resistance observed in the inter-specific families to elite cassava parents. A total of 45 BC_1 families were developed, planted in a seedling trial, and a preliminary evaluation for mites conducted last season. Selected individuals of these BC_1 families were extensively crossed to CMD resistant parents used for MAS at CIAT, to combine CMD and CGM resistance in progenitors meant for Africa, to produce BC_2 families. The BC_1 progenies were also cloned and planted in a single row trial for the evaluation of resistance to green mites during the dry spell early next year. We describe here evaluation of the putative markers in the inter-specific families, and the development of BC_2 families from selected BC_1 individuals.

Methodology

The SSR markers polymorphic in the bulks were evaluated in the 4 F_1 inter-specific families and a simple regression analysis conducted using Microsoft excel. The markers found to explain a significant part of phenotypic variance of CGM resistance in the analysis of the 4 F_1 families were then analyzed in the parents of the 45 BC_1 families.. SSR analysis of the 4 markers was as described by Mba et al (2001). The BC_1 families were also evaluated for resistance to mites, a rather preliminary evaluation since only one plant is available per genotype. Based upon the preliminary CGM resistance evaluation of the 45 BC_1 a large number of putatively resistant BC_1 progenies were crossed to CMD resistant parents for the generation of BC_2 families from which CMD and CGM resistant lines can be selected from for the generation of parents for breeding in African gene pools.

Table 1. Sexual seeds of BC_2 families produced that combines resistance to CGM and CMD resistance

ITEM	Father	Mother	Code	No. of Seeds
1	C-4	CW74-1	A1	94
2	C-4	CW236-14	A2	102
3	C-4	CW235-8	A3	135
4	C-4	CW234-12	A4	105
5	C-4	CW234-19	A5	6
6	C-4	CW235-72	A6	17
7	C-6	CW235-2	A7	21
8	C-6	CW232-8	A8	11
9	C-6	CW213-1	A9	3
10	C-6	CW234-17	A10	6
11	C-19	CW234-17	A11	6
12	C-19	CW235-8	A12	23
13	C-33	CW235-2	A13	5
14	C-33	CW234-12	A14	38
15	C-33	CW217-7	A15	12
16	C-33	CW232-8	A16	9
17	C-33	CW258-17	A17	12
18	C-33	CW235-100	A18	15
19	C-39	CW235-8	A19	8
20	C-39	CW257-25	A20	54
21	C-39	CW258-17	A21	5
22	C-127	CW234-8	A22	27
23	C-127	CW234-17	A23	12
24	C-127	CW234-19	A24	25
25	C-127	CW234-32	A25	51
26	C-127	CW235-2	A26	9
27	C-127	CW235-8	A27	16
28	C-127	CW235-51	A28	2
29	C-127	CW235-72	A29	2
30	C-127	CW235-100	A30	14
31	C-127	CW258-19	A31	16
32	C-243	CW234-17	A32	2
33	C-243	CW234-19	A33	5
34	C-243	CW235-2	A34	2
35	C-243	CW257-10	A35	10
36	CW219-3	C-127	A36	1
TOTAL				881

Results

Simple regression analysis of SSR markers NS74; NS217; NS260; SSRY330 in the 4 inter-specific families had coefficients (R^2) of 46%, 30%, 30%, and 5% in the 4 families respectively. The surprisingly low regression coefficients for resistance that is apparently controlled by a major gene (CIAT 2002) might be due to the single year evaluation of these families for CGM resistance upon which the analysis was based. CGM incidence tends to have focal points therefore a large part of phenotypic variance in an evaluation is due to the environment. The best way to avoid this is to evaluate over 2 or 3 growing cycles. A second more in depth evaluation of these families is being carried out in Santander the Quilichao over two dry seasons, a period of 18 months September 2002 until March 2004. The preliminary evaluation of the BC1 families is being repeated this year in a clonal observation trial before further regression analysis is carried out.

An evaluation of the SSR markers associated with CGM resistance in the inter-specific hybrids in the parents of the BC_1 families revealed that the bands associated with CGM resistance were not always polymorphic between the parents of the back cross populations. An effort was therefore initiated to identify more markers linked to CGM resistance, BSA, using additional SSR and RAPD markers is being used to identify additional markers for evaluation of resistance. Transfer of a gene to other crosses via MAS using a single marker associated with the gene is fraught with problems of linkage disequilibrum, based upon the frequency of that allele in the gene pool. Many more markers are required to eliminate the problems of linkage disequilibrum encountered in diverse genetic backgrounds.

Individuals of the BC_1 families that appeared to possess resistance to CGM in focal points of great CGM damage in the seedling trial were crossed to CMD resistant parents to obtain recombinants that carry CMD and CGM resistance. Table 2 shows the number of seeds by families of the new crosses. These crosses have been established in vitro from embryo axes to enable it to be shared with collaborators in Africa once CMD and CGM resistance have been confirmed by MAS and phenotypic evaluation respectively.

Conclusions

Markers associated with CGM resistance in bulks of 4 inter-specific families were evaluated in the entire families and rather low regression coefficients were found. This is most likely due to the environmental effect in the phenotypic data. BC2 families have been generated from BC1 individuals towards an introgression of this resistance into elite cassava gene pools.

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1.2.20 Depelopment of a novel approach for the analysis of diallel mating designs for better understanding the inheritance of quantitative traits

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As stated in the report from the previous year, three diallel mating designs have been evaluated for the three main target environments (sub-humid environments, acid soil savannas and mid-altitute valleys). Diallel trials were planted in 2001 and in the northern coast and acido soil savannas also in 2002. The relevance of these studies certainly lies on the information they will produce. However, it is important to emphasize that these are diallel studies that offer new alternatives to extract information and requires development of new models for their analysis. In this section a brief description of this innovative approach will be described. This research will serve as thesis work for Ph.D students from Vietnam and Uganda (a female and a male, respectively), as well as for a M.S. thesis for one of the assistants working in the project.

Theoretical model for analyzing inter and intra-family variation. The most important difference between these diallels and the traditional ones is the fact that thirty clones were used to represent each cross, and individual measurements were made for each individual genotype making up the full-sib cross. Most diallels will harvest all the individual genotypes for each cross ignoring the within-family variation. This is because the main target is identifying good families or parents. However in the case of cassava breeding the individual genotype (clone) is the central point of attention whereas in other crops such as maize, the main target is the family or F1-cross. It is important to emphasize the relevance of this distinction because the current method of selection (particularly in the first stage of selection, called *Clonal EvaluationTrials*) the cassva breeding project is trying to develop a more systematic evaluation process in which we select on the family basis (all the clones derived from a given parental line), and then the best clones from that family (the individual clone that will eventuall be released 8-10 years leater). One of the several questions these diallels aim at answering is what is the relative genetic variability between and within families. This is obviously fundamental for a more scientific process of selection. In other words, should we focus in developing good families of clones or should we concentrate on developing families of clones that have large gentic variability to be exploited.

Table 1 illustrates the progress made in developing a quantitative genetic model for the analyses of the diallels. The information presented was exposed to Dr. José Miranda Branco Filho who is an eminent quantitative geneticist who co-authored with Arnell

Hallauer the most widely used book on the subject (Quantitative Genetics in Maize Breeding, Iowa State University Press). The issue of the model and alternative ways of using the information is still underway and there are excellent perspectives that these analyses will in fact be a relevant contribution in the area of quantitative genetics. That is, the studies will hopefully not only be useful for cassava but also for other crops as well.

Selections for the Mid-altitude valleys. Tables 2 and 3 present the results of the combined analysis of variance for the two diallel evaluations conducted in the mid altitude valleys environment and the values for GCA effects, respectively. One sriking result in this analysis is the significante of SCA effects, which are related to the dominance of heterosis present in the hybrid clones evaluated in the studies.

In Table 3 a more precise estimate for GCA effects that the one presented previously in 2002 is presented. The information provided is a good example of the complexities involved in cassava breeding. The parent generating the best yielding progenies was MPER 183 (the highest positive GCA value) but it showed very deficient performances regarding Harvest Index and Dry Mtter Cntent (both with negative GCA estimates). On the other hand CM 6740-7 has an excellent GCA estimate for dry matter but intermediate performance regarding fresh root yield and harvest index. The clone SM 1741-1 has an good overall performance as a parent, particularly in relation to dry matter content.

Selections for the Sub-Humid Tropical Environment. Following the same criteria for the previous section Tables 4 and 5 present the relevant results from the diallel studies for the sub-humid environmtents. There are some interesting differences between the two data sets. The most striking one is that here SCA effects did not reach statistical siginificance (Table 4). This may be the result of the strong dry spell in this environment, which has a strong effect on dry matter content. For the other traits both GCA and SCA showed highly significant statistical differences.

Table 5 summarizes the GCA effects for each of the nine parents involved in this diallell. As in the previous cases, it is obvious that there is no perfect parental line. Good GCA for yield productivity is generally associated with a negative value for dry matter content and/or harvest index, and viceversa. It was disappointing to see the poor performance of SM 1565-17 regarding dry matter content (GCA = - 1.467). One pf the promising clones for this environment was SM 1411-5, whose breeding value based on the results in Table 5, is clearly highlighted.

Table 1. Theoretical model for the quantitative genetic analisis of diallel crosses in cassava. In addition to the usual variation among F1 crosses, within family variation has been included. They were considered fixed and random genetic effects, respectively.

Source of variation	Degrees of freedom	Mean squares expectations
Locations (L)	a-1	
Rep/alocations	a(r-1)	
Among F1 crosses	[p(p-1)/2]-1	$S^2_e + gS^2_e + grS^2_{\text{Among F1 x L}} + graS^2_{\text{Among F1}}$
GCA	p-1	$S^2_e + gS^2_e + rS^2_{\text{SCA x L}} + r(p-2)\,S^2_{\text{GCA x L}} + raS^2_{ACE} + ra(p-2)\,S^2_{GCA}$
SCA	p(p-3)/2	$S^2_e + gS^2_e + rS^2_{\text{SCA x L}} + raS^2_{SCA}$
Within F1 crosses	(p(p-1)/2)(g-1)	$S^2_e + rS^2_{\text{Within F1 x L}} + raS^2_{\text{Within F1}}$
Among F1 crosses x L	(a-1)([p(p-1)/2]-1)	$S^2_e + gS^2_e + grS^2_{\text{Among F1 x L}}$
GCA x L	(a-1)(p-1)	$S^2_e + gS^2_e + rS^2_{\text{SCA x L}} + r(p-2)\,S^2_{\text{GCA x L}}$
SCA x L	(a-1)(p(p-3)/2)	$S^2_e + gS^2_e + rS^2_{\text{SCA x L}}$
Error (a)	a([p(p-1)/2]-1)(r-1)	$S^2_e + gS^2_e$
Within F1 crosses x L	(p(p-1)/2)(g-1)(a-1)	$S^2_e + rS^2_{\text{Within F1 x L}}$
Error (b)	a(p(p-1)/2)(g-1)(r-1)	S^2_e

Where:

a = number of locations
p = number of progenitors in the diallel
$S^2_{\text{Among F1}}$ = Variation among averages for each F1 cross
S^2_{GCA} = general combining ability
S^2_e = experimental error type a
$S^2_{\text{.....x L}}$ = interaction of each effect with the environment

r = number of replications within location
g = number of clones within each F1 cross
$S^2_{\text{Within F1}}$ = Variation among clones within F1 crosses
S^2_{SCA} = specific combining ability
S^2_e = experimental error type b

Table 2. Combined analisis of variance for a diallel study conducted in the mid altitude valleys at Palmira and Jamundí (Valle del Cauca), Colombia. Mean square sfor yield (kg / plant), Harvest index and dry matter content are presented only for the among crosses component of the study.

Source of variation	df	Mean Squares					
		Fresh root yield (kg / pl)		Harves Index (0-1)		Dry matter (%)	
Locations (L)	1	3.949	ns	0.496	*	563.608	**
Rep/ L	4	17.579		0.028		19.009	
Among F1s	35	3.125	**	0.009	**	9.685	**
GCA	8	5.760	*	0.028	**	26.855	ns
SCA	27	2.461	**	0.004	**	4.597	**
Among F1s x L	35	0.645	ns	0.001	**	3.200	**
GCA x L	8	1.392	**	0.002	**	11.304	**
SCA x L	27	0.424	ns	0.001	*	0.799	ns
Error	140	0.447		0.001		0.899	

Table 3. General combining ability (GCA) effects from the diallel study in two mid-altitude valley environments. Nine progenitors were involved in the diallel design.

Clon	Fresh root yield (kg/pl)	Harvest Index (0-1)	Dry matter content (%)
CM 6740-7	0.003	-0.009	0.608
SM 1219-9	0.341	0.024	-0.572
SM 1278-2	-0.426	0.010	0.914
SM 1636-24	-0.314	-0.022	-0.464
SM 1673-10	-0.308	0.007	0.641
SM 1741-1	0.052	0.037	1.069
HMC 1	-0.313	0.015	-0.444
M ECU 72	0.341	-0.048	-1.079
MPER 183	0.624	-0.014	-0.672
St.Dev. Gi	0.172	0.0060	0.489
St. Dev. (GI – Gj)	0.257	0.0097	0.734

Table 4. Combined analisis of variance for a diallel study conducted in the sub-humid environments at Pitalito and Santo Tomás (Atlántico), Colombia. Mean square sfor yield (kg / plant), Harvest index and dry matter content are presented only for the among crosses component of the study.

Source of variation	df	Mean Squares					
		Fresh root yield (kg / pl)		Harves Index (0-1)		Dry matter (%)	
Locations (L)	1	3.341		0.258	*	118.418	
Rep/ L	4	0.732		0.022		21.699	
Among F1s	35	1.140	**	0.009	**	6.744	**
GCA	8	2.976	**	0.021	*	20.974	**
SCA	27	0.596	**	0.006	**	2.528	ns
Among F1s x L	35	0.366	**	0.003	**	2.356	**
GCA x L	8	0.797	**	0.008	**	5.642	**
SCA x L	27	0.238		0.002	*	1.383	**
Error	140	0.157		0.001		0.677	

Table 5. General combining ability (GCA) effects from the diallel study in two sub-humid environments. Nine progenitors were involved in the diallel design.

Clon	Fresh root yield (kg/pl)	Harvest Index (0-1)	Dry matter content (%)
MTAI 8	0.004	-0.011	0.053
CM 6754 - 8	-0.372	-0.001	0.241
CM 8027 - 3	-0.126	-0.005	0.858
SM 805- 15	-0.438	-0.032	0.055
SM 1565- 17	0.267	0.039	-1.467
SM 1411- 5	0.139	-0.019	0.919
SM 1219- 9	0.220	-0.010	-0.032
SM 1657- 12	0.020	0.010	-0.280
SM 1665- 2	0.286	0.028	-0.347
St.Dev. Gi	0.130	0.013	0.345
St. Dev. (GI – Gj)	0.195	0.019	0.518

Selections for the Acid-Soil Savannas Environment. Results from the diallels at CORPOICA – La Libertad for the acid soils environments (Table 6) indicate a strong interaction with the environment. In fact this makes sense because the two locations had sharp contrast regarding their soils. One had more stressful savanna conditions and the other had alluvial soils, which support excellent yields and not as severe disease development. The large interactions with the environment (error term for the main genetic

effects) resulted in some of them not being statistically significant. Individual location analysis would yield highly significant statistical differences.

Table 6. Combined analisis of variance for a diallel study conducted in the acid soil savannas at CORPOICA La Libertad(Meta), Colombia. Mean square sfor yield (kg / plant), Harvest index and dry matter content are presented only for the among crosses component of the study.

Source of variation	df	Mean Squares					
		Fresh root yield		Harves Index		Dry matter	
Locations (L)	1	172.011	**	0.263		16.988	**
Rep/ L	4	3.743		0.060		0.551	
Among Fls	44	0.631		0.015	*	0.076	*
GCA	9	1.462		0.048		0.190	
SCA	35	0.417		0.007		0.047	*
Among Fls x L	44	0.476	**	0.008	**	0.042	**
GCA x L	9	1.244	**	0.023	**	0.102	**
SCA x L	35	0.279		0.005		0.026	
Error	176	0.189		0.003		0.019	

Table 7. General combining ability (GCA) effects from the diallel study in the acid soil savannas environments. Ten progenitors were involved in the diallel design.

Clon	Fresh root yield (kg/pl)	Harvest Index (0-1)	Dry matter content (%)
CM 4574 – 7	0.184	-0.001	1.106
CM 6740 – 7	0.076	-0.008	0.182
CM 7033 – 3	-0.110	-0.006	-0.348
SM 1219 – 9	0.089	0.029	0.676
SM 1565 – 15	-0.073	-0.004	1.434
SM 2058 – 2	0.078	0.006	-0.301
SM 2219 – 11	0.276	0.041	0.593
HMC 1	-0.127	0.019	-0.032
MPER 183	-0.326	-0.077	-3.422
MTAI 8	-0.067	0.000	0.114
St.Dev. Gi	-0.067	0.000	0.114
St. Dev. (GI – Gj)	0.216	0.029	0.062

As expected CM 4574-7 had a good performance as a parent with positive GCA for yield and, particularly, for dry matter content (Table 7). However, better that CM 4574-7 was SM 2219-11 with positive GCA values for the three variables evaluated.

Illustration of the kind of additional information that this analysis could provide. Below several examples are given on the way the additional information based on the within F1 family variation could provide. The model is taking advantage of the fact that a single genotype can be vegetatively propagated. Therefore each genotype (clone) was planted in two (mid-altitude valleys) or three (acid soil savannas and sub-humid environments) locations, with three replications in each location. An error exists for the within family variation which is not ordinarily available in diallel studies. In other words, it is possible to estimate the genetic component of the within family variation. That information can be contrasted with the one obtained from the among-F1 families variation and if differences are large, then circunstancial evidence of the occurrence of strong epistatic effects can be produced.

Moreover, individual analysis of each variable can help identifying interesting events that otherwise would go unnoticed. For instance, averages for each F1 family and standard deviations (within each F1 family) can be ploted. This information can be very useful and as far as it is known the kind of analysis has never been described in the literature. The basic idea is that parental lines that have many dominant alleles affecting a given trait will yield more homogeneous progenies than a progenitor with recessive alleles. This is the principle in which the graphic approach for diallel analysis (Hayman 1954a; 1954b; 1958; Mather and Jinks, 1971).

The first example turns around the reaction to white flies evaluated in the diallel experiment evaluated in Jamundí (Valle del Cauca, Colombia). Figure 1 shows the result for the averages of the F1 crosses as well as the variation (standard deviation) among the 30 clones that made up each F1 cross.

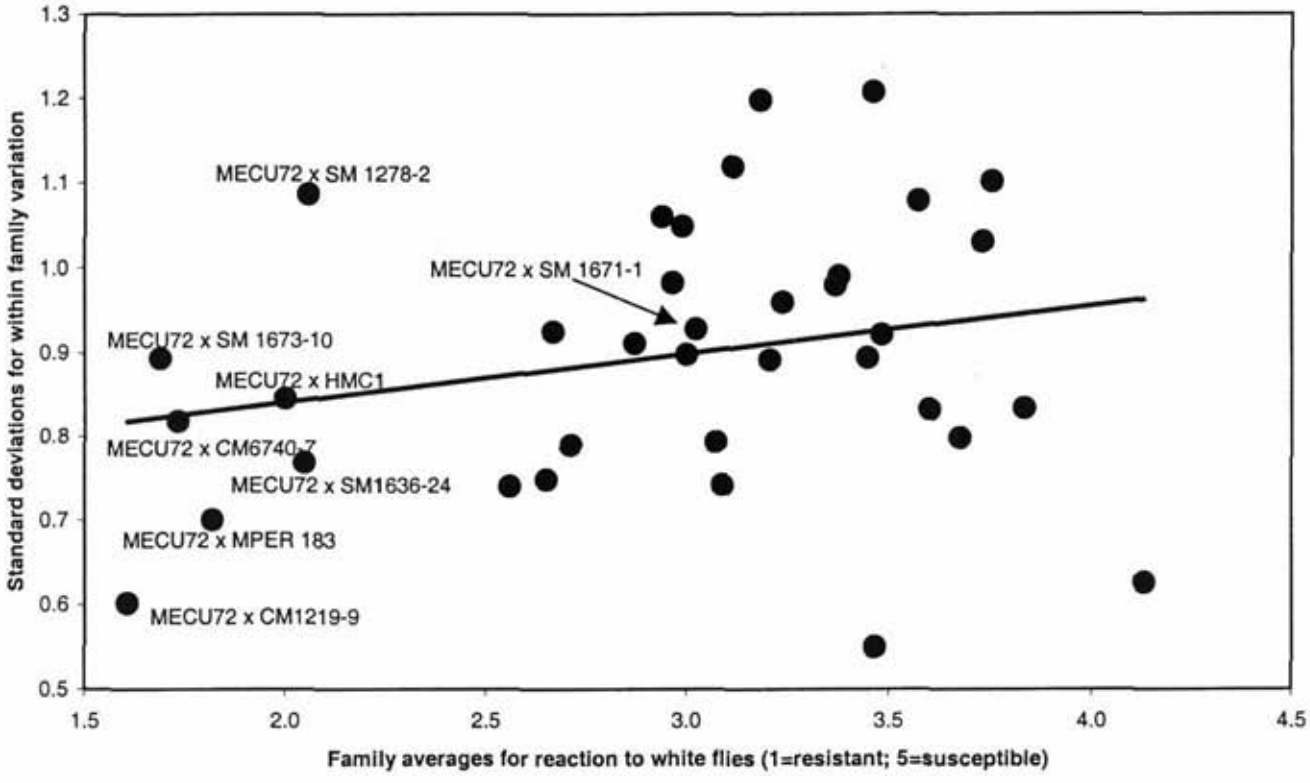


Figure 1. Averages and standard deviations for the reaction to white flies in a diallel study evaluated in Jamundí, Valle del Cauca, Colombia. Data points for the 8 different progenies with MECU72 as common parent are identified.

In Figure 1 the variation in the response to white flies attacks is clearly illustrated through the values along the horizontal axis. Lower scores meant more resistant phenotypes. Higher scores indicate susceptible reaction. It was very interesting to see the location of the progenies from MECU 72 who has been shown to have resistance (antibiosis) against the white flies. As expected most of the progenies from MECU 72 where at the left of the figure, indicating a resistant reaction to the insect. This is as much as a traditional diallel analysis could go. What is interesting and innovative is the possibility to analyze the

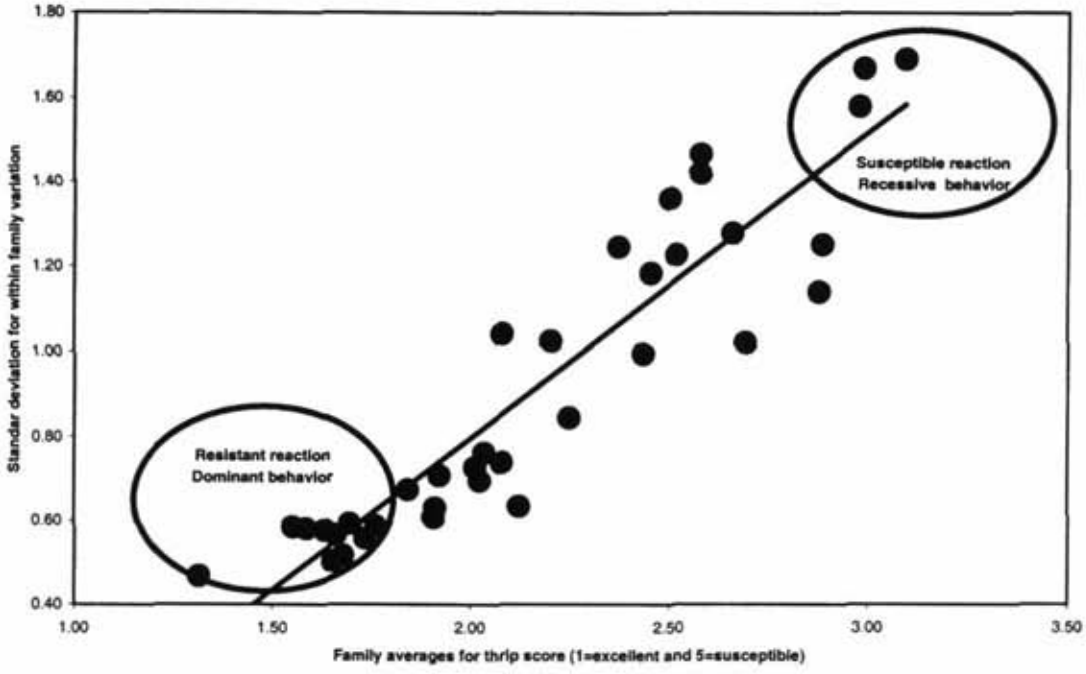
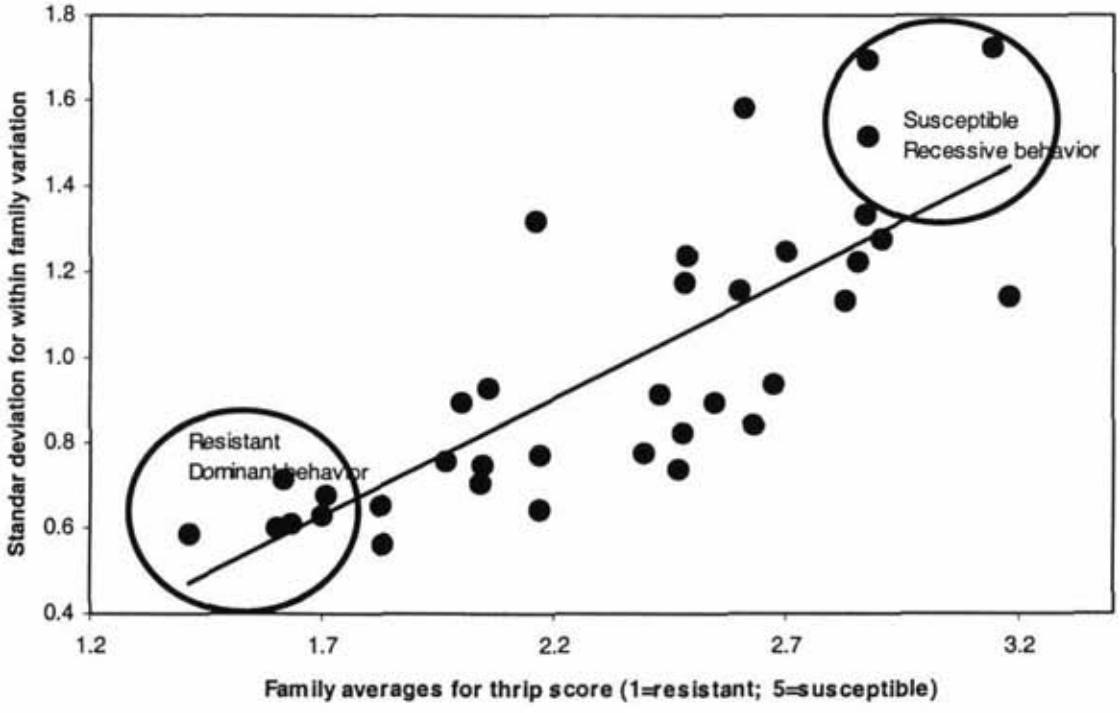


Figure 2.Averages and standard deviations from a dialllel study at Santo Tomás (above) and Ptialito



(below) in the Altántico Department for the reaction to thrips (1=resistant; 5=susceptible). Dominant and recessive "behavior" does not refer to the traditional meaingin for dominance and recessiveness. Dominance refers either to a trait where non-additive effects are important or cases where the parental lines may have loci at the homozygous dominant status.

variation around the standard deviations within families. Some crosses had very small standard deviations (MECU72 x CM1219-9) indicating a very uniform family. Other progenies (MECU72 x SM1278-2) have a much higher within familiy variation. This contrast is extremely interesting for understanding the genetic structure for relevant traits.

Continuing with reactions to pests, Figure 2 illustrates the results from the diallels targeting the sub-humid conditions (different progenitors that those for mid-altitude valleys and acid soil savannas). These results are typical of the trends that can be obtained by putting together the averages for each family and the standard deviation within families. First, there is a clear consistency between the results of the two locations, with an upward trend at the right of the figures. The results agree with the expectations, clones with lower averages (resistant to mites) show a more uniform progenies (smaller standard deviations) than the susceptible ones at the right of the figures. The tendencies observed in these plots could be explained as an statistical artifact: families with larger values are likely to have larger standard deviations. However, as will be shown below, for other traits the opposite is observed. In Figure 3 the plots for dry matter content (%) from one of the locations for the diallel for the sub-humid (Santo Tomás, Atlántico Department) and acid soil savannas environment (CORPOICA-La Libertad, Meta Department) are presented. Results for the other respective locations yielded similar results.

In this case families tended to be more uniform with higher averages. In other words the magnitude of the averages do not necessarily dictate the magnitude of the standard deviations within each family. Families with high dry matter content tended to be clearly more uniform. This could be envisioned, perhaps, as a result of the continuos selection for increased dry matter content. Most of the germplasm would have the key alleles for high dry matter, the exception being the opposite. When a progeny has an unusually low dry matter content, then the variability within family tends to increase.

It is interesting to note that similar trends were observed for this variable in the three different ecosystems and for all the locations in which they were evaluated. There is a consistency in the way each variable appear in this kind of graphs, which is further suggest that they may be an interesting new approach for analyzing genetic variability and identifying relevant cases where further studies are justified.

Harvest index is another variable that consistently showed the same tendency as dry matter content, with reduced variability within family at higher levels of the index. As in the case of dry matter content the long selection process for optimum harvest index (Kawano, 2003). Thisprocess could serve to explain why high harvest indices result in reduced variabily. In other words, high harvest index is the rule at least among the elite parental lines used in producing the diallel families.

Finally root yield showed a different tendency with increased variability within family in those F1 crosses that showed the highest averages. As in the case of the previous variables, this trend was shared by the three different ecosystems targeted and for each of the

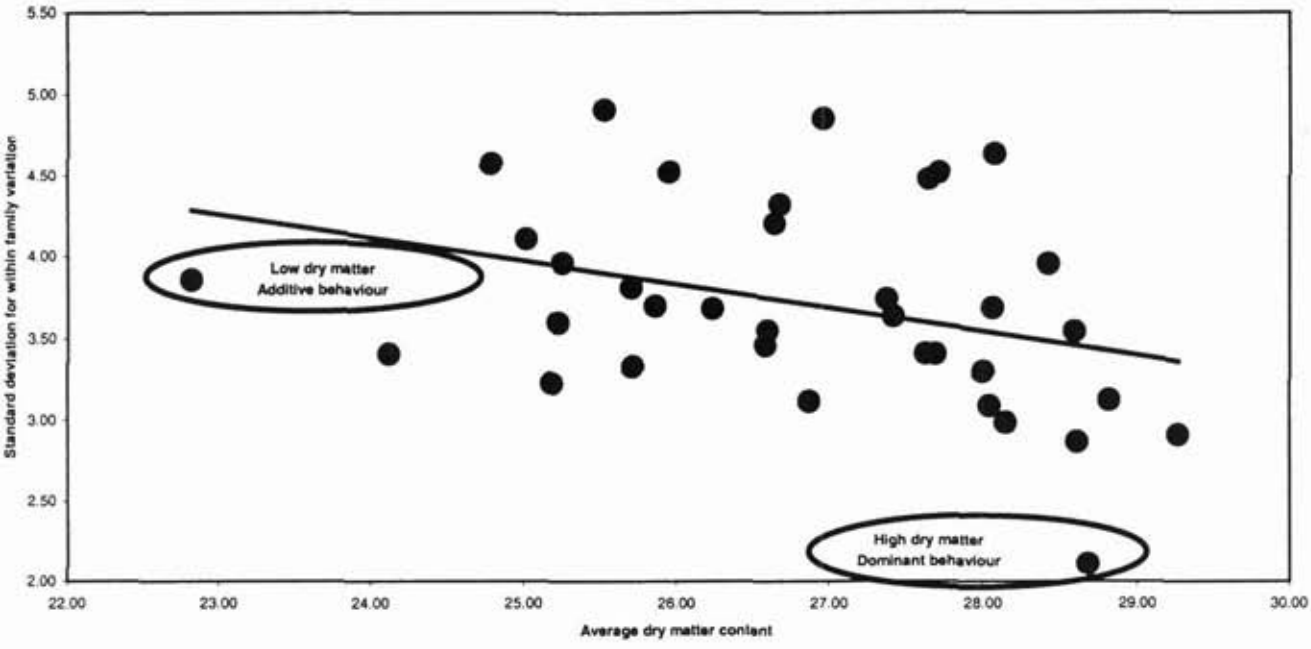
locations within each ecosystem. Figure 4 illustrates the results for root yield (kg/plant) for one of the locations at each target environment. It is clear that a common trend exists, with families with low productivity showing a "dominance behavior" that does not necessarily mean that root yield is a recessive trait.
It is clear from the information provided in Figures 3 and 4 that interesting differences are found among and within the families involved in the diallel studies. Is is also evident the consistency with which each variable responds to the analysis. Further study will be conducted by determining the identity of each family in the plos (as done for white flies).

Concluding remarks regarding diallel analysis. Our knowledge on the inheritance of agronomically relevant traits in cassava is still very limited. The most interesting result of the analyses on diallels described above can be summarized as follows:

Innovative approach from the quantitative genetics point of view. The studies will hopefully provide advances in this area of research with a more complete model for explaining genetic variability, model that can be related to actual data. It is interesting, therefore, that cassava may eventually come to provide tools for genetic analyses of other vegetatively propagated crops.

The graphic deployment of data facilitates the identification of contrasting families deserving a more detailed study. That is the case, for example, illustrated in Figure 1 among the progenies from MECU 72.

In general the statistical and graphic analysis provide an ideal group of genotypes that can be used for molecular analysis. This is for instance, what CIAT is currently doing with a group of genotypes that showed a contrasting phenotype regarding leaf retention.



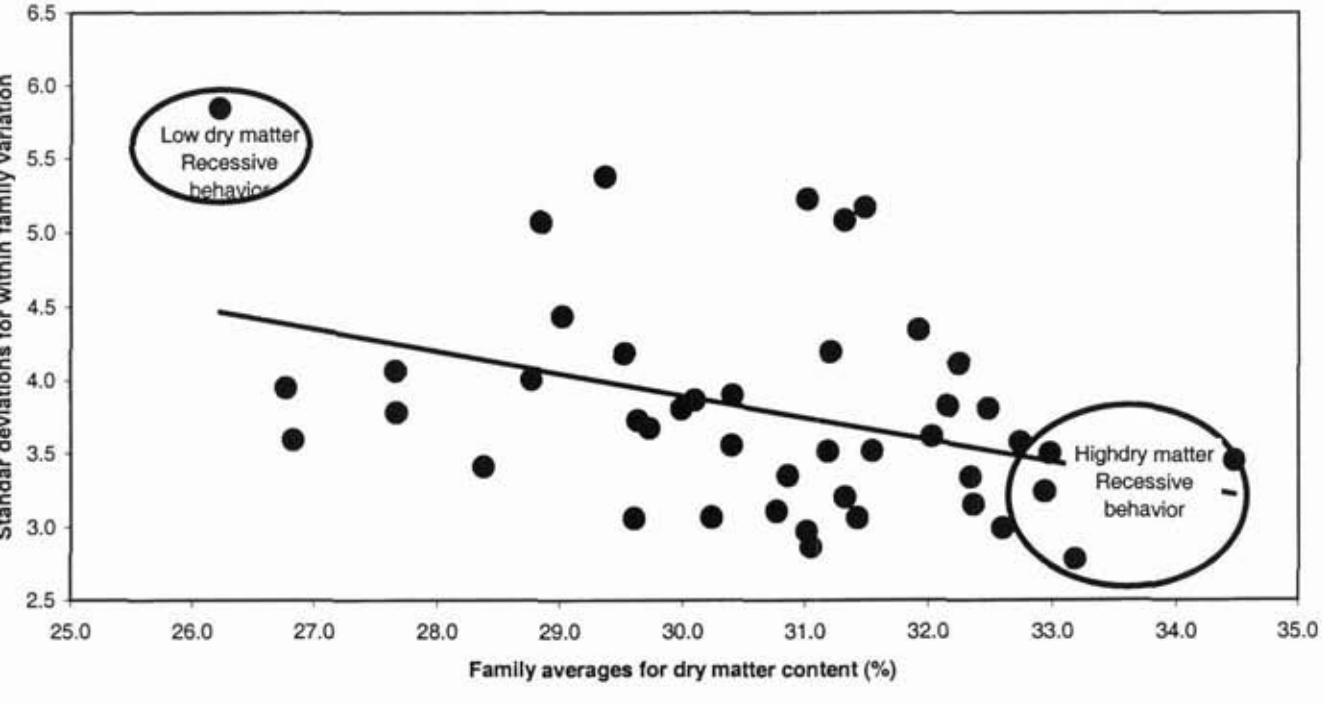


Figure 3. Averages and standard deviations from a diallel study at Santo Tomás (above) and Acid Soil Savannas (below) in the Altántico and Meta Departments, respectively.

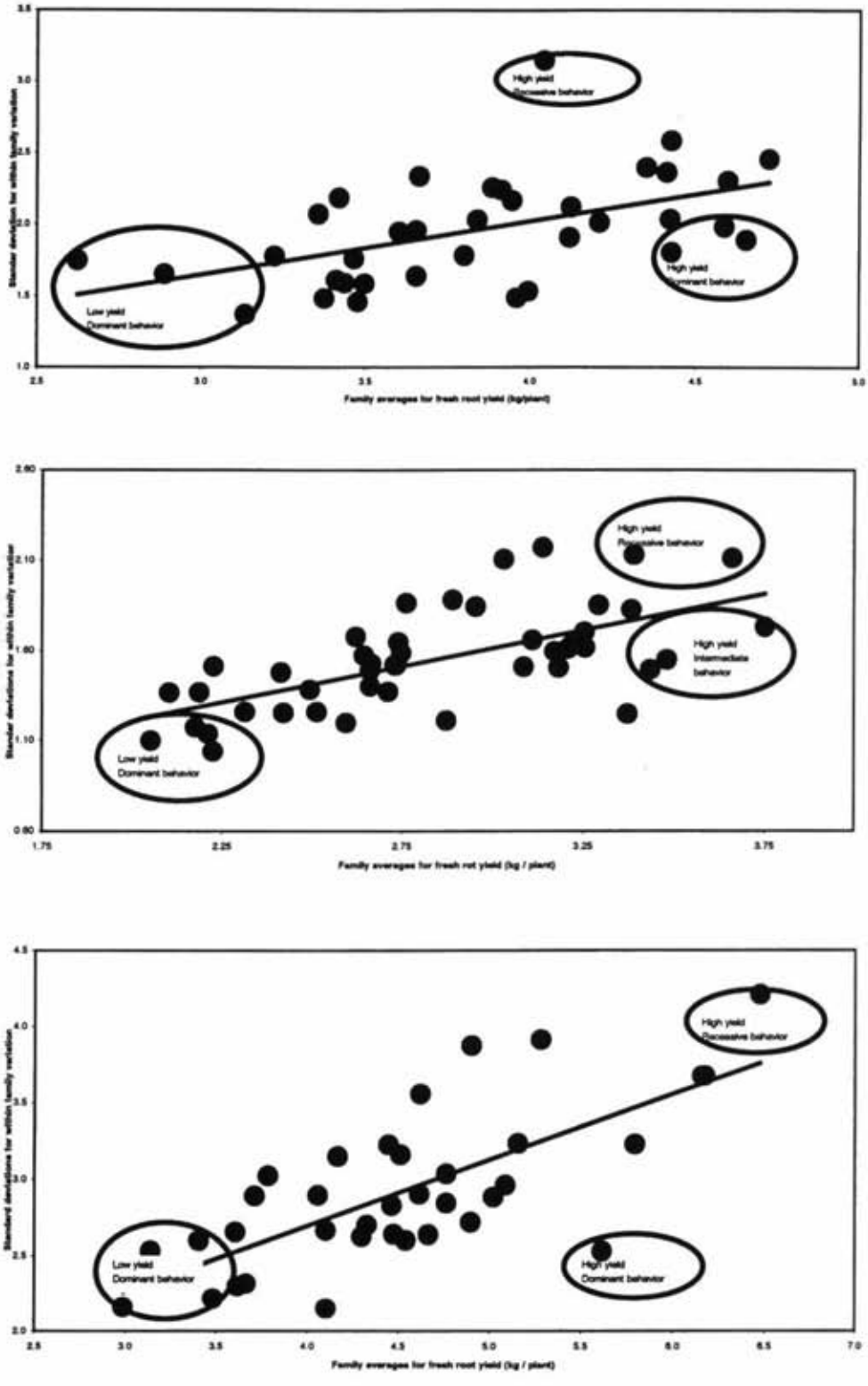


Figure 4. Averages and standard deviations from a diallel study at Pitalito (above), CORPOICA La Libertad (middle) and Palmira (below) in the sub-humid, acid soils and mid altitude valleys, respectively.

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1.2.21 Introduction of inbreeding in cassava

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For many years cassava research at CIAT has been interested in introducing inbreeding in cassava. The advantages of inbreeding can be summarized as follows:

Because no inbreeding is carried out, a sizable genetic load (undesirable or deleterious genes) is expected to prevent the crop fully achieving its actual yield potential.

Since there are no clearly defined populations (quantitative genetics sense) allelic frequencies cannot be efficiently modified.

Because the highly heterozygous nature of the crop, dominance effects are likely to play a very important role in the performance of materials being selected. The current scheme can exploit dominance effects because, once an elite clone is identified, it can be propagated vegetatively (therefore carrying along the dominance effects). However, it is the same elite clones that frequently are selected as progenitors for the production of new segregating material. In that case, the current procedure has a bias because the breeding value of these clones are unlikely to be well correlated with their performance *per se*, precisely because of the distorting effects of dominance. In other words, good clones are *just found* not *designed.*

Production of recombinant seed is cumbersome in cassava. Only 0.6 viable seeds per pollination are produced. It takes about 18 months since a given cross is planned until an adequate amount of seed is produced.

When a desirable trait is identified, it is very difficult to transfer it from one genotype to another (even if a single gene controlled the trait). The backcross scheme, one of the most common, successful and powerful breeding schemes for cultivated crops, is not feasible in cassava, because of the constant heterozygous state used throughout the breeding process.

The lack of inbreeding in cassava implies a very restricted genetic variability based on recessive traits. Commercially important mutants such as those found and exploited in maize (waxy, floury, high-quality-protein, sweet corn, popcorn, etc.) are not known in cassava. It is not clear if cassava has or not this kind of useful mutants, but it is obvious that if they existed the breeding scheme employed did not facilitate their identification since the heterozygous nature of the crop significantly reduced the chances of the homozygosity required for the expression of recessive traits.

The use of totally inbred material (as parents in the production of hybrids which will then be propagated vegetatively) would greatly facilitate the exchange of germplasm among different cassava breeding projects. Currently this exchange takes place as in vitro plants, which is expensive, time-consuming, and by its very nature restricted to a few genotypes. Botanical seed of inbred genotypes will breed true, and therefore the genotype can be trasnfered in this way without the genetic segregation that occurs from non-inbred materials. This will effectively reduce the relative isolation in which cassava-breeding projects currently operate.

CIAT has begun the production of inbred cassava material (currently at 50 and 75% homozygosity). All the elite clones identified through the cassava-breeding project are going to be used for recombination (to produce new segregating populations), but also will be self-pollinated to initiate an S2 recurrent selection process to: a) reduce the inbreeding depression in cassava (≈ reduce genetic load); b) indentify useful recessive traits of commercial (i.e. waxy roots), nutritional (acyanogenesis) or agronomic (reduced post harvest deterioration) relevance. Paralel to this a special project has been approved for the development of a protocol for the production of doubled-haploids from cassava anthers. Table 8 summarizes the results of transplanted S_2 seedlings in the Palmira Experimental Station in August 2003. A large number of seedlings (expected average of homozygosity of 75%) were produced and transplanted.

Table 8. Results of S_2 seedlings transplanted to the field. These materials are the result of a second consecutive self-pollination (average of 75% homozygosity).

Family	Progenitor	Seeds produced	Seeds germinated	Seedlings trasplanted	% Germ.
AM 247	CM 507-37	50	4	2	8.0
AM 262	CM 2772-3	80	27	21	33.8
AM 273	MCOL 72	71	37	36	52.1
AM 298	CM 6754-8	34	28	27	82.4
AM 320	MTAI 8	444	340	340	76.6
AM 321	CG 1141-1	47	29	29	61.7
AM 322	CM 4365-3	49	29	29	59.2
AM 323	CM 4574-7	14	8	8	57.1
AM 324	CM 4919-1	36	19	19	52.8
AM 326	CM 6921-3	36	30	30	83.3
AM 328	CM 7514-8	23	13	13	56.5
AM 329	SM 805-15	28	13	13	46.4
AM 330	SM 909-25	56	37	37	66.1
AM 331	SM 1219-9	520	339	339	65.2
AM 332	SM 1411-5	25	11	11	44.0
AM 333	SM 1438-2	19	12	12	63.2
AM 334	SM 1460-1	519	378	378	72.8
AM 335	SM 1511-6	493	418	418	84.8
AM 336	SM 1565-15	434	334	334	77.0
AM 337	SM 1665-2	506	444	444	87.7
AM 338	SM 1669-5	440	355	355	80.7
AM 339	SM 1669-7	241	230	230	95.4
AM 340	SM 1741-1	362	215	215	59.4
AM 341	SM 1778-45	23	21	21	91.3
AM 342	MTAI 16	35	23	23	65.7
AM 343	CM 3306-4	62	32	32	51.6
TOTAL		4649	3426	3416	62.0

During the first semester of 2004 these 3461 plants will be harvested. In the process extensive evaluations will be performed in search of useful traits. This is could eventually be a turning point for cassava research at CIAT.

1.2.22 Development of a male-sterile Nipponbare population

International Rice Functional Genomics Consortium
Cesar P.Martinez, James Carabali, J. Borrero and J.Tohme
SB-2 Project
Funding: USDA and CIAT core

Introduction

An international consortium of geneticists, molecular biologists and information scientists from Yale University, Cold Spring Harbor Laboratories, Brookhaven National laboratory, and CIAT was assembled to address the following specific goals:
To generate an extensive collection of rice lines, each containing an independent, dispersed insertion of a genetically-engineered Ds transposon;
To determine the chromosomal position of each insertion by sequence of its flanking genomic DNA;
To establish a database of that relates lines, sequences and phenotipic information;
To publicly distribute mutant lines and associated informatics.
By making use of this public information, research scientists worldwide can rapidly identify mutant alleles in genes of agronomic importance for functional genomic studies and crop improvement.

Materials and Methods

A major CIAT involvement in this project is to produce foundation seed of stable rice lines containing the Ds insertions. All experiments are carried out in Oryza sativa ssp japonica cv Nipponbare. To produce T1 seed, stock plants (provided by Yale University) will be crossed as males to wild type female plants in the CIAT nursery. Efficient outcrossing can be achieved using a male-sterile female line.

Since male-sterility in Nipponbare was not presently available a backcross- breeding scheme shown in Figure 1 was used to introgress this trait into Nipponbare. A nuclear male-sterility allele (msms) found in IR36 (provided by GSKhush from IRRI) was used as the donor parent. The Nipponbare male-sterile version will be used for the production of foundation seed from each transposition selection (T1 seed) produced in this project.

A simplified crossing method described by Sarkarung(1991) was used. A seed sample from the IR36 source segregating for male sterility was planted under field conditions in CIAT; male-sterile plants were phenotipically identified at flowering time and used as the female parent. F2 seed was harvested from F1 plants and grown in the field for the identification of male-sterile plants, which were backcrossed to Nipponbare to produce BC1F1 seed. A second BC to Nipponbare was done and the BC2F2 population was grown in the field to allow the identification of male-sterile plants, which were used to produce the BC3F1 seed, and subsequently the BC4F1. This seed was planted again to produce the

BC4F2 generation to check for segregation of the sterility trait. Male-sterile plants very similar to Nipponbare were identified. However, some segregation in terms of plant height, tillering, flowering time, and presence of awns was observed. Therefore, another BC to Nipponbare was done to obtain a more uniform population. From here on, the male-sterile Nipponbare population will be maintained by growing it in isolation to allow open pollination. Just seed from the male-sterile plants will be harvested to maintain this population.

Seed increase of several genetic stocks was also done during the course of this project, and sent to Yale University for use in transformation experiments.
Future plans: Field/greenhouse evaluations of transformed lines carrying stable Ds insertions.

1.2.23 Utilization of New Alleles from Wild Rice Species to Improve Cultivated Rice in Latin America

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SB-2 Project
Funding: CIAT core funds , Ministerio Agricultura Colombia

Summary

Statistical analysis on the performance of F8 inter specific lines from the cross BG90-2/*O.rufipogon* over eleven locations in Latin America indicated no significant difference in grain yield between Bg90-2 and its progenies. However, some of the progenies yielded 15-24 % more than BG90-2. Stability analysis showed that all lines were stable across environments. Analysis of the molecular data showed that all lines had introgressions (2.6-23%) from *O.rufipogon;* more introgressions were found in chromosomes 2, 5, 7, 12, and 3. No correlation was found between number of introgressions and grain yield. Markers RM5 and RM1 were found in some of the top yielder lines but 52% of markers detected in the F8 lines were not found in the F2 generation.

Introduction

In spite of the great impact made in rice production in Latin America (LAC) there is a need to increase it in a sustainable way to meet increasing demand. New alleles can provide genetic variability for crop enhancement. There is wide genetic variability available in rice, but limited use of this variability has been made. It has been shown (Xiao et al, 1998; Moncada et al, 2001; Tanksley and McCouch, 1997; Thompson et al, 2003) that the Oryza wild species represent a potential source of new alleles for improving the yield, quality and stress resistance of cultivated rice. These studies indicated that *O.rufipogon* possesses new alleles on chromosomes 1 and 2 with positive effect on yield and yield components. However, these studies were conducted on a limited scale since early segregating populations (BC2F2) and few replications (1-2 sites) were used. No data are available confirming that yield advantages detected in the BC2F2 generation is pass on through generations of selection in pedigree nurseries, nor over a wide range of environments. This

report focuses on the performance and stability of advanced breeding lines derived from the cross Bg90-2/*O.rufipogon* across locations in LAC.

Materials and Methods

Twenty-five lines (BC2F8) from the cross Bg90-2/*O. rufipogon*, derived from the BC2F2 generation following the pedigree method were planted in replicated yield trials in eleven locations under irrigated conditions (seven sites in Colombia and one each in Argentina, Surinam, Uruguay and Venezuela). This work was done in close collaboration with key partners from the national rice programs and private sector. Transplanting was done in CIAT whilst direct seeding was done elsewhere. A completely randomized design with three reps was used and crop management was based on recommended local agronomic practices. Varieties grown locally were used as checks. Data on main agronomic traits, including grain yield, was taken. A two-way analysis of variance was used for the analysis of grain yield, whilst a GEBEI package that implements appropiate clustering and ordination procedures and AMMI model were used in the analysis of the GxE data.
DNA of young leaves from the parental genotypes and their progenies was extracted by the Dellaporta method (McCouch et al. 1988) modified for PCR assay by CIAT Biotechnology Unit. Subsequent molecular assays were performed using 76 SSRs
Traits for yield and yield-related characters were associated with the 76 molecular markers using simple single point analysis.

Results

Data are presented in Figure 1 and Table 1. Statistical analysis showed no significant difference in grain yield between Bg90-2 and its progeny over all locations. Although none of the interspecific lines out yielded Bg90-2 in all locations, several lines performed better than Bg90-2 in each location. Analysis of the GxE (data not shown) indicated that contrasting and distinct environments were included in these trials and that the GxE interaction was high (75%). This suggests that the performance of genotypes was dependent on the climatic/soil conditions in each location and that there was a good level of genetic variability present in this group of lines, which explains the better performance of some progenies under specific conditions. This is very important for breeding purposes since the genetic variability hidden in this population was only observed when the progenies were exposed to a diverse set of climatic/soil conditions found in different rice growing areas.

Analysis of molecular data from the bulked seed sample of the BC2F8 lines shows that all of them had introgressions derived from *O.rufipogon* (Table 1). The number of introgressions ranged from 2(2.6%) to 18 (23%). More introgressions were detected in chromosomes 2(14), 5(9) and 7,12,3(7); chromosomes 4 and 10 had two introgressions. There was no correlation between number of introgressions and grain yield (Figure 1). Markers RM5 and RM1, located on chromosome 1, were found in some of the highest yielding lines.

Molecular data from the BC2F2 generation (Annual Report 2001) were compared with that of BC2F8. Only 46% of the 76 SSRs were detected in both generations, suggesting that some markers were lost during the phenotypic selection carried out through the generation advance. This was expected. However, 52% of the markers detected in the BC2F8 lines were not detected earlier in the BC2F2 generation. This could be explained because plants sampled for molecular analysis and agronomic characterization were different. On the other hand, only 48% of markers detected in BC2F8 lines are somewhat associated with yield or yield components.

Molecular data confirmed that all breeding lines had introgressions from *O.rufipogon*. This wild species is known to have a high level of genetic variability and is adapted to diverse climatic/soil conditions. Statistical analysis on grain yield and performance through eleven locations in LAC indicated that there were contrasting differences among them. Therefore, breeding lines were subjected to diverse biotic and abiotic conditions, including high disease incidence (Villavicencio, Saldaña, Jamundi), acid and infertile soils (Villavicencio, Jamundi), cold stress (Argentina, Uruguay) and good climatic conditions (Monteria, Aceituno). There were excellent and poor environments for rice production. However, all lines did better than BG90-2, the improved/ recurrent parent; some of the lines (8,13,12, 1,15, 21, 9, 19, 3) yielded between 15-24 % more than BG90-

2. The stability analysis, based on the method described by Eberhart and Russell indicated that all lines were stable across environments. Data suggest that the superior performance of inter specific breeding lines is due to favorable allele introgressions derived from *O.rufipogon*.

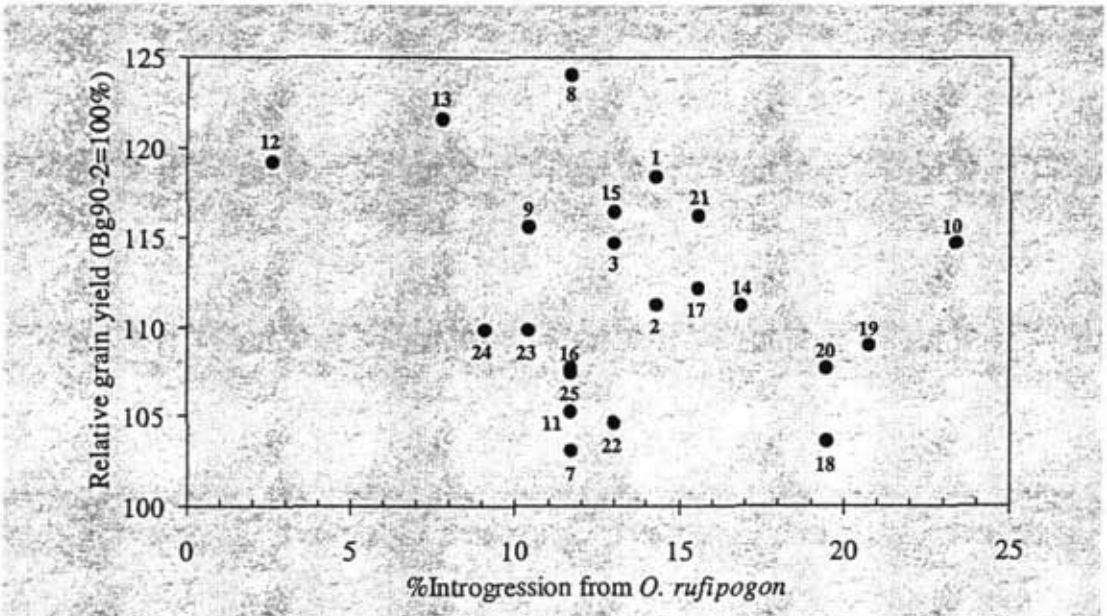

Figure 1. Relation between relative yield and percentage of introgression from *O. rufipogon* (all locations included)

Table 1. Grain yield (t/ha) and percentage of introgression from *O. rufipogon* of advanced lines from the Bg90-2/*O. rufipogon* cross.

Pedigree	Bulk sample		Yield (t/ha)											
	No. Loci *O.r*	%*O.r*	Aceituno	Armero	CIAT	Jamundí	Montería	Saldaña	Villavicencio	Argentina	Surinam	Uruguay	Venezuela	O
01.CT13941-11-M-25-1-M-M	11	14.3	11.2	4.8	5.9	7.5	11.2	6.5	6.5	10.2	4.3	4.3	6.2	7.
02.CT13941-11-M-25-4-M-M	11	14.3	10.6	5.1	5.2	7.8	9.8	7.1	5.6	9.3	3.7	3.8	6.0	6.
03.CT13941-11-M-25-5-M-M	10	13.0	11.5	5.1	6.0	6.8	11.0	7.5	6.6	10.1	3.5	2.6	5.4	6.
07.CT13941-27-M-19-1-M-M	9	11.7	10.3	4.2	4.9	6.9	9.3	5.9	4.9	11.2	3.2	1.4	6.3	6.
08.CT13946-26-M-5-3-M-M	9	11.7	11.7	4.9	5.5	5.3	9.9	7.9	6.1	12.0	3.2	6.7	8.4	7.
09.CT13946-26-M-5-6-M-M	8	10.4	11.4	4.5	4.4	7.6	10.5	6.4	5.4	11.3	3.7	4.9	6.7	7.
10.CT13956-29-M-14-1-M-M	18	23.4	10.9	4.2	5.5	7.0	10.4	5.8	6.0	11.5	3.8	3.7	7.5	6.
11.CT13956-29-M-25-7-M-M	9	11.7	10.7	3.3	4.6	4.4	10.3	5.9	5.5	9.9	3.9	4.4	7.0	6.
12.CT13958-12-M-1-7-M-M	2	2.6	12.2	5.3	4.2	4.6	9.6	8.0	5.9	12.5	4.1	5.9	7.0	7.
13.CT13958-13-M-17-5-M-M	6	7.8	11.3	5.0	5.0	7.1	11.9	7.7	6.1	10.2	3.7	6.2	6.6	7.
14.CT13958-13-M-2-1-M-M	13	16.9	11.2	4.6	5.5	4.3	9.8	6.6	4.7	10.7	3.9	3.9	7.7	6.
15.CT13958-13-M-2-3-M-M	10	13.0	11.4	3.9	5.8	6.8	10.9	6.4	4.4	10.6	3.6	5.9	7.5	7.
16.CT13958-13-M-2-4-M-M	9	11.7	11.3	3.7	5.6	6.0	9.7	6.2	4.1	10.3	4.0	4.5	6.2	6.
17.CT13958-13-M-7-5-M-M	12	15.6	11.9	4.8	5.3	4.9	10.0	7.7	5.1	8.8	4.2	3.1	8.0	6.
18.CT13958-13-M-26-4-M-M	15	19.5	11.3	3.5	4.9	5.3	9.7	6.5	4.9	--	3.5	5.8	7.0	6.
19.CT13958-13-M-26-5-M-M	16	20.8	11.5	4.9	5.5	6.2	8.9	6.8	4.4	9.4	3.5	5.1	7.2	6.
20.CT13958-13-M-33-1-M-M	15	19.5	12.0	5.4	4.9	5.3	8.8	6.3	5.3	9.5	3.6	3.8	6.6	6.
21.CT13956-29-M-29-2-M-M	12	15.6	11.6	3.9	5.6	5.9	9.5	7.3	5.5	12.6	3.4	5.1	6.5	7.
22.CT13956-29-M-8-3-M-M	10	13.0	11.0	5.3	3.9	5.3	10.2	7.4	5.4	8.9	2.8	3.2	6.4	6.
23.CT13959-3-M-10-4-M-M	8	10.4	11.3	5.6	3.9	5.5	9.1	7.3	5.0	11.6	3.7	4.2	5.8	6.
24.CT13959-3-M-10-5-M-M	7	9.1	12.2	4.7	5.0	5.3	9.4	8.0	5.0	--	4.2	5.1	7.4	6.
25.CT13976-7-M-14-1-M-M	9	11.7	11.1	4.6	4.8	4.7	8.8	7.6	4.5	10.8	3.5	5.6	4.9	6.
26.BG90-2	--	--	10.8	3.8	4.9	5.7	9.4	7.8	4.3	--	3.5	3.2	7.0	6.
27.Fedearroz50	--	--	10.5	6.5	5.4	8.6	10.0	7.8	5.0	--	--	--	--	7.
Overall Means	--	--	11.3	4.7	5.1	6.1	9.9	7.0	5.3	10.6	3.7	4.4	6.8	6.

Future plans

Molecular and statistical analysis of the experiment comparing performance of different generations (BC2F3, BC3F5 and BC3F7).

Continue the development of NILs for marker assisted selection

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1.2.24 Identification of QTLs for yield and yield components in rice: Populations derived from backcrosses between the wild species (*Oryza barthii*) and cultivated rice (Lemont).

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Funding: CIAT

Abstract

Preliminary analysis done on a BC3F2 population from the cross Lemont/*O.barthii* showed that certain regions associated with important agronomic traits (putative QTLs) were identified. Chromosome 1 carries QTLs associated with grain yield and plant height, as reported in the literature. Likewise, chromosomes 3 and 8 are important for determining panicle length, perhaps one of the most relevant traits found in advanced breeding lines derived from the Lemont/*O.barthii* cross. Markers RM227 and RM114 on chromosome 3 are associated with a region derived from *O.barthii*. Markers RM184 and RM304 on chromosome 10 are associated with days to flower; however, this is a very complex trait as has been reported by others. Advanced lines with different flowering dates have been developed out of this cross. Further molecular work is needed to confirm several interesting regions detected in this analysis.

Introduction

Rice is one of the most important food crops in the word. The 21 wild species and 2 cultivated species (*Oryza sativa* and *O. glaberrima*) represent wide genetic variability for rice breeding programs. Most of this variability has not been used so far although several studies have suggested that Oryza wild species possess new alleles for improving cultivated rice. The main objective of this study was to focus on progress made in identifying quantitative trait loci (QTL) associated with yield and yield components in a BC3F2 population derided from a cross between a recurrent parent (Lemont) and a donor parent (*Oryza barthii*). The results of applying AB-QTL analysis to 327 individuals characterized with 113 microsatellite markers are described. Marker data were used to identify QTLs associated with yield and yield components.

Materials and methods

Lemont, which was the first commercial variety developed in Texas (USA) with an excellent industrial quality, was used as the recurrent parent in this study. *O. barthii* (Accession # 104119), which served as donor parent, is a relative of *O. sativa*. There was very high sterility so three backcrosses to Lemont were done. BC3F2 plants were selected based on phenotype, discarding plants with high sterility and long awns. The best 327 individuals were selected for agronomic and molecular characterization (Fig. 1).

DNA of young leaves was extracted from the parental genotypes and the 327 BC3F2 families, using the Dellaporta method (McCouch et al., 1988). The CIAT Biotechnology Research Unit modified this method for the PCR assay. A total of 113 microsatellite markers were used to evaluate the hybrid from the Lemont/*O. barthii*. Markers used in the evaluations and the QTL analyses were selected from the rice molecular framework linkage map (10-20 cM intervals throughout the genome) (Causse et al., 1994; Chen et al., 1997).

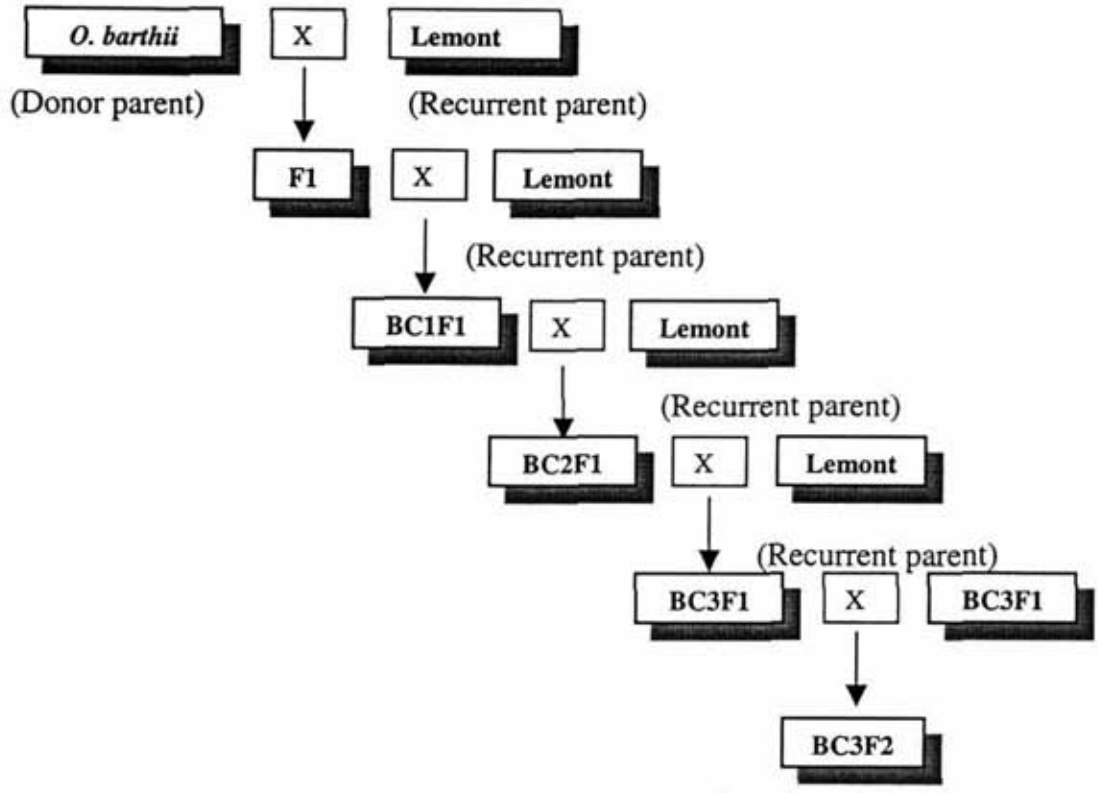

Figure 1. Generation of BC3F2 population, derived from the Lemont and *O. barthii* cross.

Results

The population of 327 BC3F2 families was analyzed using a total of 113 microsatellite markers (SSRs), distributed at approximately 10-cM intervals throughout the genome. The order of the SSRs in the molecular map was defined by the Cornell Published molecular rice map (Causse et al., 1994; Chen et al., 1997; Temnykh et al., 2000). In this experiment five agronomic traits including days to flower (dth), plant height (ph), panicle length (pl), percent sterility (ps) and yield per plant (yld) were evaluated. The association between phenotype and marker genotype was investigated using single-point analysis (SPA), interval mapping (IM) and composite interval mapping (CIM) using QTL Cartographer Vers. 1.17d software. A normality test showed that only the distribution of trait pl was normal (Fig. 2); thus it was indispensable for the correct use of aforementioned models to estimate significance thresholds with permutation tests.

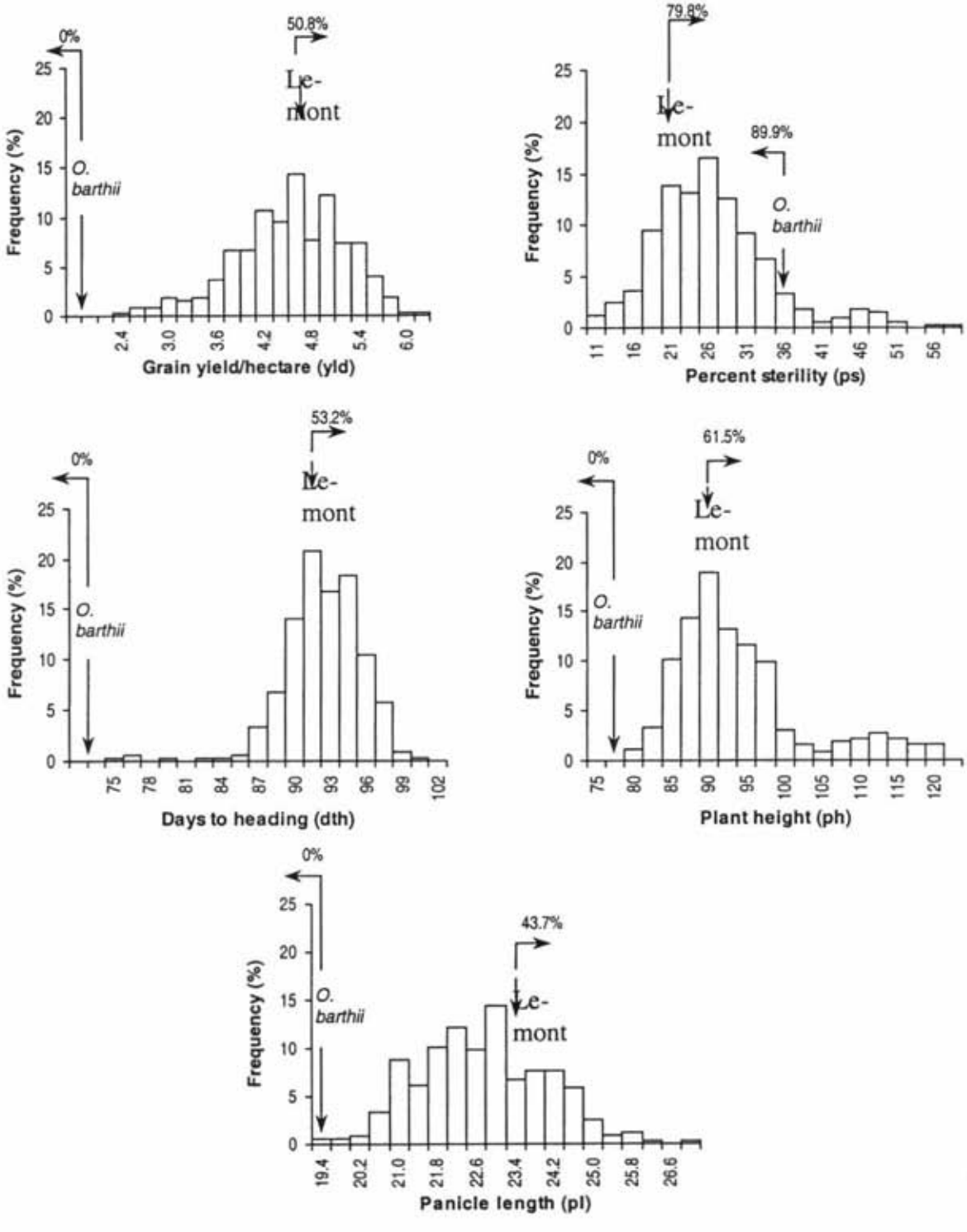

Figure 2. Frequency distribution of the five phenotypic traits.

Table 1. QTLs associated only with SPA analysis

Trait	Chromosome	Marker
dth	2	RM262
ph	1	RM259
pl	3	RM148
ps	8	RM25
yld	4	RM335

Table 2. QTLs not detected for SPA analysis

Trait	Chromosome	Marker
ph	2	RM109
	7	RM82
	8	RM42
ps	8	RM42

Moreover, permutations allow the estimation of experimentwise significance. For SPA, the threshold was established by doing 10,000 permutations, while thresholds, for IM and CIM were established by carrying out 1000 permutations. In chromosomes 4, 5, 6, 9 and 11, neither marker was associated with neither of the traits; while, in chromosomes 1, 2, 3, 7, 8, 10 and 12, there was several genetic regions associated with more than one trait. SPA detected QTLs that not were significant for IM and CIM (declared as true marker-trait associations not significant, type-I error) (table 1).Similarly, in some cases SPA did not detect QTLs that were associated for IM and CIM (low potency, type II-error) (Table 2).
The use of co-factors are particularly important because of their role in identifying some putative QTLs. Marker RM42 on chromosome 8 was associated with ph, pl and ps but not with yld. On chromosome 1, ph and yld were associated but with different markers. Table 3 shows the positions of QTLs that were identified as significant by all three analytical procedures.

Table 3. QTLs identified as significant by all three analytical procedures

Trait	Chromosome	Marker
dth	10	RM184, RM304
pl	3	RM227, RM114
	8	RM42, RM210
yld	1	RM9, RM5, RM140

The results analyzed in global form suggest that other genetic regions are also important. For example, those QTLs identified as significant simultaneously by two of the three models in contiguous zones of the same chromosome suggest a wide region that must be confirmed (Table 4).

Table 4. Genetic regions for confirming QTLs

Trait	Chromosome	Marker
ph	1	Between RM1-RM243
	2	Between RM208-RM48
	3	RM60
	7	RM82
	8	RM42
	12	Between RM247-RM117
pl	12	Between RM20-RM247
ps	8	RM42

This preliminary analysis indicates that certain regions associated with important agronomic traits (putative QTLs) in the Lemont/*O. barthii* cross have been identified. Chromosome 1 carries QTLs associated with grain yield and plant height, as reported in the literature. Likewise, chromosomes 3 and 8 are important for determining panicle length, perhaps one of the most relevant traits found in advanced breeding lines derived from the Lemont/*O.barthii* cross. Markers RM227 and RM114 on chromosome 3 are associated with a region derived from *O. barthii*. Markers RM184 and RM304 on chromosome 10 are associated with days to flower; however, this is a very complex trait as has been reported by others. Advanced lines with different flowering dates have been developed out of this cross. Further molecular work is needed to confirm several interesting regions detected in this analysis.

Ongoing activities

Cross: *O. barthii* and Lemont

Complete the characterization of agronomic and molecular data and QTL analyses to determine the number of QTLs associated with yield and alleles coming from *O. barthii*.
Evaluate more SSR markers in nonsaturated regions on the map (chromosome 5 and others) to elucidate QTLs associated with yield increase and verify which of the *O. barthii* alleles has been introgressed in this population.

Other projects

Complete the characterization of agronomic and molecular data and QTL analyses to determine the number of QTLs associated with yield increase across environments for Caiapo/*O. glaberrima* crosses.
Evaluate more SSR markers in nonsaturated regions on the map of the BC2F8 population to identify QTLs associated with yield increase and verify which of the *O. rufipogon* alleles has been introgressed into this population.
Initiate the analysis of agronomic and molecular data from different generations obtained of the Bg90-2/*O. rufipogon* cross to determine the environmental effect and to look for gene introgression from the wild species in these different populations.

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1.2.25 Associating Horn Worm Resistance in 60444 (MNG11) with Introgression from *Manihot Glaziovii*

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Introduction

The cassava hornworm (*Erinnyis ello*) is generally considered the most important pest of cassava inn the Americas (Bellotti 1981). High populations can defoliate large plantations in a short time. No naturally occurring resistance to this important pest has been found in the cassava germplasm collection. Recently, feeding experiments with leaves of the genotype 60444 (MNg11) transformed with the *Bacillus thurigensis* protein CRY1Ab, and leaves from non-transformed plants revealed a very high mortality of the hornworm when fed with non transgenic plants (Chavariaga et al. 2003, unpublished data). The above observation suggests a naturally occurring resistance to hornworm in the genotye 60444. This genotype was developed at the Moor plantation, Ibadan, Nigeria in the 1950s using third back cross derivatives of the inter-specific cross between cassava and *M. glaziovii* for the production of CMD resistant materials. Resistance to the hornworm could have been inadvertently introgressed from *M. glaziovii* (Bellotti 2003, personal communication).

Another progeny of the *M. glaziovii* back cross derivatives, TMS30572, was used in constructing the molecular genetic map of cassava. Regions suspected to be introgression of large chunks of the *M. glaziovii* genome, as demonstrated by suppression of recombinantion have been noted (Fregene et al. 1997). One of these regions on linkage group D has been shown to bear resistance QTLs for CMD and CBB. A study has therefore been initiated to associate this region of the genome with resistance to hornworm resistance.

Methodology

The genotype 60444 has not been used in breeding at CIAT and there are no remnant seeds from previous crosses for genetic studies of hornworm resistance, segregating populations will therefore be developed. The breeding program at the Moor plantation, Ibadan utilized principally open pollinated seeds in their breeding activities and the likelihood that some inbreeding may have occurred in 60444 cannot be ruled out. The development of segregating populations for mapping resistance to hornworm resistance has to take into consideration several possibilities of gene action. In light of this, a single full-sib family, by crossing MNg11 and the hornworm susceptible variety MCol 2215, and an S1 family, by selfing 60444 will be developed.

Results

Ten *in vitro* plants of the genotype 60444 were hardened in the screen house and transferred to the field for genetic crosses for the development of mapping populations. It is expected that seeds from the populations will be ready by May/June next year and sexual seeds from the families will immediately be germinated and transferred to the field. Once woody stakes will be harvested from the progenies once they become available and planted in the green house for the feeding studies.

Conclusions

A project to associate the linkage group D region of the cassava genome in the genotype 60444 with resistance to hornworm resistance has been initiated. It involves the generation of a full-sib and S_1 families from 60444 and their evaluation for hornworm resistance and molecular markers from the D region of the cassava genome.

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Activity 1.3 Development of molecular techniques for assessing genetic diversity and mapping useful genes

1.3.1 Development of strategy for abiotic stress project in CIAT

Manabu Ishitani and Joe Tohme

SB-2 Project

Introduction

Environmental stresses including drought are the major constraint on crop yield. Genetic variability of the traits for environmental stress tolerance exists in different crops but is often complex and multigentetic. Our task is to facilitate breeding for the traits by developing molecular tools and isolating molecular determinants. In this report we will describe the following two points to efficiently implement molecular components into breeding toward to genetic improvement of yield under different stresses conditions: 1) overall strategy for abiotic stress project and 2) prioritization of existing and future projects.

Overall Strategy for Abiotic Stress Project. The following are the key components to develop successful stress project which allow focusing on specific determinants of the traits in efficient and cost-saving manner. All physiological, morphological and biochemical changes that confer stress tolerance in crops must have a molecular genetic basis. Thus, analyzing physiological/biochemical determinants of yield response to environmental stresses is the prerequisite to molecular dissection of the traits since we are not focusing on stress traits (e.g. drought) per se, rather focusing on these physiological/biochemical traits that contribute to improved productivity under stress conditions.

Key components for the success:

Availability of genetic resources

Understanding physiological/biochemical aspects on traits of interest

Availability of molecular tools

Development of tools for "proof of concept (POC)"

Minimize efforts

Public information

Strategic relationships with academic and private institutions

Synergies between different traits

Challenge lying in molecular work to determine molecular components for the traits is how to filter thousands of genes into the manageable number of candidate genes. As shown Figure 1, several key steps will be required for the process from gene pool to candidate genes. Physiological and biochemical screening will allow us to focus on specific aspects (e.g. deeper root, ABA content) on a trait followed by molecular screening using high throughput tools (e.g. microarray). The resultant data will be combined with other parameters such as QTL to help to select candidate genes. Tools for proof of concept (POC) will facilitate determination of candidate genes. This could be achieved by forward and reverse genetic study if the genetic tool (e.g. knock-out lines) will be available and appropriate for a trait. Transgenesis will be an alternative way to prove gene function. In the end, selected candidate genes will be examined in crops for further application including marker assisted selection. The tools mentioned here need to be established in the early phase of the project since it is time consuming to establish the tools in a cost-efficient as well as high-throughput manner.

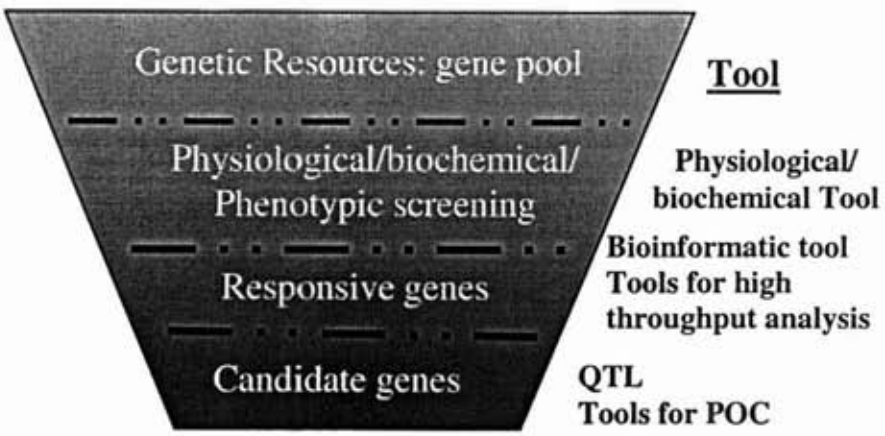


Figure 1. Filtration of genes using different tools

Prioritization of traits in crops. CIAT major commodities include four crops: common bean, forage, cassava and rice with all different traits as listed below. Prioritization of traits in crops is required to meet our needs in crop improvement in an effective manner. The risk rate (high, medium and low) to achieve the goal shown in the table is a relative score which is based on complexity of individual trait (e.g. single or multi genetic traits) as well as other factors such as genotype x environment (G x E) interaction. There are two ways to

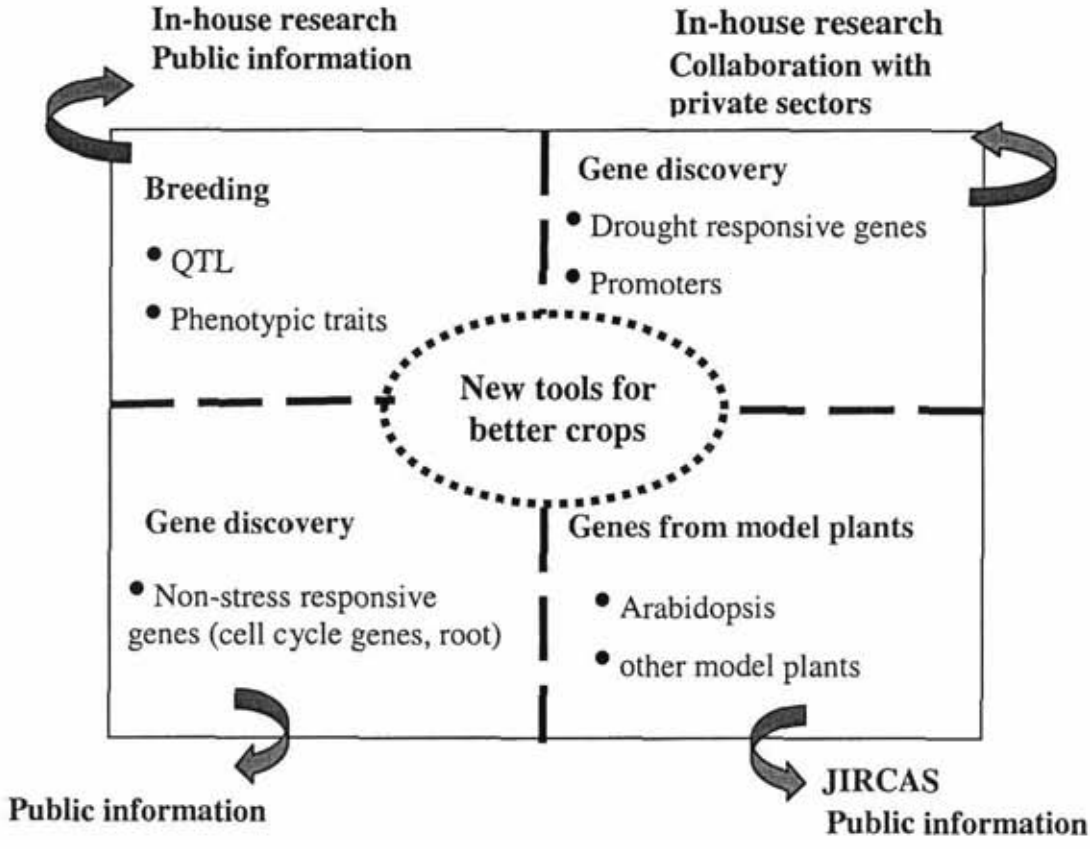
prioritize traits which are 1) same trait among the crops and 2) each trait in each crop. The table below showed prioritization of trait in each crop, which mainly based on 1) availability of genetic resources, 2) impact on trait improvement 3) availability of tools to conduct molecular work. Recent advanced genetic and genomic tools allow us to compare genetic diversities across the crops. In this sense, it makes more sense to establish workplan for a same trait in different crops.

Crop	Trait	Priority	Risk/Complexity
Common bean	Yield/Drought	1	High
	Low-P	2	Low
	Aluminum	3	Low
	Zn and Fe	2	High
Forage	Aluminum	1	Low
	Low-P	1	Low
	Nitrification inhibition	2	?
	Drought	2	High
	Biotic stresses	3	Medium
Cassava	Beta-carotene	1	Medium
	Yield/Drought	2	High
	High protein	1	High
	Biotic stresses	2	Medium
Rice	Yield/Drought	3	High
	Nutritional quality	1	High
	Biotic stresses	1	Medium

Projects for traits in red are currently funded.

An example: Requirement to develop strategy for drought tolerance in common bean

There are four activities to ensure meaningful deliverables such as trait genes for the crop improvement as described below. The philosophy to develop such strategy is to make best use of in-house, public and non-public resources to identify different molecular components for the trait. Breeding activity includes QTL and phynotyping which should link to other activities in the course of work. Others are all related to gene discovery activities which are divided to three sub-activities based on different target genes as described. Establishing collaboration with public and private sectors will ensure access to advanced technologies (e.g. whole transcriptome ESTs) which strengthen different activities of the project. It should note that genes from model plant would be appreciable to the crop if the genes are related to physiological and biochemical traits that are also found in the crop. Detailed workplan for the trait will be developed by implementation of these components in different level.



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1.3.2 Development of a molecular marker: DREB for drought tolerance in common bean

Leonard Galindo, Mathew Blair, Steve Beebe, Manabu Ishitani and Joe Tohme

SB-2 Project

Introduction

Aim of this study is to examine involvement of DREB gene in common bean for drought tolerance and develop DREB gene as a molecular marker for drought tolerance.

A transcription factor, DREB gene has been isolated from different plants including rice, maize, tomato, barley, *Arabidopsis* as a key regulatory component for drought and freezing tolerance. The DREB gene is called "master switch" for drought and freezing tolerance because the gene regulates its downstream genes which are related to stress response (Seki et al., 2001). Over-expression of the gene in different plants has resulted in increased stress tolerance under defined stress conditions (Kasuga et al.,1999; Pellegrineschi et al., 2002). Moreover, it was suggested that in wheat DREB gene is linked to the frost tolerance locus (Vagujfalvi et al., 2003). Thus, DREB gene is up to now only the one that is proven as a stress tolerance gene by different laboratories. Therefore, these evidences strongly support an assumption that DREB gene is one of key regulatory components for drought and freezing tolerance in plants. Despite accumulated information in DREB gene in plants there is no report of function of DREB genes and its regulation in common bean under stress conditions. Therefore, it is essential to investigate how the DREB genes are regulated under drought stress, and to find linkage of the expression with drought tolerance using drought-tolerant and -susceptible lines in common bean.

Material and Methods

For plant stress experiments at greenhouse level we used 5 parental lines of common bean: G19833, G21212, DOR364, BAT881 and BAT477 because they have different physiological and biochemical traits including deep rooting and photosynthate transport for drought tolerance. These parental lines were used in BMZ project for "Bean Genomics for Improved Drought Tolerance in Central America". The population includes parental lines as well as the Recombinant Inbred Line populations (RIL) of three crosses (BAT 477 x DOR364; G 21212 x BAT 881; SEA-5 x MD 23-24). Since field test for gene expression study is not established and we would like to see DREB gene is stress- responsive in common bean we exposed about two week-old plants to drought and cold shock as described below. Plant seeds were sowed in pods in numbers of 3 or 4 seeds per pod and grown for 10 to 14 days under greenhouse conditions. For drought stress plants were taken

from pods, cleaned with water to eliminate dirt from roots and placed on the bench top for 3, 6 or 12 hours. After each treatment roots were separated from aerial parts and plant sections were harvested to store at -80°C. For cold stress treatment plants in pods were placed in a cold room at 4°C for 6, 12 or 24 hours. After each time point plants were harvested as described above.

Markers for Marker Assisted Selection (MAS) will be sought among Simple Sequence Repeat (SSR) markers already developed at CIAT, or among single nucleotide polymorphism (SNP) within different orthologs of DREB gene to be developed in this work. Breeding of drought tolerant varieties is underway and mapping all DREB orthologs in common bean might be enhanced on marker-facilitated breeding and selection.

Results and Discussion

Our first goal is to isolate DREB/CBF genes from Common bean. We decided to use a PCR approach using Thermal Asymmetric Interlaced (TAIL)-PCR technology because 1) previous study suggested DREB/CBF genes do not contain intron, 2) the transcript expression pattern is unknown in Common bean. To design primers for TAIL-PCR a search for several DREB/CBF proteins in monocotyledons and dicotyledons was performed in the Genbank and 8 sequences representing 6 different plants were chosen to perform a nucleotide alignment (Figure 1). Multiple sequence alignment was performed using AlignX of the VectorNTI (Informax Inc.) package and conserved regions were determined to design primers for TAIL-PCR. An additional search in specialized soybean and ESTs databases was also performed to find closely related or uncharacterized bean DREB/CBF sequences but no homologous sequences were found. Eight primers (R-CBF1-1:TMCKRCCMGCYGGYTTYTTCG; R-CBF1-2: GRTGMCG GTYTCHCGAAACTT; F-CBF2-1: AYTTCKCYGAYTCKGCTTGGMG; F-CBF2-2: GYYTSAAYTTCKCYGAYTCKGC; R-ap2-internal: KKRAAAGTHCCRAGCCA AATCC; F-ap2-internal: GGATTTGGCTYGGDACTTTYMM; R-ap2-flank: CTTCA SAMACCCAYTTWCCKGA; F-ap2-flank: CRGCTMGWGCTCAYGACGTSGC) were designed from conserved DREB/CBF and AP2 consensus domain sequences. The primers are degenerate at several positions since alignments showed differences in some of the bases of the conserved regions.

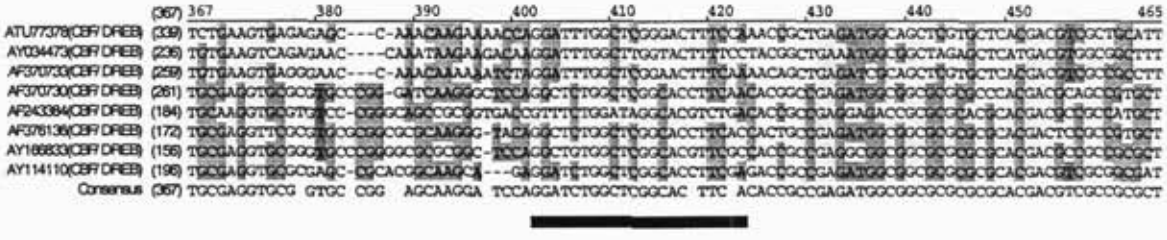

Figure 1. Multiple sequence alignment of 8 DREB/CBF sequences from 6 plants: ATU77378 (*Arabidopsis thaliana*), AY0343473 (*Lycopersicon esculetum*), AF370733 (*Brassica napus*), AF370730 (*Secale cereale*), AF243384 (*Oryza sativa*), AF376136 (*Triticum aesvestium*),

AY166833 (*Oryza sativa*), AY114110 (*Oryza sativa*). The sequences were aligned using AlignX. Only a section of the alignment is shown with one of the primers (named: R-AP2-internal) indicated with a black thick bar below.

For amplification of putative CBF/DREB genes we used protocols previously described (Liu & Whittier 1995) with some modifications. Several amplifications were attempted but failed to amplify specific fragments for DREB/CBF gene. This could be due to use of degenerated primers for the PCR. We are currently testing different conditions for the primers.

Future activities

- Conduct expression analysis of DREB gene in the parental lines treated with different stresses using Real-Time PCR
- Map DREB genes in common bean.

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1.3.3 Dissection of a cluster of Resistance Gene Candidates (RGCs) associated with resistance to angular leaf spot (ALS) in common bean (III)

I. Acosta, C. Romero and J. Tohme
SB-2 Project

Introduction

Using Resistance Gene Candidates (RGCs) as molecular markers, we have previously detected a locus (RGC7) associated with resistance to angular leaf spot (ALS) in common bean (López et al., 2003 Phytopathology 93:88-95). This result prompted us to sequence a large genomic region that contains a multifamily cluster of RGCs highly similar to the original RGC associated with the ALS resistance locus. (BRU, Annual Report, 2002). The sequencing of the BAC clone 57-M14, even though is derived from the ALS susceptible cultivar Sprite, provides a valuable platform for the genetic dissection and cloning of the corresponding resistance genes (R-genes) in the source of ALS resistance, the variety G19833. Furthermore, this strategy allows both a) the identification of additional molecular markers in order to assess more precisely the fate of these genes when used in breeding programs; and b) the study of R-gene cluster organization and structure, which may be informative as to the evolution and generation of new specificities of R-genes in general. The comparison of the sequences and genomic structures of diverse cultivars under study should contribute to explain their observed phenotypic differences on ALS resistance. Here, we report the final phase of the BAC sequencing project and the current status of the isolation of R-gene coding sequences from the resistant cultivar.

Materials and methods

BAC sequencing finishing
Initially, sequencing of the BAC clone 57-M14 was attempted by a transposon insertion strategy using a kit from Epicentre (BRU, Annual Report, 2001). However, the insertion events were biased to occur in regions highly enriched in retroelement-type sequences, which hindered the coverage of the entire BAC sequence. To overcome this, a sub-cloning library was constructed. After the entire sequencing of the two libraries we reached 70 kb of genomic sequence distributed in about 20 contigs, but the coding sequences of four of the five RGC contigs found were still incomplete (BRU, Annual Report, 2002).

At this point, we decided to focus in extending the R-gene contigs through the design of primers in the edge or boundary of the known regions. These primers were used in two ways:
Primer walking, which is the use of primers for direct sequencing from the BAC clone DNA as a template.

Vectorette PCR or Genome Walker (Hagiwara, K. & Harris, 1996) that consists in the amplification of unknown DNA sequences adjacent to a known sequence, in our case the available contigs. This method is based in the separate digestion of genomic or BAC DNA with different restriction enzymes that leave blunt ends. The fragments are ligated to an adaptor and become the template for PCR amplification (Fig 1) with a specific primer that targets the end of a known sequence and a primer corresponding to the adaptor. The PCR products from different digested templates will have dissimilar sizes, allowing us to gain new sequence data from different portions of the unknown region.

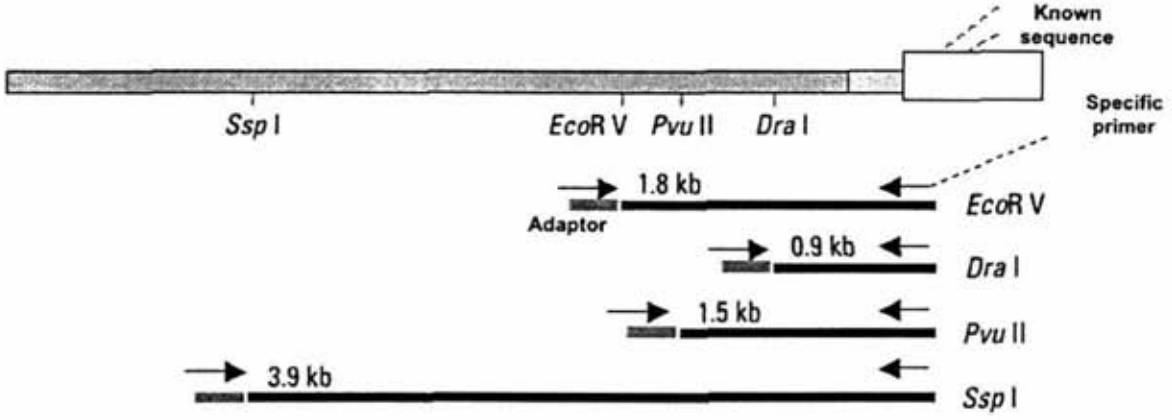

Figure 1: The upper bar shows the restriction sites of interest in the unknown region (grey) adjacent to the known sequence (white). The lower bars represent the PCR products from each different digested template (adapted from Universal Genome Walker Kit, User Manual, Protocol # PT3042-1 Version # PR03300, Clontech Laboratories, Inc.)

In brief, the BAC clone was digested separately with *Dra*I, *Eco*RV, *Msl*I, *Pvu*II, *Sma*I and *Ssp*I and ligated with an asymmetric-bubbled adaptor 224M13 (Hagiwara, K. & Harris); this configuration avoids the amplification of adaptor-adaptor large fragments. The ligation was diluted and an aliquot used to perform PCR. The amplification product was purified and sequenced with the primer M13. Additionally, we designed two specific "consensus primers" targeting conserved motifs in the already known regions of the R-genes copies. These consensus primers were also used in vectorette PCR.

RGCs were annotated using the following softwares:

- GenScan (http://genes.mit.edu/GENSCAN.html) and NetPlantGene (http://www.cbs.dtu.dk/services/NetPGene/) to identify open reading frames (ORFs) and to make predictions of splice sites (intron-exon boundaries)

- BLASTX searches and BLASTP searches in the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were performed to identify the protein domains.

Search for new molecular markers

To detect microsatellites present in this genomic region we used the software Simple Sequence Repeat Identification Tool (SSRIT at http://www.gramene.org/db/searches/ssrtool) and designed PCR primers with PRIMER3 software in adjacent regions to target identified microsatellites. They were PCR amplified using DNA from the varieties G19833 and DOR364 and the products were separated in a 6% poly-acrylamide gel to search for polymorphisms.

Results and discusión

BAC sequencing finishing

Besides to facilitate the cloning of resistance genes, the sequencing of these large genomic regions contributes to the understanding of the molecular evolutionary events that had resulted in the generation of specific pathogen recognition and the subsequent resistance capabilities.

Currently, we have 12 assembled contigs that span a total of 90.3 Kb. The primer walking, the vectorette PCR and the Genome Walker methods have been useful to fill sequence gaps that the previously developed libraries were not able to cover. Indeed, we added about 20 Kb of new sequence and joined several contigs (we had 20 contigs before). In general the gaps were regions of repetitive DNA with a high AT content, which may explain the difficulty to fill those gaps.

We were able to finish the sequence of four out of the five copies of RGCs contained in the BAC. A diagram in Figure 2 illustrates the structure of the predicted proteins from these RGCs (RGC7-A to RGC7-E), reflecting the current status of the annotation. Though a larger analysis is still required, several interesting observations have already being noted.

RGC7-A is the member on this cluster that still requires additional sequence information. Indeed, no sequence has been obtained further the predicted TIR and NBS domains so that we are not certain about the presence of LRR in this copy. However, this member is pseudogene because it has a stop codon at the end of the known (and incomplete) sequence of the NBS domain and contains a non-LTR retroelement-type sequence inserted in a predicted intron in the middle of the N-terminal TIR domain. This splicing site is not predicted in the other members of the family.

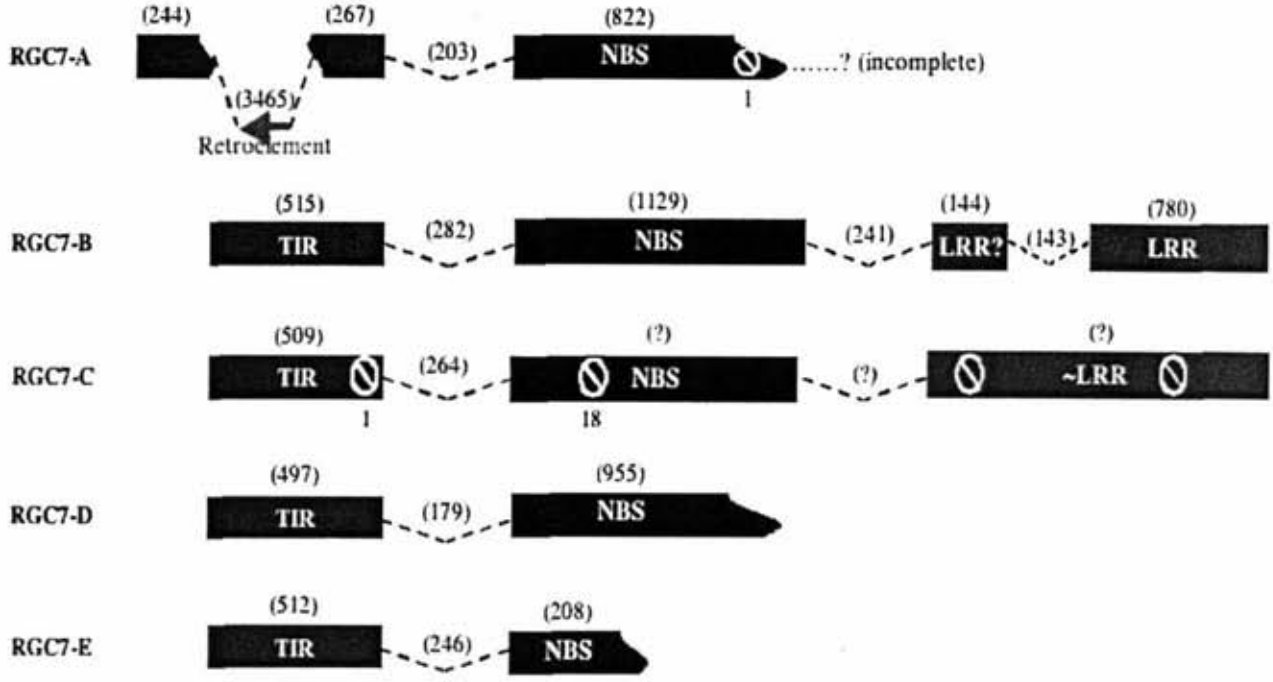

Figure 2. Domain distribution of the Resistance Gene Candidates found in the common bean BAC clone 57-M14. Predicted exons are shown in colors and predicted introns as dashed lines. The presence of the TIR domain (red) and the donor splicing site for exon 1 are conserved between copies. The NBS domain is truncated in RGC7-D and RGC7-E and is not completely known in RGC7-A. Only 'pseudo-exons' is distinguishable in RGC7-C because it exhibits various stop codons (indicated as stop symbols with the number of restrictive codons below them when known).

RGC7-B is the only member of the cluster with the complete and defined structure of a typical TIR-NBS-LRR R-gene. With a total size of 3233 bp, it contains 4 predicted exons. Interestingly, exon 1 contains the entire TIR domain and the exon 2 NBS domain, a feature shared with the other copies. In fact, the position of intron 1 (intron 2 in RGC7-A) in all the members is well conserved. Additional analyses are on the way to determine the structure of the LRRs and other characteristics of the domains.

Although when the translated sequence of RGC7-C shows similarity with the TIR, NBS and LRR domains, the coding sequence does not contain a true ORF. The presence of the first conserved intron was predicted but the apparent exons actually contain a large number of stop codons. Thus, RGC7-C is a perfect example of a pseudogene. When its nucleotide sequence is compared to that of RGC7-B (data not shown), several deletions are detected in RGC7-C which may account for the stop codons but further consideration of the pattern of these deletions is still in process.

RGC7-D and RGC7-E are truncated TIR-NBS R-genes. They not only lack the LRR domain but also have an NBS domain interrupted abruptly before the end of the second exon. This occurs to a greater extent in RGC7-E which has only 208 nt in such exon (compare with the equivalent exon in RGC7-B). More interestingly, both RGC7-D and

RGC7-E show a striking palindromic sequence right after the end of the gene that may represent a mark of recombination events giving origin to (imperfect) duplicated sequences.

Genetic mapping of BAC 57-M14

Even though the BAC clone 57-M14 was identified using the original RGC7 from G19833, we decided to verify that the physical region encompassed by the BAC mapped at the same genetic locus represented by RGC7 and linked to ALS resistance. This was necessary because sometimes the high level of gene duplication in eukaryotes like plants causes that gene families are located in more than one region in the genome. Therefore, we looked for molecular markers in the sequence of the BAC clone, mainly microsatellites (SSRs), which are considered the more informative towards such a goal. Strikingly, despite of the high amount of repetitive DNA sequences contained in the BAC, we only found three perfect SSR in the total 90.3 Kb. One of them, SSR07, was a trinucleotide repetition located in the very beginning of the coding sequence of RGC7-C so that it was very promising for our purposes. Indeed, primers targeting this SSR07 were designed and upon amplification on DNA from G19833 and DOR364, the parentals in our mapping population, a size polymorphism was detected and evaluated in the whole progeny. The segregation of this marker was quite similar to that of the original RGC7 and when Single Marker Analysis (Qgene software) was applied to assess its correlation with the phenotype of resistance to ALS, the SSR07 explained even a higher proportion of the resistance than the original RGC7. This microsatellite explain 75% and 69% of the resistant phenotype to two different isolates of the pathogen. Thus, we can conclude that the physical region we have sequenced in common bean must contain (a) major component(s) of the resistance to ALS. Besides, in the serach for SNPs we have already detected the presence of one polymorphism that is currently under confirmation. Now, the goal is to identify it (them) from the actual resistant variety: G19833.

Ongoing Work

To design primers for each candidate gene based on the sequence information of BAC 57-M14 (obtained from the susceptible variety Sprite), in order to amplify the respective ortholog genes from G19833.

To complete the annotation of the BAC 57-M14 and to analyze de structure of the other main component of its sequence, the putative retroelements.

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1.3.4 Identification of candidate genes for mineral uptake and storage in cDNA libraries from common beans

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[1]SB-2 Project-CIAT ; [2]Clemson University Genomics Institute

Introduction

This study starts with the hypothesis that the identification of candidate genes for mineral uptake and storage would allow a better understanding of the mechanism and inheritance of mineral accumulation by associating QTLs for mineral content with the map location of known genes involved in mineral transport and storage in the plant and seeds of common bean. The objective of this research was to start the analysis of candidate genes by screening two cDNA libraries from common bean roots for genes involved in micronutrient uptake. Our immediate targets were the genes for ferritin, the major iron storage protein in plants, for zinc transporters from the ZIP family and for enzymes involved in the synthesis of nicotianamine, an iron chelator.

Methodology

Library Screening. Two cDNA libraries were screened: PV-DEd and PV-DEe. Both libraries were constructed from mRNA isolated from the roots of DOR364 plants grown hydroponically under phosphorous sufficiency or deficiency for 5 days, that was cloned into the UniZAP – cDNA vector (Stratagene) as described in the 2002 Annual Report. A total of 36,000 clones were screened (18,000 for each library) on gridded Hybond N+ membrane filters. Positive clones were identified from the six fields / 96 position / double-replicate 4 x 4 pattern.

Probes and Hybridization conditions. A series of soybean cDNA clones were selected based on a keywords search of the Genbank database (ferritin, zinc transporters and nicotianamine). The most promising 15 clones were ordered from Invitrogen. These were mini-prepped from single colony cultures using standard techniques and sequenced with T7, Sp6 and M13 reverse or forward primers depending on their cloning vector, to confirm sequence identity and gene homology. A total of five soybean clones were used as probes. In addition, a single common bean cDNA clone with homology to ferritin was isolated from a leaf cDNA library and also was used as a probe. Probes were random-hexamer labeled with radioactive P^{32} using the DECAprime II DNA labeling kit from Ambion. The root cDNA filters were pre-hybridized for 8 hours and hybridized overnight at 60 C in a modified "Overgo" hybridization buffer. Filters were washed three times in 1X SSC buffer and placed into cassettes with Phosphoimager screens which were scanned the following day with a Storm 480 from Molecular Dynamics. Positive hits were found by

looking for double hybridization pattern. Positive clones could then be identified by their position in the double-replicate pattern found at each grid axis in the address system.

Results and Discussion

The number of positive hits per hybridization gave a good idea of the relative levels of gene expression of each clone given the total number of clones screened which ranged from 18,432 to 73,728 clones depending on the probe used (Table 1). Gene expression as analyzed by the library screening effort reflects the gene expression in roots of young plants grown in hydroponic culture with sufficient or deficient phosphorus, since these were the tissues used for mRNA extraction used to make the library. Gene expression of ferritin homologues was noted to be relatively high and similar in both the high P and low P libraries (Figure 1). Meanwhile the homologues of nicotianamine (NA) synthase and transferase genes had very low expression levels (1200 times less than ferritin) that were also more dependent on the library source: while all the NA synthase positive clones were from the low P library, the NA transferase positive clones were found in both the high and low P libraries. Since average insert size for the root cDNA libraries is around 1.5 - 1.8 kb, we are hopeful that a full length clone will be obtained.

Future plans

Sequence positive clones for full-length insert sequences.
Develop SNP (single nucleotide polymorphism) based assays to follow inheritance of common bean orthologs of micronutrient related genes.
Hybridize the root cDNA libraries with additional probes for other candidate genes, especially for iron reductase in collaboration with USDA-Houston (see additional section, this Annual Report).

Table 1. cDNA probes used to identify orthologs of candidate genes for mineral accumulation in two root cDNA libraries of common bean.

Probes	Library Screened	Number of clones screened	Number of Positives Clones (tissue specific)	Percentage of expressed genes
Phaseolus Putative Ferritin	HP	18,432	9	0.048
Soybean Putative Ferritin	LP	18,432	13	0.071
Soybean Putative Zinc Transporter 1	HP	18,432	0	0
Soybean Putative Zinc Transporter 2	LP	18,432	0	0
Soybean Putative Nicotianamine Synthase	LP,HP	73,728	3 (LP)	0.00004
Soybean Putative Nicotianamine Transferase	LP,HP	73,728	2 (HP/LP)	0.00003

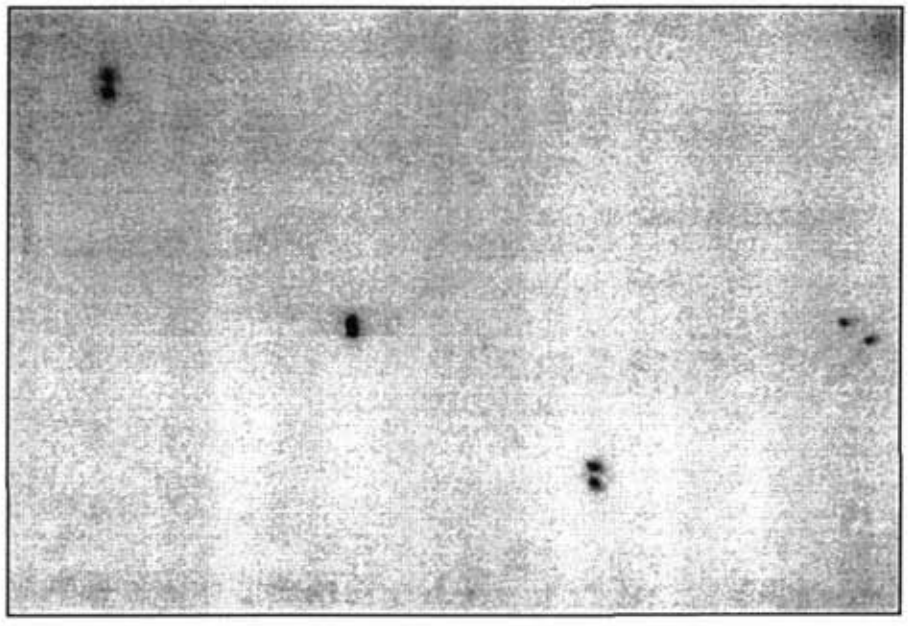

Figure 1. Hybridization with a probe for soybean ferritin for part of a 4X4 gridded high density filter of the root cDNA library, showing 4 positive clones out of a total of 3,072 clones in this field of view.

1.3.5 Generation and analysis of cassava ESTs: towards the identification of a unigene set

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This research was supported by grants from Agropolis (France). Sequencing facilities were provided by Génopole Languedoc Roussilon.

Introduction

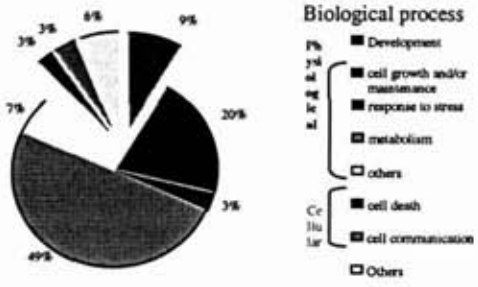
Cassava (*Manihot esculenta* subsp. *esculenta* Crantz), is a major food crop in the tropics. In addition to being a basic food in the human diet, cassava constitutes one of the more common raw materials for starch production. The yield, dry matter content (indirectly starch content) and planting materials are affected by *Xanthomonas axonopodis* pv. *manihotis*, the causal agent of cassava bacterial blight (CBB). One tool which holds a lot of promise in unraveling the complexities in gene expression is Expressed Sequence Tags (ESTs). In this study, we have targeted 2 economically important characters, starch content and CBB resistance to generate several types of libraries including subtracted ones. A large collection of EST was generated. We report here the single pass sequencing of 18202 cDNA clones from the 5'end and the analysis of the cassava EST collection.

Main results

Characterization of a unigene set. After trimming a total of 13080 sequences was obtained. These were assembled in a unigene set of 6046 sequences, including 2032 Tentative Contigs (TC) and 4014 singletons. This unigene set was searched against the GenBank database using the BLASTX algorithm. Twenty-five percent of ESTs (1457) do not show similarity to sequences in the non-redundant protein database.

Comparing the cassava unigene set with other plant species. . To identify genes that are highly conserved across plant species and sequences that might be specific to cassava, we compared the cassava unigene set to the EST database of *Arabidopsis*, *Medicago*, tomato, soybean and potato using TBLASTX. Conceptual translation of 18% of the sequences did not show any similarity of available EST of other plant species. They might represent new cassava-specific genes, although some of them might be simply 5' or 3' untranslated sequences.

Functional categories. Functional categories were defined using the Gene Ontology (GO) classification scheme (http://www.geneontology.org). A total of 69% of unigenes did not have a significant match. Thus, only 31% of the unigene set was assigned a putative function using this method (Fig below).



Genes unique to libraries infected with Xam. To identify important genes involved in the defense response, we identified sequences that were only present in the libraries inoculated with *Xanthomonas*. We obtained a unigene set of 1677 sequences (1514 singletons and 163 TC). Some of these genes show similarity to genes previously identified and involved in defense mechanisms, i.e homologs to R-proteins, chitinase and receptor-like protein kinases. A high number of sequences (56%) did not show similarity to proteins in the database or match unknown or hypothetical proteins.

Library specific sequences. A unigene set representing 2296 sequences (1319 singletons, 977 TC) was found only in the root libraries. 2616 (2256 singletons and 360 TC) sequences were found only in the stem libraries and 521 (439 singletons and 82 TC) to the leaves libraries. These sequences can be considered as candidates of tissu-specific genes, it will be necessary to validate them by other strategies. *Detecting SNPs.* A high number of ESTs was available with two cultivars CM523-7 and MPer183 and therefore we explored the applicability of our EST data set for identifying coding SNP (cSNP). Among the 1809 contigs, 285 contained four or more sequence reads with CM523-7 and 129 with MPer183. 7.7% (CM523-7) and 17% (MPer183) of these contigs contained SNPs with at least two reads for each. The estimated cSNP frequency was 1 polymorphism per 121 bp.

Conclusions

The cassava EST data presented here is the first effort in the large scale sequencing of the cassava expressed genome and also in cataloguing cassava genes.A unigene set of 6046 sequences was identified and a putative function assigned to 31% of unigenes.
A number of ESTs were found to be present only in the *Xam* challenged libraries. he EST data represent a valuable source for the identification of SNP and further mapping analysis. The EST resource will increase the density of gene markers on the cassava genetic map.

Future plans

Information obtained here will be used to develop microarray technology for further cassava gene expression studies. Based on this, developing new cassava varieties having high dry matter content and durable resistance to CBB will be the next challenge.

1.3.6 Identification of genomic regions responsible for conferring resistance to white fly (*Aleurotrachelus socialis)* in cassava

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SB-2 Project

Introduction

The whitefly (*Aleurotrachelus socialis*) is one of the most serious pests and disease vectors that affect agricultural production around the world. In cassava (*Manihot esculenta* Crantz), the whitefly causes from 70 to 80 percent economic losses. The most important source of resistance genes is the genotype M Ecu 72. Due to the whitefly's importance as a pest, it is necessary to understand the nature of genes that confer resistance to the whitefly in genotypes such as M Ecu 72. For this purpose F1 segregation and the genetic expression of the cross M Ecu 72 (resistant genotype) x a very susceptible genotype (M Col 2246) and molecular markers are being used. This will help accelerate selection of whitefly-resistant materials, as well as isolate resistance genes (R genes). Genetic and molecular studies (Richter and Ronald, 2000) have shown that the R genes are clustered in the genome of several species. They display an apparent multiallelic structure, or they group as genetically separate loci. Different genes determining resistance to insects and nematodes have been reported within the same cluster in tomatoes (Rossi et al., 1998). R genes are thought to be functionally and evolutionary related. The sequences of several R-gene clusters from rice, tomatoes and lettuce have now shed light on the molecular mechanisms leading to their evolution. As suggested by Lefebvre and Chèvre (1995), the genes governing quantitative resistance could share homologies with the clone's R genes, making the candidate gene approach feasible for the study of possible association between resistance gene analogs (RGAs) and quantitative trait loci (QTLs) controlling pest resistance. In this work M Ecu 72 and M Col 2246 were amplified with RGA primers designed by C. Lopez in cassava (pers. com.), to find putative loci related with whitefly resistance. An additional step toward a better understanding of the attack response of the whitefly to cassava was the establishment of a cDNA library, which was developed with a new, highly effective method known as differential subtraction chain (DSC). Using this approach, two mRNA populations, extracted from both resistant and susceptible genotypes, were examined to elucidate the differential gene expression between them.

Materials and methods

For this work an F1 cross (family CM 8996, 276 individuals) between M Ecu 72 (as the resistant parent) and M Col 2246 (as the susceptible parent), elite cassava cultivars from Ecuador and Colombia, respectively, was used. The parents and their offspring were evaluated in the field at two sites: Nataima (Tolima) and Santander de Quilichao (Cauca). The purpose of this evaluation was to identify gene segregation in the offspring and select the resistant and susceptible materials. Both parents were evaluated with 343 cassava SSRs (simple sequences repeat) (Mba et al., 2001) including 156 cDNA SSRs (Mba et al., submitted). AFLPs (Vos et al., 1995) are being used to find markers associated to resistance for mapping and ultimately cloning the resistant genes. Silver staining is being used to visualize the allelic segregation of the markers. Cassava RGA primers were done in the parentals, and the polymorphics were mapped in the F1. For the isolation of expressed sequences, 21 forty-day-old plants were used, 7 of each genotype (M Ecu 72 and M Per 334 resistant and M Col 2246 susceptible). These plants were taken to the greenhouse, where they were infested with 300 whitefly adults per plant, for a population of 2100 whiteflies per cage. Leaves were collected at six different times for RNA extraction. For the differential subtraction chain (DSC), the follow strategy was used: Genotype M Ecu 72 was infested for use as the tester, while genotype M Col 2246 was used as the driver. At present the DSC technology is being performed according to Luo et al (1999). The representational difference analysis of cDNA was divided into several phases:

Generation of a PCR amplicon, which is representative of the original mRNA from M Ecu 72 and M Col 2246. Subtractive hybridization of this amplicon M Ecu 72 (tester) and M Col 2246 (driver), during which amplified portions of differentially expressed genes are enriched and common sequences are depleted

Cloning and screening of the resulting products

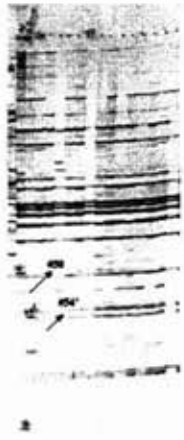


Figure 1. Silver-stained polyacrylamide gel showing combination ACA CTT of AFLP of both parents M Ecu 72 (resistant), M Col 2246 (susceptible) and 25 individuals of progeny. Note the polymorphic bands # 50 and #54'.

Results

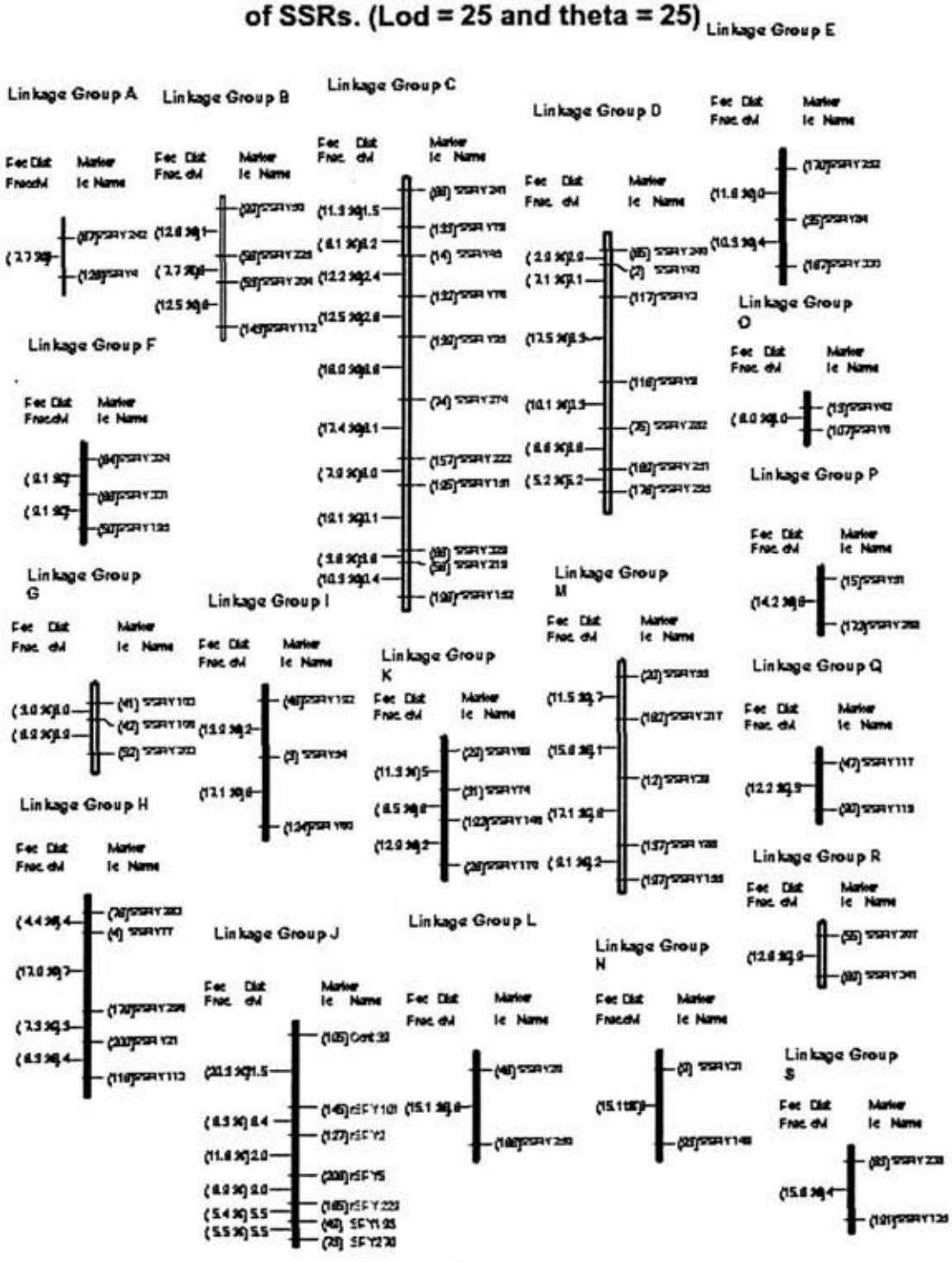
Field evaluation. The field evaluation showed high pressure being exerted by the pest in Nataima, where test materials had high damage rates; however, some materials had lower levels of damage in the evaluations. We can conclude that these genotypes show a resistance level similar to parental M Ecu 72.

AFLP analysis. An analysis was made of 128 combinations of primers with both parentals (M Ecu 72 and M Col 2246) and both bulks of 10 whitefly-resistant DNA and 10 susceptible DNA. We obtained 53 polymorphic combinations, in which there were 425 polymorphic bands between the resistant and the susceptible. All combinations were amplified in the F1 (Fig. 1).

Mapping. Approximately 55 of the SSRs evaluated were polymorphics in the parentals and were evaluated in the F1 (286 individuals). To construct the linkage map, 103 SSRs were analyzed, of which 71 were anchored. A genetic linkage map of cassava was constructed with 71 SSR markers segregating from the heterozygous female parent (M Ecu 72) of an intraspecific cross. The map consists of 19 linkage groups, which represent the haploid genome of cassava. These linkage groups spanned 550.2 cM, and the average marker density was 1 per 7.9 cM. The position of the 71 SSRs markers is shown on the framework (LOD = 25, *theta* = 25) of the molecular genetic map of cassava (Fig. 2). Map distances are shown in Kosambi map units. Of these markers, 26 (shown in green, Fig. 2) had been placed previously on the cassava framework map (Fregene et al., 1997); the other 45 SSRs are new. Of the 71 SSRs, 31 were cDNA sequences (Mba, in prep.), while the others were genomic DNA.

Association between molecular markers and resistance. The molecular data are being analyzed using QTL packages (QTL cartographer Q gene) to determine linkages between the SSR markers and phenotypic characterization. Preliminary analysis (X^2 at the 5% level) was done using SAS. Putative associations were found between 43 SSRs markers and the field phenotypic characterization (score 1.0 to 2.0 of the damage levels and populations).

Fig. 2: Preliminary Cassava framework Map of MEcu-72 for Resistance to White Fly, consisting of SSRs. (Lod = 25 and theta = 25)



Cassava RGAs. We obtained eight polymorphic RGA primers in the parentals (Fig. 3). To date, we have mapped three Bac primers in the F1 to find associations with yield QTLs. Polymorphism of the presence and absence bands between the parentals was found in Bac 9, 31, 35, 45, Contig 39 and Contig 43d and polymorphism of different bands in Bac 36 and the RT.

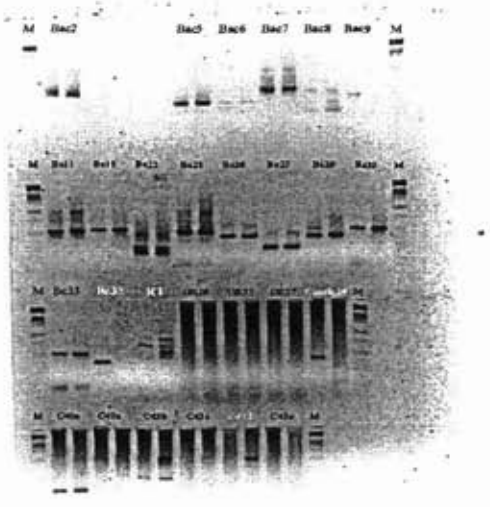


Figure 3. DNAs of M Ecu 72 and M Col 2246 amplified with RGA primers.

Differential subtraction: RNA extraction. RNA was isolated from young leaves of M Ecu 72 (E), M Per 334 (P) and M Col 2246 (M), colleted in the greenhouse. To isolate total RNA, the Rneasy Plant Mini Kit QIAGEN™ was used. Genomic DNA was removed prior to isolation of poly $(A)^+$ RNA with DNAse I. The SV Total Isolation System of Promega™ was used. The generation of cDNA was done using poly A+ mRNA as the substrate, which was isolated using the protocol Oligotex mRNA Spin Column of QIAGEN™. First-strand cDNA synthesis and cDNA amplification were done using SMART PCR cDNA Synthesis kit" de Clontech™ (Fig. 4)

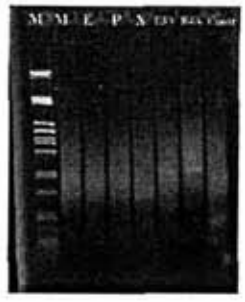


Figure 4. M: λ digested with Pst I. cDNAs amplified with kit SMART™.

The PCR products from the amplification of cDNA, were purified using QIAquick PCR Purification kit QIAGEN™. Then digestion ligation was done, where the cDNA was digested with DpnII, and then adapters (BamI and BamII) were ligated. Finally, the amplicon generation was done for the hybridization reactions of the subtraction. For "tester" M Ecu 72 (E), 150 ng was obtained; and for "driver" M Col 2246 (C), 15 µg.

Ongoing activities

- Saturation of linkage map of M Ecu 72, using AFLPs
- Isolation, cloning, sequencing and mapping of AFLPs polymorphic bands between resistant and susceptible genotypes
- Design of SCARs for marker-assisted selection
- QTL analysis for whitefly resistance
- Mapping of cassava RGA polymorphics (BACs Primers, Gene Resistance Primers) in F1 (276 genotypes)
- Isolation of expressed sequences during the defense response of M Ecu 72 to whitefly attack.

In order to identify differentially expressed sequences, a new technology known as DNA chips or microarray can scan a significant number of clones. Microarray-expression profiling will be used to identify putative early response regulatory and/or signaling genes and to test the function of selected candidate genes using reverse genetics.

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1.3.7 Gene expression profiling of cassava responses to *Xanthomonas axonopodis* pv. *manihotis* infection

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Introduction

Cassava (*Manihot esculenta*) is currently the fourth energetic food in the world after rice, maize, and wheat and feeds more than 1000 millions people. Cassava Bacterial Blight (CBB) caused by *Xanthomonas axonopodis* p.v *manihotis* (*Xam*) is a destructive disease in the South America and Africa (Lozano, J.C. 1986) and yield losses range between 12 and 100%. The most suitable approach for controlling CBB is through host-plant resistance (Verdier, V. *et al.*, 1998). Cytochemistry and biochemistry of plant defense responses to CBB have been studied (Kpémoua, K. *et al.*, 1996). However, plant response to pathogen attack remains uncharacterized at the molecular and cellular level. In cassava, using the cDNA-AFLP technique Santaella *et al.*, (2002) identified genes involved in the cassava – Xam interaction. Sequences were isolated and showed homologies with plant genes related with resistance genes, signal transduction pathway, disease and stress responses and senescence associated genes. At the moment high-troughput techniques, such as cDNA microarrays, provide a genome-wide description permiting the characterization of the expression profiling of thousands of genes in a single experiment.

The objective of this research was to identify genes associated with cassava defense response to *Xam* combining subtractive library construction and cDNA microarrays.

Materials and methods

Plant material inoculation and cDNA synthesis. Young plants (4-week old plants) from two resistant varieties (MBRA 685 and SG 107-35) were inoculated by stem puncture with *Xam* strain CIO 151. Stem tissues were collected at 6, 12, 24, 48 and 72 hours post inoculation (pi), and 7 days pi. The controls were healthy non-inoculated plants and plants inoculated with sterile water. mRNA was isolated using Oligotex mRNA Midi kit (QIAGEN, CA). cDNA was synthesized using SMART™ PCR cDNA synthesis kit (CLONTECH, CA) from 400-500 ng of mRNA as starting material.

cDNA subtractive library. To identify differentially expressed genes during pathogen attack subtractive hybridization was performed, using the Differential Subtraction Chain method. A pool of cDNA obtained from inoculated plants was used as "tester" and a pool of cDNA obtained from healthy non-inoculated plants and plants inoculated with sterile water was used as "driver".

cDNA Microarrays preparation. Cassava clones from subtractive libraries, cDNA-AFLP analysis (Santaella *et al.*, unpublished results) and other clones were collected, amplified by PCR and printed on glass slides. The resulting microarray contained 3072 elements with each cDNA printed eight times as replicates. For control pourposes, a set of spot controls from tomato, potato and cassava housekeepings genes, human genes and spiked controls were arrayed.

Fluorescent probe preparation and Hybridization. Total RNA was isolated using SV total RNA isolation system (Promega Corp.) cDNA was synthesized using SMART™ PCR cDNA synthesis kit (CLONTECH, CA). Fluorescent-labeled probes were prepared using Klenow labelling (for indirect labelling) and microarray hybridization were performed with cDNA pool from 24-48 pi. v.s cDNA from healthy plants, using two duplicate slides with reverse labeling (dye-swap).

Data Analysis. Spot intensities from scanned slides were quantified using the ArrayPro 4.0. software. Background correction was realized, only signals that were higher than two standard deviation from local background were considered for further data analysis. Intensity-dependent normalization LOESS was performed and the differentially expressed genes were detected using SAM (Significance Analysis of Microarrays).

Results and Discussion

Two different strategies were used to identify pathogen-induced defense genes, cDNA subtractive libraries and cDNA Microarrays. Using the subtractive libraries, 768 cDNA clones were isolated for each resistant variety. Of these, 110 randomly selected clones were sequenced and a homology search was conducted using the BLAST program. The sequence analysis showed that 16 cDNA clones shared homology with plant genes involved in defense responses (Table 1), 70 clones were either homologous to plant genes of unknown function or showed no homology, and the remaining 24 clones showed homology with other plant genes. A hypothetical model of gene expression changes that occur in cassava-*Xam* incompatible interaction was proposed (Figure 1).

Ca^{2+} signals. Specific signal transduction pathways are activated. Calcium fluxes are an early event in signaling pathway and trigger plant defense responses. Previous studies revealed that the rapid accumulation of cytosolic Ca^{2+} is necessary for the production of oxidative burst and PR genes activation (SUS06, SUS07). Clone SUS05 is homologous to a fungus-inducible Calmodulin *Mlo* family protein isolated recently from rice.

Table 1. Sixteen cDNA clones from subtractive libraries shared homology with plant genes involved in defense responses

Clone	Highest homology	E-value
SUS01	Pectin methylesterase [*Nicotiana tabacum*].	4E-30
SUS02	Catalase CAT1 [*Manihot esculenta*].	3E-43
SUS03	Nucleoside diphosphate kinase 3 (ndpk3) [*Arabidopsis thaliana*]	1E-38
SUS04	Glutaredoxin [*Ricinus communis*].	4E-38
SUS05	Calmodulin [garden pea].	4E-44
SUS06	Lipid transfer protein [upland cotton].	2E-14
SUS07	Bet v I allergen family protein	2E-14
SUS08	Ankyrin-like protein [*Arabidopsis thaliana*].	5E-16
SUS09	Xyloglucan endotransglycosylase protein [*Arabidopsis thaliana*].	9E-47
SUS10	Zinc-finger protein [*Arabidopsis thaliana*].	9E-57
SUS11	MYB transcription factor - like protein [*Arabidopsis thaliana*].	2E-08
SUS12	Similar to eukaryotic translation initiation factor 2B	4E-12
SUS13	Polyubiquitin (ubq10) [*Arabidopsis thaliana*].	5E-36
SUS14	Ubiquitin-like protein (UBQ12) [*Arabidopsis thaliana*].	1E-31
SUS15	Matrix metalloproteinase [*Arabidopsis thaliana*].	5E-13
SUS16	DnaJ protein [*Hevea brasiliensis*].	7E-24

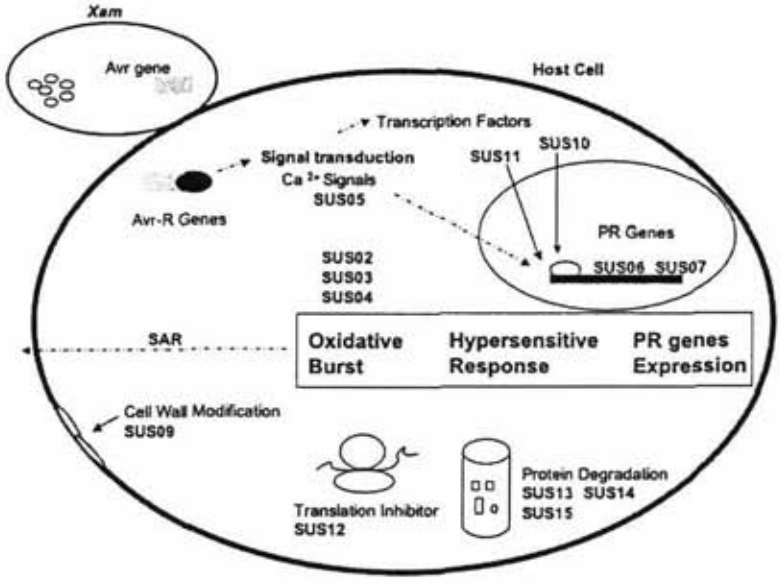

Figure 1. Hypothetical model of the cassava defense response to Xam infection. In green are the cDNA clones from subtractive libraries involved in defense responses.

Oxidative Burst, PR genes and HR. During infection, cassava produce a biphasic or polyphasic oxidative burst that is regulated by proteins such as Catalase and NDPKs (SUS02, SUS03), these genes can inhibit Reactive Oxigen Intermediates (ROIs) in specific time-points over infection, Clone SUS02 encodes a protein with significant homology (80% identity) to Ngcat1, a gene that was previously reported to be expressed following pathogen infection and salicylic acid treatment (Yi, SY. *et al.* 1999). Clone SUS03 shares a homology (68% identity) with AtNDPK2 that appears to play a regulatory role in ROIs-mediated MAPK signaling over biotic and abiotic stresses (Moon, H. *et al.*, 2003). PR and other defense-related gene expression are probably regulated by transcription factors, such as MYB and Zinc Finger Protein (SUS10, SUS11). Ca^{2+} signals, ROIs and PR genes can activate the HR, thus *Xam* may be confined to dead cells. This local response could trigger nonspecific resistance throughout plant (SAR).

Other defense responses. Associated with oxidative burst, HR and SAR, a number of other active defense responses are activated. These responses are also effective against *Xam*. A clone homologous to a brassinosteroid-regulated gene Xyloglucan Endotransglycosylase (XET)(SUS09) was reported. Brassinosteroids (BRs) were found to induce disease resistance in plants. BRs increase the abundance of mRNA transcripts for wall-modifying proteins such as XET that incorporate new xyloglucan into the growing cell wall. The pathogen spread is then hindered by physical strengthening of cell walls. Translation inhibition and protein degradation appear to play a role in cassava defense response, SUS12, SUS13, SUS14 and SUS15 genes activity may be coupled to HR development. Clones homologous to genes involved in glycolisis, vesicular transport (data not showed) and traffic protein (SUS16) were also identified. In addition, clone SUS08 encodes a protein with homology to an ankyrin from *Arabidopsis*. Ankyrin repeats are involved in the protein-protein interaction and have been implicated to function in some defense signaling pathways such as *Arabidopsis* NPR1.

cDNA Microarrays. cDNA Microarray enriched for genes involved in the cassava defense responses was constructed and hybridized to a cDNA pool from 24-48 pi. v.s cDNA from healthy plants. Data analysis revealed that some clones showed significant differential expression in response to pathogen attack (Figure 2). For up-regulated clones, the difference between experimental and control expression levels were up to 11-fold. These results indicate that some gene transcripts were induced between 24-48 hours after inoculation. It would be interesting to evaluate the microarray with other different time points throughout the infection. Several differentially expressed genes revealed by the microarray such as Glutaredoxin (SUS04), Lipid Transfer Protein PR gene (SUS06), Zinc-Finger Protein transcription factor (SUS10) Dormancy-associated protein (DOR) and Ubiquitin (SUS13) are associated with oxidative burst, defense signaling regulation and protein degradation in the plant-pathogen interactions. Other differentially expressed clones were homologous to *Arabidopsis* genes of unknown function (*A.th*) or showed no homology with sequences in the databases (NH), representing a new source of genes potentially involved in cassava defense responses.

Conclusions

Functional genomic tools such as subtractive libraries and microarrays permitted to give a comprehensive overview of the molecular basis of the cassava defense response to the bacterial blight pathogen. Many defense signal transduction pathways lead to responses like oxidative burst, cell wall modification, protein inhibition, protein degradation, metabolic changes and subsequent HR and SAR induction. The determination of genes that are up and down regulated at different times after the inoculation can help in understanding the defense mechanism of cassava to different pathogens.

Ongoing Activities

Confirm the differential expression of the characterized clones by Real-Time PCR analysis.

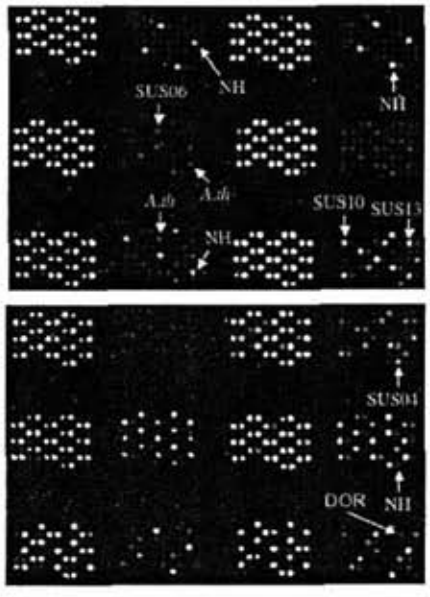

Figure 2. Three colors images showing differentially expressed clones SUS04, SUS06, SUS10, SUS13, Dormancy-associated protein (DOR) and *Arabidopsis* genes of unknown function (*A.th*) or without homology with sequences in the databases (NH).

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1.3.8 Development of a Diversity Array Technology (DArT) Chip for Cassava

Andrzej Killian[1], Peter Wenzel[1];Carmen deVicente[2] Edgar Barrera[3], Ana-Maria Correa[3], Martin Fregene[3]

[1](CAMBIA); [2](IPGRI); [3](CIAT)

Introduction

Genetic resources, mostly held by small farmers represent a critical resource for the future productivity and stability of production of the crop. How to evaluate and use in a systematic manner the vast amount of variability present in cassava is still a challenge to most cassava breeding programs. Genotyping micro-array technologies offer the highest throughput available up to date. One of them diversity array technology, DArT ™ (CAMBIA), is sequence-independent (low-input) and allows the fingerprint of an individual's genome based on a high number of polymorphic sites spread over the genome. These screening procedures should allow testing of thousands of individual samples in a speedy manner. We describe here a proof of concept on using the DArT tool as a cost-effective way for measuring and characterizing genetic diversity of cassava germplasm.

Methodology

The project was initiated in early October 2002, with the shipment of cassava DNA samples from CIAT to CAMBIA. Plant materials used for the generation of the DArT chip was chosen to represent a broad as possible diversity of the cultivar, a few genotype of its wild progenitors and 2 wild species were included to capture a large number of polymorphic fragments. They include 14 accessions from Brazil, 14 from Colombia, 4 from Guatemala, 2 each from Nigeria, Cuba, and Ecuador, Peru and Thailand respectively.

Others include, one accession each from Argentina, Bolivia, Costa rica, Fiji islands, Indonesia, Mexico, Panama, Venezuela, and USA. Six and 2 improved varieties were included from CIAT and IITA respectively. The wild species accessions were 29 of Manihot esculenta sub spp Flabellifolia, 7 of M. carthaginensis and 1 of M. walkerae. DNA isolation was according to Dellaporta et al. (1983) followed by two washes of phenol/chloroform.

A critical step in DArT is the complexity reduction step. Work at CAMBIA with several other plant genomes has shown that digestion with PstI restriction enzyme (RE) in combination with more frequently cutting RE is an efficient method to reduce complexity. A preliminary experiment was therefore conducted to determine the best enzyme combinations, mixture of genomic DNA from twenty cassava genotypes was digested with PstI, ligated to adapters and further digested with several frequently cutting RE (BstNI, ApoI, TaqI and BanII), followed by amplification with an adapter-specific primer. The cassava genomic PstI fragments lacking the recognition site for the frequent cutting RE (BstNI, ApoI and TaqI) were individualized by transformation into E. coli, amplified from bacterial colonies and micro-arrayed. From each of the libraries 760 clones were arrayed. Genomic representations prepared in the same way (RE digestion/ligation followed by amplification) from each of the twenty genotypes separately, were labelled with Cy3-dUTP and hybridized together with Cy5-dUTP-labelled reference DNA to these microarrays. Slide preparation, hybridizations, washes and scanning are as described by Andrzej et al. (2002). Images generated by the scanner were used to extract quantitative fluorescence signal data for each array feature using our proprietary software. Same software was used to binarize the data (score as 0/1) for all slides. Binary scoring table was used to prepare the Hamming distance matrix and to obtain a dendogram. Library expansion to obtain more polymorphic clones was carried out using the enzyme combinations PstI/BstNI, and PstI/TaqI, and 80 DNA samples from CIAT. About 3000 clones were evaluated for polymorphism from the 2 libraries. The arrays were printed and DArT analysis carried out as described earlier.

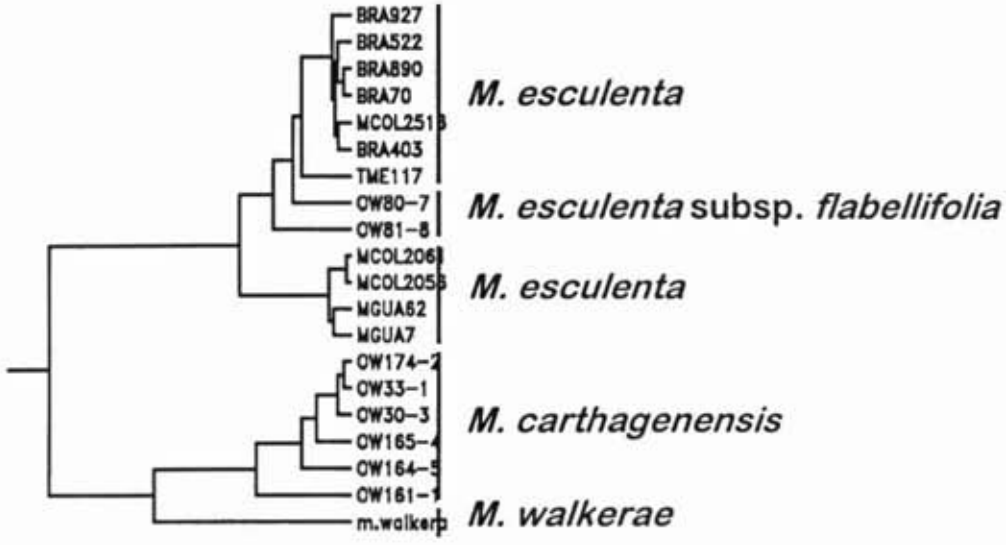

Figure1. Genetic relationship among the cassava accession used to identify polymorphic clones in the PstI/BstNI, Pst/TaqI and Pst/ApoI array. The dendrogram was created using the distance table based on 296 polymorphisms and the UPGMA clustering algorithm

Results

Among the three libraries /PstI/BstNI, PstI/ApoI, and PstI/TaqI) tested in the preliminary experimnt, PstI/BstNI gave the largest number of polymorphic clones (132), followed by TaqI (112) and ApoI (69). In total, 313 candidate polymorphic clones were obtained in the initial experiment to determine the best enzyme combination. DArT analysis based on 296 polymorphic clones without a single missing data point was used to generate a binary matrix and obtain a dendrogram, based on Hammings distance, representing genetic relationship between the 20 samples (Fig. 1).

The libray expansion with 80 clones yielded 440 polymorphic clones (14.3%), for the PstI/TaqI array, consistent with the results obtained with the initial, smaller array (14.6% polymorphic clones). A dendrogram based upon the analysis of the 80 cassava samples with the polymorphic clones is presented in Figure 2.

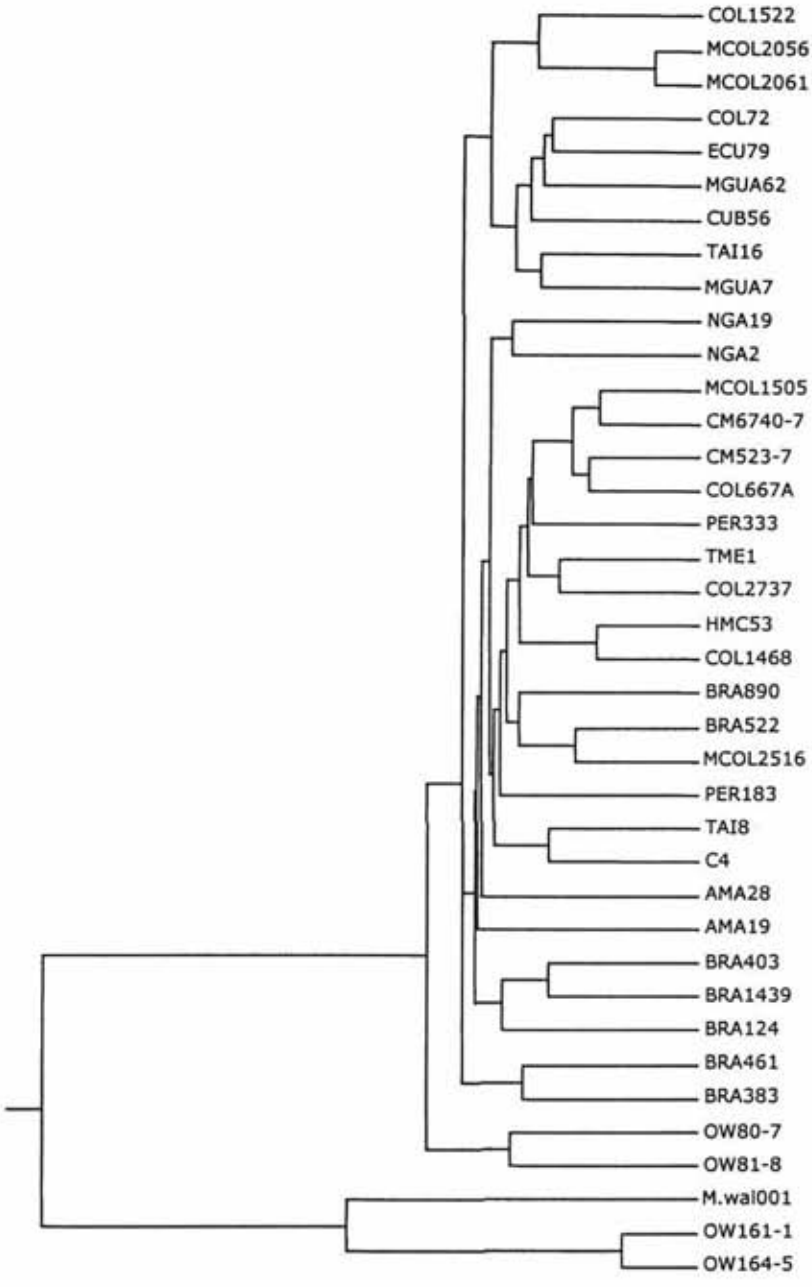

Figure 2. Genetic relationship among the cassava accession used to identify polymorphic clones in the PstI/TaqI array. The dendrogram was created using the distance table based on 440 polymorphisms and the UPGMA clustering algorithm.

In the PstI/BstNI array 554 polymorphic clones (18.0%) were identifid, also consistent with the polymorphism frequency in the smaller PstI/BstNI array in the first project phase (17.2%). The dendrogram resulting from this array is shown in Figure 3.

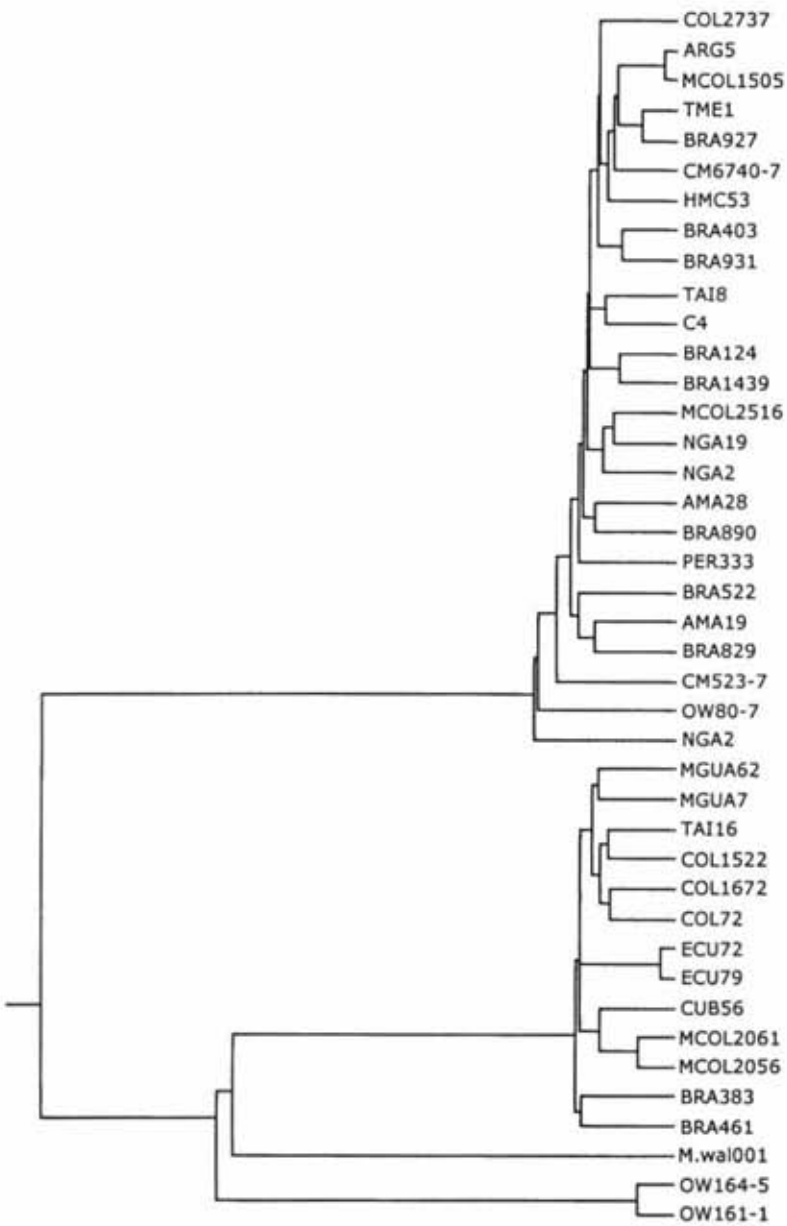

Figure 3. Genetic relationships among the cassava accessions used to identify polymorphic clones (markers) in the PstI/BstNI array. The dendrogram was created using the distance table based on 554 polymorphisms and the UPGMA clustering algorithm. Topology and branch lengths of this dendrogram are biased due to the presence of significant numbers of polymorphic clones derived from repetitive sequences.

There is a difference between the two dendrograms obtained with the PstI/BastNI and the PstI/TaqI array, respectively. A thorough inspection of the data suggests that the BstNI array contains a higher proportion of clones derived from repetitive sequences than PstI/TaqI array. Typing using repetitive sequences introduces a bias in genetic diversity analysis due to over-representation. The PstI/TaqI array does not show a high proportion of clones with repeated sequences and can be used as a routine genotyping tool for genetic diversity analysis. Work is ongoing to analyze a larger sub set of accessions from the CIAT core collection with at least 800 polymorphic clones

Conclusion and Perspectives

This feasibility project has resulted in the design and validation of a reliable complexity reduction method that is uncovering several hundred polymorphic DArT markers in cassava germplasm. Because DArT markers can be scored in parallel in a single analysis, a high throughput, cost-effective whole genome genotyping is now available for determining high-density genome profiles of cassava. As this study has demonstrated, the availability of such a method has the potential to improve dramatically the recognition, conservation and exploitation of cassava genetic diversity. This platform is now available for research on genetic diversity and genetic mapping studies in cassava. With current improvements of DArT's throughput and its cost reduction it is feasible to molecularly characterize very large germplasm collections within a reasonable period of time and cost.

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1.3.9 Diversity Array Technology (DarT) for potato diversity análisis

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[1]SB-2 Project; [2]CIP, Peru

Introduction

Potatoes are the fourth most important crop in the world after wheat, rice and maize (FAO, 1995). The annual potato production is about half that of all roots and tubers grown worldwide. Potatoes are consumed by more than a billion people in the world, including 500 million from developing countries.

There are about 500 potato species, which represent series from diploid to hexapod genomes. There are two principal centers of diversity: one in Mexico and the other in Peru, Bolivia and NW Argentina (Hanneman, 1995). Genetic diversity of potatoes includes 4000 cultivars, conserved and maintained at CIP (Centro Internacional de la Papa) in Peru. There are 200 wild species that have some traits of interest due to their resistance to diseases, pests and extreme environmental and stress conditions.

Genetic resources are the foundation for generating new varieties and are a reservoir of genetic stability and adaptability. They are important to deal with environmental changes and pest attacks. To know about diversity is essential in order to preserve and use it for improving current cultivars. Diversity studies traditionally employ molecular markers based on gel platforms such as isozymes, SSR, RFLP and AFLP. These techniques are important because they have generated a lot of information, but they are linked to some problems such as being labor-intensive, the gel is expensive, not much information results from each experiment, sometimes it is difficult to resolve bands on gels, and with some manufacturer's products, it is necessary to know the DNA sequences.

DArT is a technology employing microarrays, capable of solving these problems. It is based on the generation of DNA segment arrays with unknown sequences (panels) of two or more genomic DNA individuals. These segments are hybridized with fluorescent-labeled DNA probes (representations) of two individuals that can be used to create the panel (Jaccoud et al., 2001). In our case panels were created with six potato genotypes including two individuals of a potato genome map. This technology has some applications such as tracking changes in DNA methylation, fingerprinting, diversity studies and elucidating complexes mixtures of DNA (Jaccoud et al., 2001).

Materials and methods

A total of 96 potato genotypes from the CIP potato germplasm collection were collected, and DNA extracted. Six of them were used to make diversity panels.

*Generation of diversity panels.*Total DNA (100 ng) of six potato genotypes was digested with 2 units of *PstI* or *MseI* enzyme in separate reactions. Digestion products were ligated to their specific adapter and were PCR amplified. The amplification product was column purified, viewed in a 1% agarose gel and quantified. Then 400 ng DNA from the PCR of each genotype was mixed and 10.5 ng was bonded to pGEM T easy vector and used to transform competent *Escherichia coli* strain DH5∝ by electroporation. The positively transformed cells (white colonies) were transferred onto a freezing medium, where they grew at 37°C overnight. The culture was PCR-amplified usingT7 and Sp6 universal primers. The PCR products were dried using an incubator chamber at 50°C to obtain 2µL of the PCR total initial volume, which was re-suspended with 23µL of a 3X SSC, 1.5M betaine spotting buffer and used to spot microscope slides.

Generation of representation. DNA (100 ng) of six genotypes used for building diversity panels was digested with *MseI* enzyme, bonded to its own adapter and PCR-amplified. The product was then column purified and labeled with Cy3 or Cy5 dyes using an Amersham Megaprime Labeling Kit, except for the enzyme. The enzyme used was Kleenow fragments, exonuclease-free, polymerase I. Five enzyme unites were employed for each labeling reaction.

Hybridization. All Vanderbilt protocols were used to make panel hybridization which involves three steps: microarrays prehybridization, washing and hybridization. For the prehybridization a buffer containing 5X SSC, 0, 1% SDS and 1% BSA was used to submerge microarrays for 45 min at 56°C. After washing, slides were air dried for 30 min and hybridized in a Clontech hybridization chamber for 17 h at 42°C with a solution containing representations.

Scanning and image analysis. Microarrays were scanned in a Virtek Chip Reader scanner, using a 2.0 version software. Images were analyzed using VersArray Analyzer version 4.5 for Windows (BIORAD), which is being demonstrated at CIAT.

Net intensity spot values were normalized by using a loess regression that adjusts spot signal values according to their position on the slide and between signal channels. New signal values were then analyzed, detecting sequences present in each potato genotype.

Results

Two genomic libraries were constructed using 6 out of 96 potatoes genotypes. One of these was made using *Mse*I digestion enzyme; the other with *Pst*I enzyme. Each library contained 768 clones.

Ten panels for potato-*Mse*I library were generated, including all library clones and control potato clones from TIGR. Six of those panels were hybridized with representations generated with PCR, using primers without an additional selective nucleotide at the 3´end. No polymorphic clones were identified in the *Mse*I panel using those representations.

The other four panels were hybridized with representations generated with PCR using primers with an additional selective nucleotide, adenine or thymine, and with a bulk of six different genotype representations generated with PCR using primers without an additional selective nucleotide. Those experiments are still in analysis.

Ongoing work

- Hybridize *Mse*I panels with different genotypes representations obtained by using PCR primers with selective nucleotides

- Evaluate *Pst*I library for polymorphic clones by hybridizing *PstI* panels with different genotype representations

- Hybridize all potato genotypes with panel of genomic library with more polymorphism

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1.3.10 Microarray gene expression analysis as a functional genomics tool for postharvest physiological deterioration in cassava

Cortés, D.F[1].; Reilly, K.;[2] Beeching J.;[2] Tohme, J[1].
[1]SB-2 Project; [2]Dept. of Biology and Biochemistry, Bath University, UK

Introduction

The rapid postharvest physiological deterioration (PPD) of cassava roots is a major constraint that impacts negatively on the livelihoods of poor farmers, processors and consumers alike. Extending the shelf life of cassava to one or two weeks is perceived as a goal that would be beneficial, particularly with respect to contributing to the sustainable livelihoods of small-scale rural farmers, as well as poverty alleviation.
Genomics and bioinformatics tools are increasing our knowledge of plant genome structure, organization and gene function. Novel technologies such as expressed sequences tags (ESTs) and cDNA microarrays are proving rapid ways to identify genes and to link sequence information to biological function.

The objective of this project is to identify the full set of major genes involved in the postharvest physiological deterioration by exploiting the powerful high-throughput analysis of cDNA microarrays. This will not only help understand the problem but also provide the tools (clones) that could serve as components of gene constructs to modulate PPD. This study is an important step in making this goal achievable, utilizing a cDNA library from different time points during the PPD process in cassava roots.

Results and discussion

Genomic library construction. A genomic library consists of a collection of clones that together encompass the entire genome of cassava. A genomic library cloned onto Lambda DASH II/BamHI (Stratagene) has been made at Bath from cv. M Col 22. With a titer of 10^5 cfu/ml, the library is ready for screening with cDNA clones differentially expressed during a postharvest deterioration time course and subsequent isolation of corresponding genes including their regulatory elements (promoters).

Microarray experiments with PPD-related cDNA libraries. A total of 7680 clones from the early library (0, 6 and 12 h after harvest) and 3072 clones from the late library (24, 48 and 96 h after harvest) were processed. Slide spotting for the microarray experiments was

carried out using the Hitachi SPBIO 1.55 system available at CIAT with PL-100C-Poly L lysine slides (CEL Associates). To have slides containing 4 technical replicates of each spot and containing 4 replicates of a control plate containing control genes and target spike genes, the early library was distributed onto 5 slides and the late library onto 2 slides. The total number of slides used in each hybridization was 7 (early slides 1-5, late slides 1-2) as shown in Table 1.

Table 1. Hybridization strategy for microarray experiments.

	Hybridization	Probes	Slides						
Time-course experiments	1	0 vs 12 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	2	0 vs 24 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	3	0 vs 48 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	4	0 vs 72 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	5	0 vs 96 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
Range experiments	6	12 vs 24 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	7	24 vs 48 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	8	48 vs 72 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2
	9	72 vs 96 h	Early slide 1	Early slide 2	Early slide 3	Early slide 4	Early slide 4	Late slide 1	Late slide 2

The probes used were cDNA probes prepared from RNA extracted at different time points from cassava roots of CM 2177-2, the same cultivar used to make the spotted cDNA library. For all experiments the earliest time point was labeled with Cy3 and the latest with Cy5; thus upregulated genes appear red. The spike genes used were TIGR E1-E5 (Cy 3 + Cy 5, should appear yellow); TIGR E6+E7 (Cy3 only, should appear green); TIGR E8+E9 (Cy5 only, should appear red).

Scanning was carried out using the VersArray Chipreader (Biorad) available at CIAT. Initial analysis of all these hybridizations was carried out at Bath University, using ArrayVision software. Both normalized and raw data files were saved (126 analysis files). Subsequent processing of these analysis files is in progress using Cluster and Treeview software and has been completed for the early slide 1 time-course experiments (shown in gray in Table 1). Table 2 shows a partial list with 18 of 165 upregulated genes of interest identified to date. An initial example of hierarchical cluster analysis of these genes is shown in Figure 1.

Recommendations and future work

- Analysis has now been completed, and clones of interest transferred to Bath for sequencing analysis.

- Given the number of clones of interest, it would be very labor intensive to confirm expression profiles by Northerns for this number of candidate genes. Thus it would be advisable to repeat the hybridization experiments at CIAT so that the microarray data could "stand alone" as expression data in any publications/presentations.
- Other microarray experiments to identify genes with specific tissue activity could be carried out using different plant tissues as the source for RNA isolation.

Genes of Interest – PPD Time-Course Experiments

				(log 2) Fold Upregulation					Flags					Sum
Library	Slide	Spot Label	Clone Name	0 vs 12	0 vs 24	0 vs 48	0 vs 72	0 vs 96						
Early library	Early slide 1	p 2 B8 (4)	Early plate 1 B8	0.64	1.20	1.97		1.58		1	1		1	3
		p 2 G20 (4)	Early plate 1 G20	0.43	0.11	1.63		1.01			1		1	2
		p 2 M21 (4)	Early plate 1 M21	-0.13	0.38	1.22		1.08			1		1	2
		p 3 A2 (4)	Early plate 2 A2	0.45	0.39	1.34		1.02			1		1	2
		p 3 A4 (4)	Early plate 2 A4	1.45	1.68	2.00		0.86	1	1	1			3
		p 3 D19 (4)	Early plate 2 D19	1.22	0.70	1.11		0.62	1		1			2
		p 3 I4 (4)	Early plate 2 I4	-0.36	0.52	2.02		1.11			1		1	2
		p 5 K14 (4)	Early plate 4 K14	1.00	1.00	2.23		1.51	1	1	1		1	4
	Early slide 2	p 2 C20 (4)	Early plate 5 C20	1.40	-0.08	0.82	0.52	1.34	1				1	2
		p 2 D20 (4)	Early plate 5 D20	1.38	0.07	0.68	0.44	1.34	1				1	2
		p 2 J13 (4)	Early plate 5 J13	0.45	-0.04	0.95	0.19	1.29			1		1	2
		p 3 C2 (4)	Early plate 6 C2	0.66	0.43	1.68	0.40	2.26			1		1	2
		p 3 I8 (4)	Early plate 6 I8	0.07	-0.07	1.08	0.68	1.47			1		1	2
		p 3 K4 (4)	Early plate 6 K4	0.95	0.12	0.68	-0.26	0.99	1				1	2
		p 3 L21 (4)	Early plate 6 L21	1.02	0.16	0.72	-0.14	1.10	1				1	2
		p 4 E16 (4)	Early plate 7 E16	0.25	0.39	1.06	0.47	1.40			1		1	2
		p 4 F16 (4)	Early plate 7 F16	0.49	0.28	1.05	0.22	1.40			1		1	2
		p 4 N14 (4)	Early plate 7 N14	0.55	0.14	1.18	-0.04	1.31			1		1	2

Table 2. Partial list of clones of interest selected on the basis of upregulation > +1 fold in 2 or more arrays, yellow indicates visually confirmed: Early library.

Figure 1. Initial hierarchical cluster analysis for clones of interest – Early library.

1.3.11 Development of molecular techniques for assessing genetic diveristy and mapping of useful genes

Development of a Diversity Array Technology (DArT) Chip for Cassava

Andrzej Killian[1], Peter Wenzel[1];Carmen deVicente[2] Edgar Barrera[3], Ana-Maria Correa[3], Martin Fregene[3]
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Methodology

The project was initiated in early October 2002, with the shipment of cassava DNA samples from CIAT to CAMBIA. Plant materials used for the generation of the DArT chip was chosen to represent a broad as possible diversity of the cultivar, a few genotype of its wild progenitors and 2 wild species were included to capture a large number of polymorphic fragments. They include 14 accessions from Brazil, 14 from Colombia, 4 from Guatemala, 2 each from Nigeria, Cuba, and Ecuador, Peru and Thailand respectively. Others include, one accession each from Argentina, Bolivia, Costa rica, Fiji islands, Indonesia, Mexico, Panama, Venezuela, and USA. Six and 2 improved varieties were included from CIAT and IITA respectively. The wild species accessions were 29 of *Manihot esculenta* sub spp *Flabellifolia,* 7 of *M. carthaginensis* and 1 of *M. walkerae.* DNA isolation was according to Dellaporta et al. (1983) followed by two washes of phenol/chloroform.

A critical step in DArT is the complexity reduction step. Work at CAMBIA with several other plant genomes has shown that digestion with PstI restriction enzyme (RE) in combination with more frequently cutting RE is an efficient method to reduce complexity. A preliminary experiment was therefore conducted to determine the best enzyme combinations, mixture of genomic DNA from twenty cassava genotypes was digested with PstI, ligated to adapters and further digested with several frequently cutting RE (BstNI, ApoI, TaqI and BanII), followed by amplification with an adapter-specific primer. The

cassava genomic PstI fragments lacking the recognition site for the frequent cutting RE (BstNI, ApoI and TaqI) were individualized by transformation into E. coli, amplified from bacterial colonies and micro-arrayed. From each of the libraries 760 clones were arrayed. Genomic representations prepared in the same way (RE digestion/ligation followed by amplification) from each of the twenty genotypes separately, were labelled with Cy3-dUTP and hybridized together with Cy5-dUTP-labelled reference DNA to these microarrays. Slide preparation, hybridizations, washes and scanning are as described by Andrzej et al. (2002). Images generated by the scanner were used to extract quantitative fluorescence signal data for each array feature using our proprietary software. Same software was used to binarize the data (score as 0/1) for all slides. Binary scoring table was used to prepare the Hamming distance matrix and to obtain a dendogram. Library expansion to obtain more polymorphic clones was carried out using the enzyme combinations PstI/BstNI, and PstI/TaqI, and 80 DNA samples from CIAT. About 3000 clones were evaluated for polymorphism from the 2 libraries. The arrays were printed and DArT analysis carried out as described earlier.

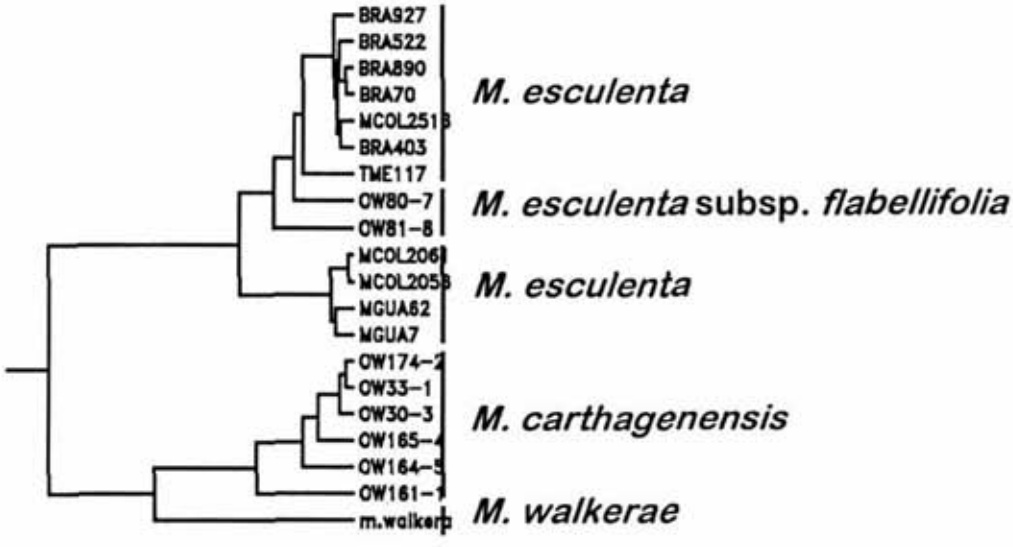

Figure1. Genetic relationship among the cassava accession used to identify polymorphic clones in the PstI/BstNI, Pst/TaqI and Pst/ApoI array. The dendrogram was created using the distance table based on 296 polymorphisms and the UPGMA clustering algorithm

Results

Among the three libraries /PstI/BstNI, PstI/ApoI, and PstI/TaqI) tested in the preliminary experimnt, PstI/BstNI gave the largest number of polymorphic clones (132), followed by TaqI (112) and ApoI (69). In total, 313 candidate polymorphic clones were obtained in the initial experiment to determine the best enzyme combination. DArT analysis based on 296 polymorphic clones without a single missing data point was used to generate a binary

matrix and obtain a dendrogram, based on Hammings distance, representing genetic relationship between the 20 samples (Fig. 1).

The libray expansion with 80 clones yielded 440 polymorphic clones (14.3%), for the PstI/TaqI array, consistent with the results obtained with the initial, smaller array (14.6% polymorphic clones). A dendrogram based upon the analysis of the 80 cassava samples with the polymorphic clones is presented in Figure 2.

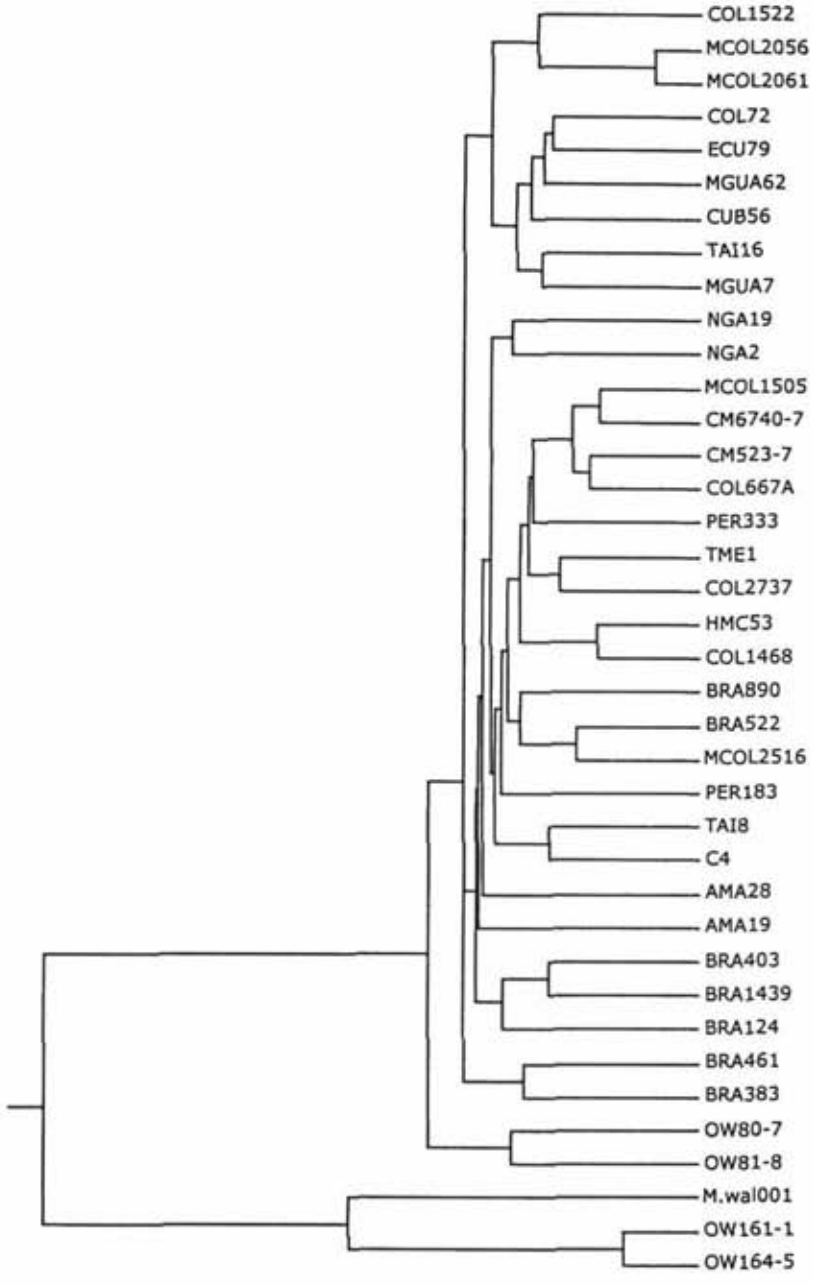

Figure 2. Genetic relationship among the cassava accession used to identify polymorphic clones in the PstI/TaqI array. The dendrogram was created using the distance table based on 440 polymorphisms and the UPGMA clustering algorithm.

In the PstI/BstNI array 554 polymorphic clones (18.0%) were identifid, also consistent with the polymorphism frequency in the smaller PstI/BstNI array in the first project phase (17.2%). The dendrogram resulting from this array is shown in Figure 3.

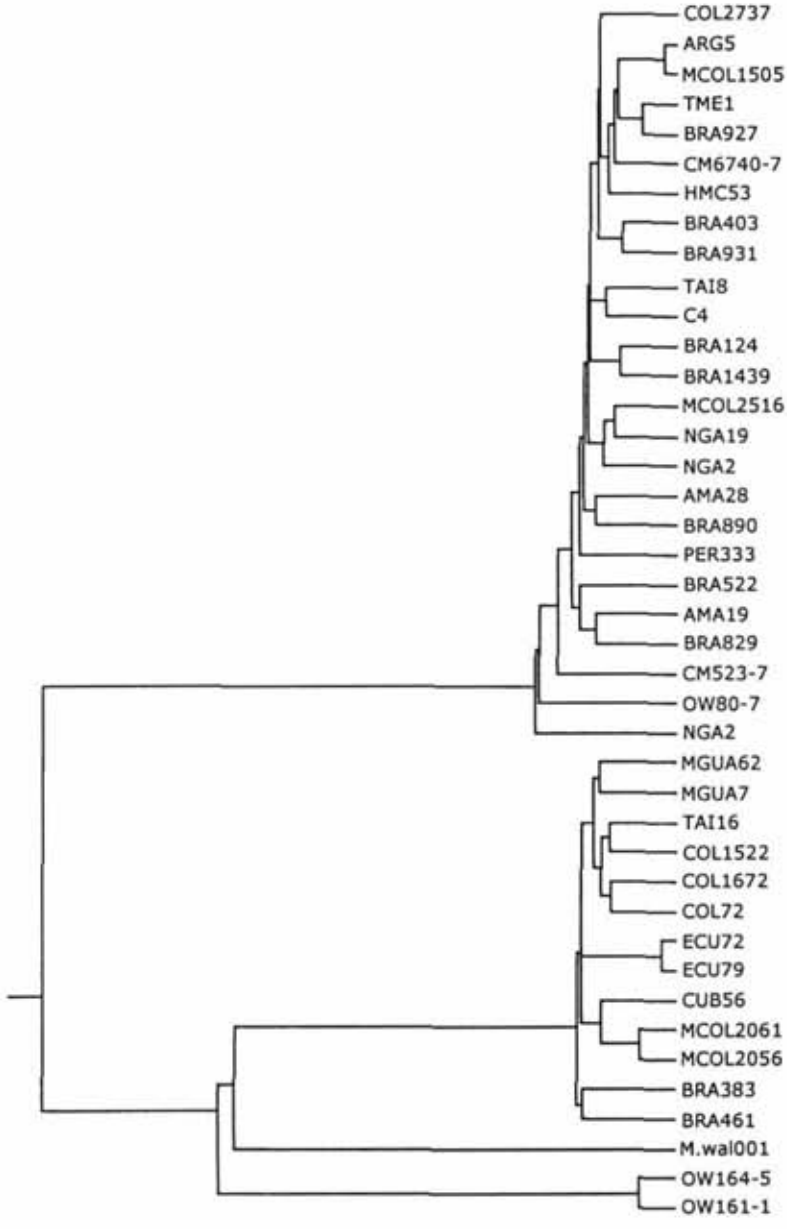

Figure 3. Genetic relationships among the cassava accessions used to identify polymorphic clones (markers) in the PstI/BstNI array. The dendrogram was created using the distance table based on 554 polymorphisms and the UPGMA clustering algorithm. Topology and branch lengths of this dendrogram are biased due to the presence of significant numbers of polymorphic clones derived from repetitive sequences.

There is a difference between the two dendrograms obtained with the PstI/BastNI and the PstI/TaqI array, respectively. A thorough inspection of the data suggests that the BstNI array contains a higher proportion of clones derived from repetitive sequences than PstI/TaqI array. Typing using repetitive sequences introduces a bias in genetic diversity analysis due to over-representation. The PstI/TaqI array does not show a high proportion of clones with repeated sequences and can be used as a routine genotyping tool for genetic diversity analysis. Work is ongoing to analyze a larger sub set of accessions from the CIAT core collection with at least 800 polymorphic clones

Conclusion and Perspectives

This feasibility project has resulted in the design and validation of a reliable complexity reduction method that is uncovering several hundred polymorphic DArT markers in cassava germplasm. Because DArT markers can be scored in parallel in a single analysis, a high throughput, cost-effective whole genome genotyping is now available for determining high-density genome profiles of cassava. As this study has demonstrated, the availability of such a method has the potential to improve dramatically the recognition, conservation and exploitation of cassava genetic diversity. This platform is now available for research on genetic diversity and genetic mapping studies in cassava. With current improvements of DArT's throughput and its cost reduction it is feasible to molecularly characterize very large germplasm collections within a reasonable period of time and cost.

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1.3.12 Positional Cloning of CMD2 the Gene that Confers High Level of Resistance to the Cassava Mosaic Disease (CMD)

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CIAT

Introduction

Previous work revealed that the SSR markers SSRY 28 and NS158 are the closest markers to the gene CMD2 that confers resistance to the cassava mosaic disease (CMD), and are located at distances of 9 and 3 cM respectively (Akano et. al 2002; Zárate 2002, CIAT

2002). High resolution of the CMD2 region of the genome was therefore initiated in this region. The experimental approach involves a search for recombinants between CMD2 and the above markers using a large full-sib population, followed by an analysis of the recombinants with thousands of readily assayed markers to identify additional markers more closely linked to the gene. This requires the use of several types of marker systems that can achieve whole genome screens with a reasonable level of effort. Molecular markers identified that are closest to gene CMD2 will be used to screen a BAC library for the construction of a BAC contig that transverses the region of CMD2 via successive steps of BAC end sequencing, mapping and more BAC library screening.

Methodology

The fine-mapping population was 1690 individuals from a cross between TME3, the source of CMD2 and the improved variety TMS30572. The cross was evaluated in the 2002 growing season for CMD resistance in the field at IITA, Ibadan, under heavy natural pressure of the disease. DNA was isolated from the individuals of the cross, using the Dellaporta et al. (1983) method, and diluted 10X in TE without quantification for molecular marker analysis was at CIAT. The population was evaluated with the 2 SSR markers according as described by Mba et al. 2001 and recombinants between the markers and CMD2 identified. DNA from 10 resistant recombinants and 10 susceptible recombinants were combined to form 2 bulks which were then evaluated with several markers system including AFLPs, ISTRs, RAPDs and SSRs in a modified bulk segregant analysis (BSA) method.(Michelmore et al. (1991). Evaluation with AFLP markers (Vos et al. 1995) was using a commercial ALFP (Invitrogen Life Technologies, Gaithersburg, MD) following the manufacturer's instructions.. All 64 possible combinations were used in the evaluation. For ISTRs (Inter Sequence Tagged Repeat), the method described by Rohde et al (1996) was used with all possible 64 combinations of the 8 F and 8 B universal retro-elements (retro-transposons) sequence primers. Evaluation with RAPD markers was using 768 commercial primers (Operon Technologies Inc, CA) and a modified Williams et al. (1990) protocol. PCR conditions were 1X PCR buffer, 2.5mM of $MgCl_2$, 0.4mM of DNTPs, 0.8uM of each primer, 1.0U of enzyme taq polymerase, and 50 ng/ul of DNA template in a of 2oül. The amplification program was an initial denaturation cycle at 94^0C for 5min; 35 cycles of 94^0C for 30s, 36^0C for 1 min, 72^0C for 1 min 30s; and a final extension cycle of 72^0C for 5min. The amplified fragments were separated on 1.5% agarose gelsand visualized by staining with Ethidium Bromide and examination under UV light. A set of 146 newly developed SSR markers (CIAT 2002) were also used to evaluate the recombinant bulks using PCR and PAGE electrophoresis conditions described by Mba et al. (2001). Markers that were polymorphic in the recombinant bulks were then analyzed in individuals of the bulks. A polymorphic RAPD fragment in the individuals of the recombinant bulk was cloned into pGEMT-easy (Promega inc, Madison) and sent for sequencing at the University of Iowa sequencing facility. Primers were designed from the sequences and are being used as SCAR marker for MAS.

Results

The evaluation of the fine-mapping population with the SSR markers SSRY28 and NS158 allowed the identification 112 recombinant individuals. The evaluation of the resistant and susceptible recombinant bulks with AFLP, SSR, ISTR produced several candidate markers that were polymorphic in the bulks but the polymorphism was not consistent when individuals of the bulks were analyzed separately (opened bulks) . However, analysis with RAPD markers produced 2 polymorphic candidate markers, AC-15 and RME-1 that remained consistent in the individuals of the bulks (Fig 1). Evaluation of the two markers in the entire fine map progeny revealed that AC-15 is at least 2cM from CMD2, while RME-1 is less than 1cM from the gene. The polymorphic fragment in RME-1, a 800bp fragment was cloned into the pGEMT-easy and sequenced. Homology comparison between the sequence of the RAPD band and sequences in public database using BLAST (www.ncbi.nlm.nih.gov) revealed the sequence is similar to the minor caspid protein of bacteriophage T3 suggesting that the fragment is single copy gene. This is being verified at the moment via southern hybridization to total cassava DNA. SCAR primers have been designed from the sequence for the use of the RAPD marker in MAS since it is closer than to the gene than NS158, the closest marker to date to CMD2. The above result provides a molecular marker closely linked to CMD2 that can be used to screen a BAC library BAC of cassava for the construction of BAC contigs which is the next stage of the positional cloning of the CMD resistance gene.

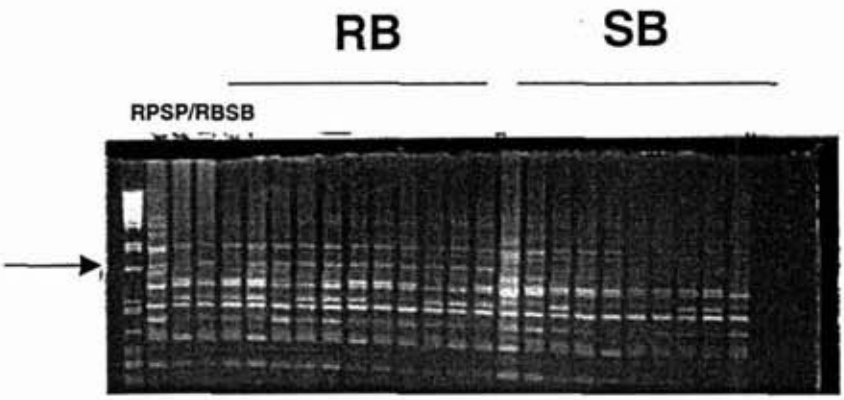

Figure 1. Ethidium bromide stained agrarose gel of individuals from the recombinant bulks evaluated with the RAPD marker RME-1. A fragment at around 800bp (arrow) can be observed in the resistant parent (RP), and resistant bulks (RB) that is absent in the susceptible parent (SP) and susceptible bulk (SB)

Conclusions

A high resolution map with 4 markers, one at less than 1cM has been constructed around the genome region of CMD2. The cloning of this gene is now proceeding to the next stage of BAC library screening and the construction of a BAC contig around CMD2.

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1.3.13 Saturation of the Molecular Genetic Map of Cassava with PCR-based Markers: Progress on the Mapping of a New Set of 140 New SSR Markers

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CIAT

Introducción

The wide-spread utility of the molecular genetic framework map of cassava published six years ago (Fregene *et al.*, 1997) was delayed by the preponderance of the RFLP's markers used in constructing that map. Over the past 3 years, efforts have been geared to the development and genetic mapping of SSR markers to make markers on the cassava map more accessible to cassava researchers worldwide especially in the NARS of Africa, Asia, Latin America and the Caribbean. An initial effort led to the mapping of 77 markers (Mba et al., 200, CIAT 2001), other efforts have led to mapping of 57 SSRs (Zarate 2002) and 45 SSRs from cDNA sequences (Garcia 2002). We describe here the screening of another 140 SSR markers, identified in BAC end sequences, in the parents of the mapping population (Niogeria-2 and CM2177-2) and a preliminary report of the mapping of 26 polymorphic markers.

Methodology

Primers were earlier designed from a total of 141 BAC end sequences found to contain SSR motifs (CIAT 2002). A Dellaporta *et al.*, (1983,) modified protocol was used to extract DNA extraction from the parents and the progeny of the mapping population. DNA quantification was done using the Dyna Quant TM.200 Fluorometer. Hoefer Pharmacia Biotech. PCR amplifications were carried out in 25 µl reactions containing 50 ng of DNA, buffer 1X, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs , 0.2 mM of each primer and 0.25 U of Taq polymerase. The PCR profile was changed for some markers, a reduction of the annealing temperature, to achieve amplification. The products of amplification were electrophoresed on 6% polyacrylamide gels and visualized by silver staining. For the survey of the parents, the following samples were included: Nigeria-2, CM2177-2, K150, TME3, TMS30555, CMD resistant and susceptible bulks. SSR markers that showed polymorphism in the survey of the parents, i.e. having a unique allele in either or both

parents of the mapping population, i.e. polymorphic, were used to screen the 150 progenies of the mapping population.

Results

A total of 35 polymorphic markers were found in the survey of the parents (Fig1). Of this 26 markers have been evaluated in the F_1 progeny (Fig 2). The level of is about 25% polymorphism and it is much lower than that found for SSR markers developed from an enriched library (60%) and that from a cDNA library (about 40%). The low level of polymorphism found here could be due to the source of the markers, BAC ends, or other unknown reasons.

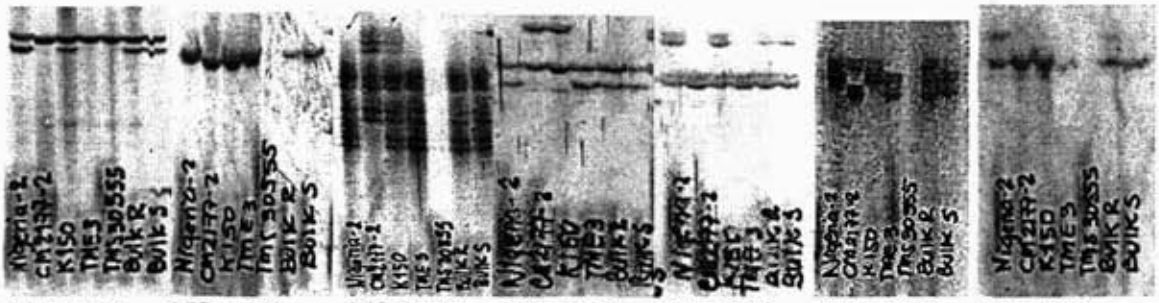

Figure1. Silver stained gel polyacrilamide showing PCR analysis of seven markers NS used to survey the 2 parents of the mapping population, including another genotypes : the progeny k150, TME3 and resistant (R) and susceptible (S) bulks. Each marker have changes in the annealing temperature (left to right : 52, 45, 52, 60, 60, 53, 55^{0}C, respectively.) The polymorphism between in the male and female genotypes can be observed.

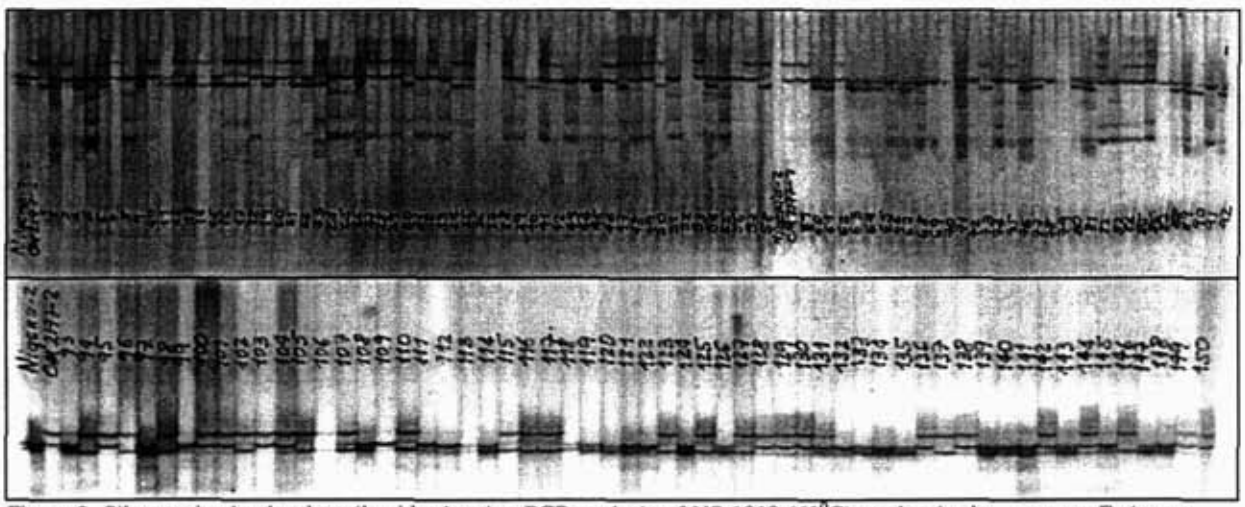

Figure 2. Silver stained gel polyacrilamide showing PCR analysis of NS 1019 (60^{0}C) marker in the progeny F_1 (cross Nigeria-2 x CM 2177-2). The segregation of dates for this marker in a ratio 1:1, presence: absence of the unique parental allele (67/79 for the female and 76/70 for the male framework respectively) can be observed.

Conclusions

A new set of 140 SSR markers identified from more than 2000 BAC end sequences of the white fly resistant variety MECU72 was evaluated in the parents of the mapping population and 35 polymorphic markers identified, of this 26 have been evaluated in the F1 progeny.

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1.3.14 Study of gene expression during pistil development in apomictic and sexual *Brachiaria* spp.

D. Bernal, D. Cortes, J. Tohme
SB-2 Project

Introduction

Apomixis is a reproductive mode through seeds that contain an embryo produced without the fusion of the egg and sperm cells; therefore it allows the fixation of any genotype, regardless of its complexity, ***through seeds***. Perpetuating improved hybrids in this way could result in potential socioeconomic benefits that would challenge those of the Green Revolution (Vielle-Calzada, 1996). Currently, the most accepted point of view is that apomixis and sexuality are closely interrelated development pathways, where the former can be considered a deregulation of the sexual process in both time and space (Tucker, 2003). Sexuality is the reproduction mode in the 460 or so angiosperm families. There are several types of apomixis, of which one or more are present in at least 33 of the angiosperm families. Apomixis has been found to be a derived anomaly of polyphyletic origin (Carman, 1997). If just one or a few alterations do indeed change a sexual

development pathway to an apomictic one, then its polyphyletic origin could be understood from the standpoint of having arisen independently several times from different sexual ancestors (Spillane, 2001). Perhaps depending on the specific time and/or space deregulation presented, a particular type of apomixis appeared in each origin event.

This project seeks to determine if there are different transcripts between apomictic and sexual plants; and if that is so, to typify them. Characterization of differences in the transcriptome of sexual and apomictic plants should give us insight into the mechanisms involved in the deregulation of the sexual reproductive developmental pathway, which end up transforming it in to an apomictic reproductive one.

Methodology

Using 13 plants from an interspecific *Brachiaria* hybrid population produced by the Tropical Pastures Program at CIAT, gene expression was compared among closely related apomictic and sexual genotypes in order to identify genes specific to the apomictic development.

cDNA synthesis. Pistils are the organs containing the female gametophyte, where genes regulating the reproductive developmental pathway are assumed to be expressed. Pistils take around 18 days to develop, from their differentiation until they are ready for pollination at floret anthesis (Dusi, 1999). Obtaining clean pistils from *Brachiaria* plants is time consuming due to their small size and protected location within the floret; however it is worth the effort as the pistil accounts for only 0.001% of the floret's weight, which means that by extracting RNA only from pistils, one eliminates a large set of uninteresting transcripts.

The 13 plants were classified as apomictic or sexual using the whole plant progeny test. About 300 pistils were dissected out of each plant, comprising the reproductive development from the 3rd to the 20th developmental day. cDNA was synthesized from mRNA fractions extracted from these pistils, from several individual plants (7 apomictic and 6 sexual plants), and two full-length cDNA libraries were constructed from the apomictic and sexual bulks as reported earlier (Cortes, 2002).

Subtractive hybridization. Any phenotypic variation found between two biological samples is due in part to their transcriptome variation. Consequently, mRNA sequences found in greater abundance or found only in one of two mRNA fractions coming from different biological samples are particularly interesting to investigators as they could be responsible for their differences. If we are interested in understanding a particular biological condition such as stress responses or differentiation mechanisms, we must isolate those transcripts present only in that specific condition. Subtractive hybridization is a molecular method designed to isolate those target mRNA sequences. The biological sample that contains them is called the tester, and the biological sample that does not have the condition but is similar to the biological sample is called the driver. For instance, if we were studying the response to heat shocks, we would use heat-shocked plants as tester and non heat-shocked

plants as driver. To identify differentially expressed genes between apomictic and sexual pistils, three different subtractive hybridization methods were used as each one has different advantages and disadvantages. The methods were subtractive suppression hybridization (SSH), mirror orientation selection (MOS) and differential subtraction chain (DSC). In each method the apomictic and sexual cDNA bulks used to build the two full-length cDNA libraries, were used both as tester and driver in order to isolate the cDNA sequences found only in the apomictic cDNA bulk (forward subtraction) and those found only in the sexual cDNA bulk (backward subtraction). The subtracted cDNA ultimately obtained with each method was used to construct subtractive libraries of 384 clones each.

Microarrays. cDNA microarrays are a group of DNA probes spotted on to a solid substrate, usually a microscope glass slide. There may be around 10,000 spots or slightly more per slide. Hybridization of the microarrays with two complex cDNA fractions, each one labeled with different fluorochromes, allows the researcher to monitor the relative level of expression of the 10,000 probes simultaneously between the two complex cDNA fractions, by comparing the intensity of the fluorescence emitted by the fluorochromes at each DNA spot. Subtractive hybridization methods are not absolutely efficient, and subtractive libraries do contain clones that are not differentially expressed between the two biological samples. Hence, microarrays were used to identify those clones from the subtractive libraries that are indeed differentially expressed between apomictic and sexual plants. Six hybridizations were performed between apomictic and sexual cDNA fractions. One hybridization was performed between two apomictic cDNA fractions obtained from different apomictic species, *Brachiaria decumbens* vs *B. brizantha.* This hybridization was used as negative control, assuming that those sequences that differ between these two species are not involved in apomixes, since both of them are apomictic species. The hybridizations were performed as follows (each arrow represents a hybridization; the two-headed arrows show that a repetition of each hybridization was done swapping the fluorochrome used to label each cDNA fraction.):

1. Apomictic cDNA bulk ⟷ Sexual cDNA bulk
   B. ruziziensis cDNA
2. *B brizantha* cDNA ⟷ Sexual cDNA bulk
   B. ruziziensis cDNA
3. *B. decumbens* cDNA ⟷ Sexual bulk cDNA

B. ruziziensis cDNA

4. *B. decumbens* cDNA ⟷ *B. ruziziensis* cDNA

After hybridization, each microarray was scanned with VersArray™ ChipReader to obtain a 16-bit tagged image file (TIF) for each fluorochrome, which was analyzed using Versarray™ Analyzer Software. Normalization between the two images was necessary to make biologically valid comparisons, thus the Loess intensity-dependent normalization method was applied as proposed by Yang et al. (2002). The program SAM (significance analysis of microarrays) from Stanford University was used to determine which spots were differentially expressed in each hybridization, choosing those whose ratio between the intensity of the fluorochromes is different from 1, keeping the false discovery rate below 7%. Those clones found to be differentially expressed in at least one repetition of the six hybridizations between apomictic and sexual cDNA, and were not found differentially expressed in both repetitions of the hybridization between the apomictic cDNA fractions, are considered candidates responsible for the differences between apomictic and sexual reproductive development.

Subsequently, these differentially expressed clones were sequenced the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, with some modifications introduced by the CIAT Biotechnology lab, and the automated ABI PRISM 377 Sequencer (Perkin Elmer). Sequencing analysis software was used to obtain sequences, which were semi automatically cleaned (removal of ambiguous bases, adaptors and vector) using Sequencher™ 4.1. Cleaned sequences were compared against the All nonredundant GenBank CDS translations+PDB+SwissProt+PIR database, and against the GenBank non-mouse and nonhuman EST entries.

Results and discussion

Six subtractive cDNA libraries were obtained, doing a forward and reverse subtraction with three subtractive hybridization methos. Each one with 384 clones:

1. Apomictic cDNA minus sexual cDNA using SSH
2. Sexual cDNA minus apomictic cDNA using SSH
3. Apomictic cDNA minus sexual cDNA using MOS
4. Sexual cDNA minus apomictic cDNA using MOS
5. Apomictic cDNA minus sexual cDNA using DSC
6. Sexual cDNA minus apomictic cDNA using DSC

The subtractive libraries 1, 3 and 5 are enriched in fragments found only, or more abundant in the apomictic pistil cDNA bulk. Each one of them was obtained using one of the subtractive hybridization methods aforementioned: SSH, MOS and DSC. The other three subtractive libraries, 2, 4 and 6 are enriched in fragments found only, or more abundant in the sexual pistil cDNA bulk. Each one was also obtained using SSH, MOS and DSC.

Thirty cDNA microarrays containing 10,752 spotted probes each were constructed. Every cDNA microarray contains 4 repetitions of the 2,304 clones from the 6 subtractive libraries, and some other control probe spots used to analyze the microarrays properly.

Based on these analyses, 65 out of the 2,304 clones from the subtractive libraries were found to be more abundant in the apomictic cDNA fraction than in the sexual one in all six hybridizations performed between apomictic and sexual cDNA, and they were not found to be in different quantities among *B. decumbens* and *B. brizantha* cDNA, which are both apomictic species. Figure 1 shows the distribution of the 65 clones among the 6 subtractive libraries. No clone was found less abundant in the apomictic cDNA fraction than in the sexual cDNA in the 6 hybridizations performed.

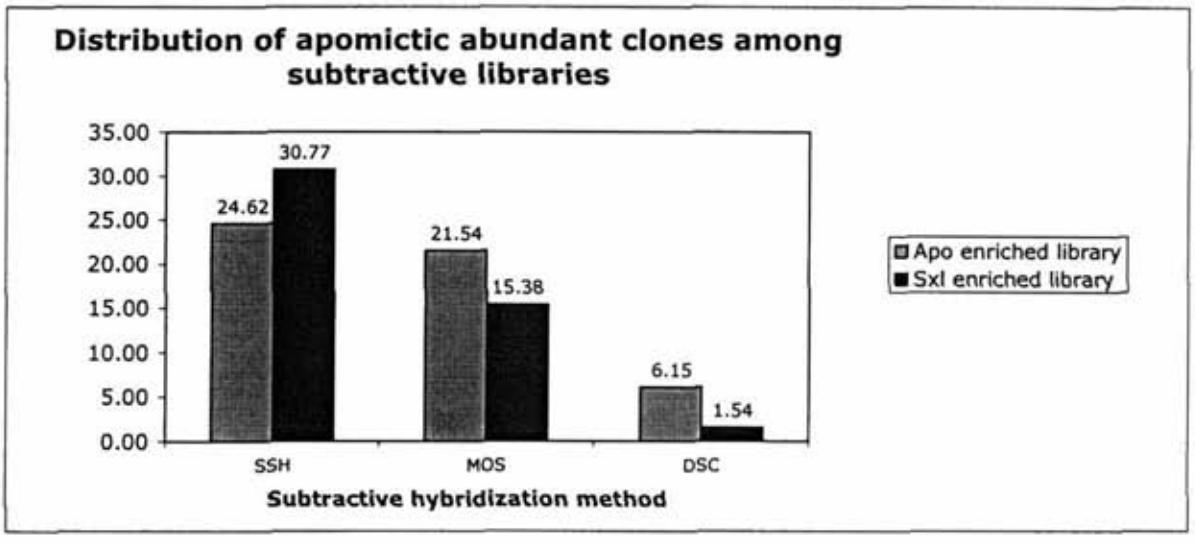

Figure 1. Distribution of 65 clones found in higher quantities in apomictic than in sexual cDNA according to microarray analysis among 6 subtractive libraries.

Finding some clones at higher levels in the apomictic cDNA fraction and none at higher levels in the sexual one supports the hypothesis that apomixis is a deregulation of the sexual pathway, where this deregulation is controlled by the expression (and not lack of expression) of certain genes only in the female gametophytes with apomictic reproductive development.

Finding clones at higher levels in the apomictic cDNA fraction from the subtractive library where the sexual cDNA was used as tester (i.e., apomictic cDNA was subtracted from the sexual cDNA fraction) is contradictory. A plausible explanation for this contradiction is that since apomictic plants are usually facultative apomictics (meaning they produce both apomictic and sexual seeds whereas sexual plants only produce sexual seeds), it is likely that the apomictic cDNA fraction contained sexual sequences in addition to the apomictic sequences. If this is the case, when we take the apomictic cDNA fraction we are really taking apomictic and sexual sequences, and when we take the sexual cDNA we are taking only sexual sequences. Then, when we subtract the sexual cDNA fraction from the apomictic cDNA fraction, we end up with those sequences that belong only to the apomictic cDNA fraction. On the other hand, when we perform the subtractive hybridization backwards (i.e., subtracting the apomictic cDNA fraction from the sexual

cDNA fraction), what we are really doing is taking sexual sequences and subtracting from them both apomictic and sexual sequences. Therefore, this subtractive hybridization is inefficient, because most likely there are no sequences found in the sexual sequences and not found in the apomictic plus sexual sequences. For that reason, this subtractive hybridization leaves us with random clones. Some of these random clones are found only, or at higher levels in the apomictic cDNA fraction according to the microarray analysis.

Another explanation could be that there are indeed no clones found only, or at higher levels in the sexual cDNA, so this backward subtractive hybridization would also be inefficient, also leaving us with random clones, and some of those clones are found only, or at higher levels in the apomictic cDNA fraction according to the microarray analysis. However, just as among those random clones, there are clones found more abundantly in the apomictic fraction, there should be clones found more abundantly in the sexual fraction, hence the fact of not having found any clones only or more abundantly in the sexual cDNA fraction according to the microarray analysis, supports the second explanation, that is that there are indeed no clones found only, or more abundantly in the sexual fraction.

According to the microarray analysis, there are 65 clones differentially expressed between apomictic and sexual plants, all of them found at higher levels in the apomictic fraction; 47 clones have been sequenced so far. Earlier, 150 clones were sequenced to assess the redundancy and kind of sequences we had obtained in the subtractive libraries. Currently, we have 197 sequences, of which only about 12% are redundant, meaning there is a big possibility that some sequences from the subtractive libraries were not cloned.

The comparison of the 47 sequences against the All nonredundant GenBank CDS translations+PDB+SwissProt+PIR database yielded either random hits or homologies with hypothetical, putative, unknown or known proteins, distributed as shown in Figure 2a. The sequences homologues to putative, hypothetical and known proteins are distributed among functional categories as shown in Figure 2b.

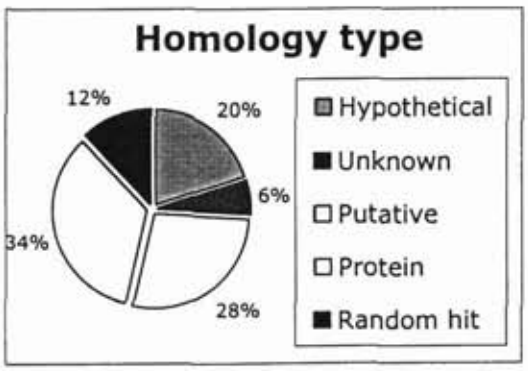

Figure 2a.

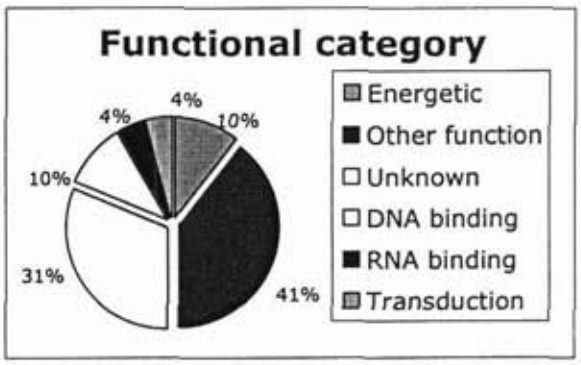

Figure 2b.

Distribution of the apomictic abundant clones among homology types and functional categories.

As Figure 2a shows, most of the hits are with real proteins (light aquamarine), around 12% show random hits, and the other hits were with sequences that are classified as proteins based on prediction with suitable software or on homologies with known proteins or ESTs. Therefore we might have some new sequences, putting together those that had random hits and those that gave hits with unknown proteins.

Looking at the sequences with hits, which tell us something about their function (Fig. 2b), we have some sequences that probably control the identity of cells, being them DNA or RNA binding proteins or transduction factors. We also have a big portion of unknown function proteins, and a big portion of energetic or other function proteins such as transport or proteosome proteins.

Given that apomixis might be a deregulation of sexuality in space or time, these sequences which code for transcription, translation or transduction factors are very interesting, since they regulate the fate of cells. A more in-depth analysis of their functions is being carried on.

Future actions

- More fragments from the subtractive libraries should be cloned, as the redundancy found in the sequenced fragments is very low.
- The 65 clones classified as more abundant in the apomictic cDNA fraction by the microarray analysis will be used to find the full-length mRNA in the full-length cDNA libraries constructed earlier.
- The full-length cDNA libraries will be amplified with clones from pistils less than 3 days old.
- A subtraction library and microarray analysis should be done with this new full-length cDNA from pistils less than 3 days old.
- A hundred and fifty clones that are different between the two apomictic species will be sequenced to gain basic knowledge about the evolutionary paths that are being taken by two closely related apomictic species.

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1.3.15 Phenotypic characterization and seed multiplication of a collection of rice T-DNA insertional mutants

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[1]SB-2 Project ; [2]IP-4 Project
Project funded by the Génoplante consortium

Introduction

In the framework of its work plan for functional analysis of cereal genomes, the Génoplante consortium decided to construct a rice T-DNA insertional mutagenesis collection. Rice was chosen because of its small genome and because of all genomic resources available for this species (ESTs, genetic maps, complete sequence, etc.). The lines are produced in Cirad laboratories, and grown in Cirad and IRD greenhouses, in Montpellier, France. The present work carried out at CIAT as a collaboration with Génoplante consists in (i) a systematical phenotypic evaluation of the mutant collection, with production of an associated phenotypic database, and (ii) the multiplication of seeds for the entire collection, for later distribution to all laboratories interested in rice functional genomics.

What is a T-DNA mutant collection? A T-DNA mutant collection is a library of lines obtained from transformation by *Agrobacterium tumefaciens*, using a specific construct called the *T-DNA insert*. If the T-DNA is inserted in a gene or its regulatory region, the insertion event results in alteration or disruption of the functionality of this gene, which can even be completely silenced. Each line contains one ore several insertions, resulting in the silencing of one of several genes in the line. Typically, dozens of thousands are produced. As the transformation events can be considered to be uniformly dispersed along the genome, one may expect that an important fraction of the genes contain an insertion. Additionally, the insertion sites can be individually characterized in sequencing the

genomic region flanking the T-DNA insertion site. We then dispose of a *Flanking Sequence Tags* (FST) database. Hence, in the case of completely sequenced genomes like *Arabidopsis* or rice, the insertion(s) of each line can be placed on the annotated genome through sequence comparison and thus related to a specific gene or gene region.

Utility of a T-DNA mutant collection. A T-DNA mutant collection is a powerful tool for discovering *gene functions*. Indeed, the disruption of a given gene or its promoter and/or transcription factors may result in alteration of the corresponding phenotype in relation to the wild phenotype. This phenotype can be observed at different levels - morphologic, developmental, physiologic, etc. As the entire collection gives access to genotypic-phenotypic association at the whole genome level, the scientific studies based on such a material fall into the field of *functional genomics*.

Basically, there are two main approaches for discovering gene functions using a such library:

• the collection can be used for *forward genetics* screens through systematical evaluation of all available T1/T2 families for a given trait. The mutant lines showing phenotypic variation in comparison to the wild phenotype can then be later analyzed for their T-DNA inserts (location on the genome, annotation on the sequence containing the insert) and association between the observed phenotype and the affected gene may be possibly established.

• the other approach is called *reverse genetics*. One desire to test hypotheses about *candidate sequences* suspected to have a role in the control of the trait under study. Reverse genetics screens allow identification of the mutant(s) disrupted in a given (gene) sequence based on (i) PCR based amplification product in 2D or 3D DNA pools, (ii) hybridization signals on flanking regions spotted on medium density filters or (iii) by homology search in the FST database, supposing that the entire collection has been sequenced at least for one of the two flanking regions of each T-DNA insert. The identified mutants can then be screened for the targeted trait to evidence genotypic-phenotypic associations. The main advantage of the reverse genetics approach is that it saves large-scale phenotypic screenings. It is specially interesting for time-consuming and/or high-cost phenotypic screenings.

Characteristics of the Génoplante T-DNA rice mutant collection. The collection is based on the cultivar Nipponbare (*Oryza sativa* L. ssp. Japonica), because (i) it is relatively easily transformed, and (ii) its genome was used as matrix for the rice genome sequencing project.

The lines are generated through Agrobacterium-mediated transformation through an efficient protocol (Sallaud et al., 2003). The objective is to ensure a primary coverage of the rice genome with enhancer trap T-DNA insertion sites (n = 40,000 lines). Individual and parallel characterization of insertion sites by systematic sequencing of the genomic

region flanking the left border of the T-DNA is carried out. Later, the enhancer trap T-DNA will be equipped with a non autonomous *Ds* element which can be further mobilized in trans for creating mutants alleles in genes in the vicinity of T-DNA insertion sites. The lines are also be individually characterized by GUS assays.

All production and phenotypic (including GUS assays) data will be gathered from the different Génoplante partners (including the present work) using automated entry in a phenotype database software. A sequence database software will integrate finished flanking sequence data, survey against known DNA sequences as well as a functionally oriented representation of their location in the rice genome. All these data will be integrated in the future public database Oryza Tag Line.

General features of T-DNA integration in this collection include (i) 1 to 4 copies were integrated at 1 to 2 loci, (ii) we observed 30-40% of single-copy transformation events (TE), (iii) the T-DNA segregated according to a 3:1 ratio in 95% of single copy TE, as well as 50% of multiple copy TE, (iv) a preferential integration in the gene-rich fraction of the genome was observed.

Phenotyping of the rice mutant collection. Here, we present one of the several projects carried out on the Génoplante rice mutant collection. In its first phase, it consisted the following main activities:

- to carry out screenhouse and field phenotyping of a first set of 5,000 lines,
- to produce a mutant phenotypic database,
- to produce T2 entire panicles for further detailed analyzes of grain filling,
- to multiply T2 seeds to constitute a rice T-DNA mutant stock center for future distribution and collaboration with partners.

Materials and Methods

Screenhouse. Five thousand T0 plants were produced at Cirad and grown in Cirad and IRD glasshouses in Montpellier, France. Twenty-five T1 seeds per T0 plant were received at CIAT and were sawn in a screenhouse.
Sawing was carried out in four batches of 1,250 lines, with about three weeks delay between two batches. Seeds were pre-treated by heat for three days at 50 °C to brake dormancy.
The nurseries were realized in plastic trays with a mixture of CIAT (67 %) and Santander de Quilichao (33 %) soils.
Germination was counted at ten days after sawing (DAS). The first phenotypic observations were carried out at 18-20 DAS, with counting of the number of individuals presenting the mutant phenotype. A list of possible phenotypic traits was established from data mining of several rice phenotypic databases (www.gramene.org, www.grs.nig.ac.jp/rice/oryzabase, www.irri.org/genomics), and was used as a guide for

observations. An English-Spanish-French lexical of botanical and agronomic terms was established to facilitate phenotype identification.

Field. A 2 hectares field was set up following the requirements of the ICA (Instituto Colombiano Agropecuario). In particular, the entire field was covered by nets to avoid damage and seed dissemination by birds.

Plantlets were transplanted at 25 DAS. A basic fertilization was applied, composed of Mono-Ammonium Phosphate, Iron Sulfate, Potassium Chloride and micro-elements. Irrigation was applied two times a week.

Control lines of Nipponbare cv. were planted each 10 T-DNA lines in order to facilitate the comparison with wild phenotype.

Phenotypic analyses were carried out at different ages, using the list of possible traits as a guide. A first round of observation was run on approximately 45 days-old plants. A second round was run at flowering time, while the ultimate observation was done at maturity. This permitted to maximize the chances to detect phenotypic variations, as various traits could be observed at one of these stages only. Moreover, this permitted to follow the evolution of a suspected phenotype at early stage and possibly confirm or invalidate it.

Harvest and storage conditions. Harvest was organized as followed:

- Three panicles per plant of each line were collected. This material is to be sent to Biogemma, a private partner of the Génoplante consortium. Biogemma will conduct phenotypic analyses on this material in the framework of a project on functional genomics of grain filling . Seeds from these panicles will later be sent to Cirad to constitute the main mutant stock center.

- The seeds from three to six panicles – depending on the level of sterility – were collected from each plant of each line. This will constitute the replicate of the mutant stock center hosted by CIAT.

- When possible, for each line showing a mutant phenotype, all remaining panicles from one plant with the phenotype were collected in order to facilitate segregation studies based on wild x mutant crosses.

Results

Legal aspects. All issues relative to legal aspects, i.e. importation permit, quarantine and authorization for field trial of transgenic plants were fixed.

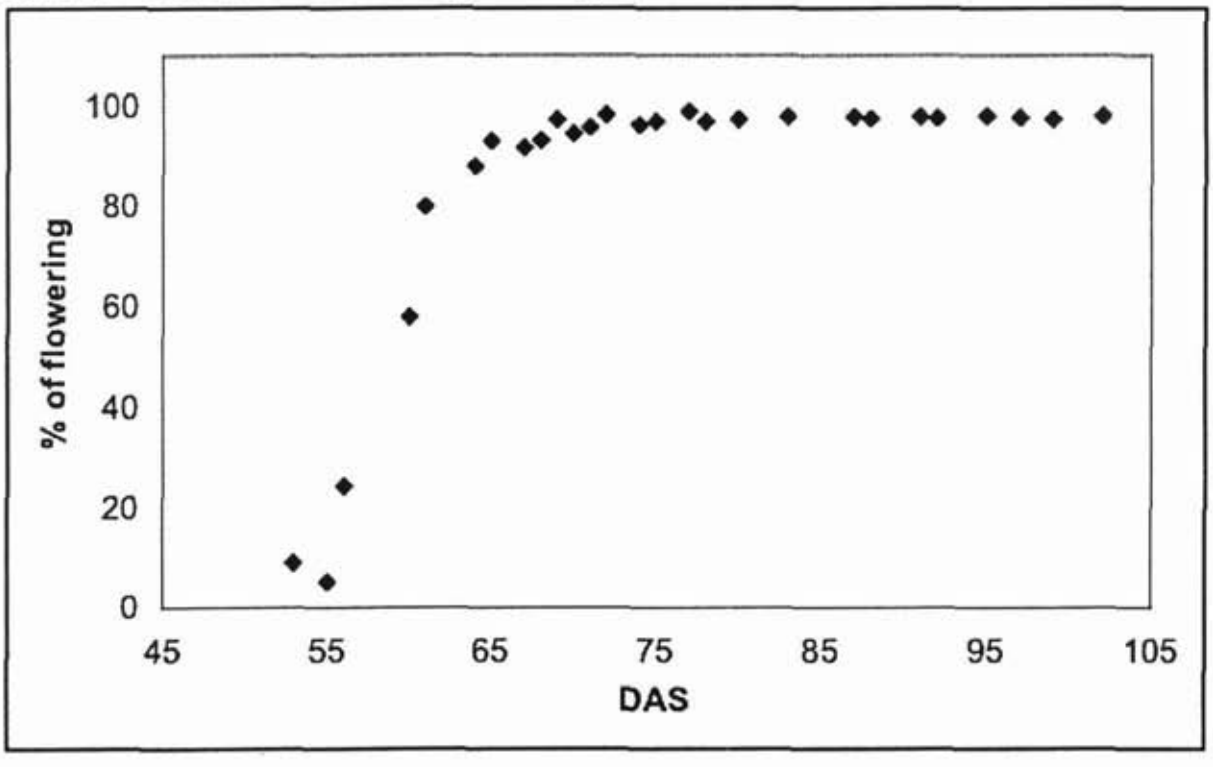
Germination rates. Germination rate of controls (Nipponbare cv.) was close to 100 %. For T-DNA lines, the mean germination rate was around 61 %. A trial without heat treatment showed a mean germination rate of about 35 % (Figure 1).

Growth. Rice plants generally grown normally. The Nipponbare variety showed a normal phenotype, however, due to short-days conditions inducing a short cycle, plants showed slight reduction in height and tillering than under long-days conditions.

Cycle. Nipponbare is a photosensitive cultivar. Thus, under short-days conditions (about twelve hours of daylight per day at Cali), its cycle is reduced in length. At 60 DAS, 50 % of the plants had flower, while the 90% of flowered plants was attained at 64 DAS.
The complete cycle of Nipponbare (sawing to maturation) under our conditions was around 93 days.

Diseases. Plant health was generally very good and close to optimum. Only very localized attacks of *Hydrelia* and Hoja Blanca Virus were observed. Also, zinc deficiency symptoms were observed in various locations of the field.

Figure 1. Evolution of the % of flowering in the Nipponbare control



Fertility/Seed set. Seed set of the controls was good. The mean fertility was around 81 %.

Mutant phenotypes. In the screenhouse, about 18 % of the lines showed phenotype variation in comparison to the wild type. This is much more than currently observed in

other mutant collections, where about 3 to 5 % of mutants are identified by visual phenotypic screening. T-DNA insertion is probably not responsible for all the variation observed. Indeed, it is well known that other sources of mutation like the Tos 17 retrotransposon are positively activated by *in vitro* culture of rice. Moreover, discrepancies in germination dates and seed quality, mainly due to the growth conditions of the T0 plants, may be responsible for apparent mutations, notably Growth-Retardate (GR), Tillering, Height. Also, redundancy of phenotypes was frequently observed between two or more lines. We think that, for lines showing close serial numbers, this could result from the same transformation event. It will be necessary to verify if those lines proceed from the same callus, thus bearing the same transformation event.

The rate of lines which showed any type of mutant phenotype was 18 %, which should be, as in the screenhouse and for the same reasons, an upper bound. Also, we chose to include even doubtful data as it is preferable to eliminate false-positive data after more detailed analyses for a specific trait than to miss real data. As it is impossible to describe all the data here in details, we chose to present only a few examples.
Numerous lines showed chlorotic or albino plantlets, with associated deficiency in leaf development (Figure 2a, b). General abnormal development was also frequently observed (Figure 2c, d).

The most common phenotype included several types of dwarfism more or less pronounced (Figure 3a). Some lines also showed a mutation for increased size. Figure 3b shows an example combined with awn spikelets.
Several lines showed reduced or increased tillering. Notably, a phenotype with very high tillering was observed for several lines (Figure 4b). The Growth-Retardate phenotype was frequent, with associated reduced tillering and height, and late flowering (Figure 4b).

Database set up. A local database of all data relative to growth conditions, germination, flowering, and phenotypic observations was set up. This database is used as a working tool to facilitate data entering and compilation. It also can be used for data browsing as it permit to display information by mutant bar code number or CIAT number. This database also displays photos of the mutants (Figure 5).
From this database, a flat datafile will be extracted in order to fill the Génoplante database which will be later made publicly available.

Conclusion

The overall process of seed multiplication and phenotypic analysis worked very well. The timetable was respected, and valuable phenotypic data were produced. This first trial should constitute a good basis for conducting a larger project.

Future plans

To extend the phenotypic analysis and seed multiplication to the entire collection (35,000 lines).

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Figure 2. Examples of mutant phenotypes observed in the rice T-DNA collection in the screenhouse. a: Chlorotic, b: Albino, c and d: Development defects

a

b

c

d

Figure 3. Examples of mutant phenotypes observed in the rice T-DNA collection. a: dwarf, b: increased size associated with spikelet awning

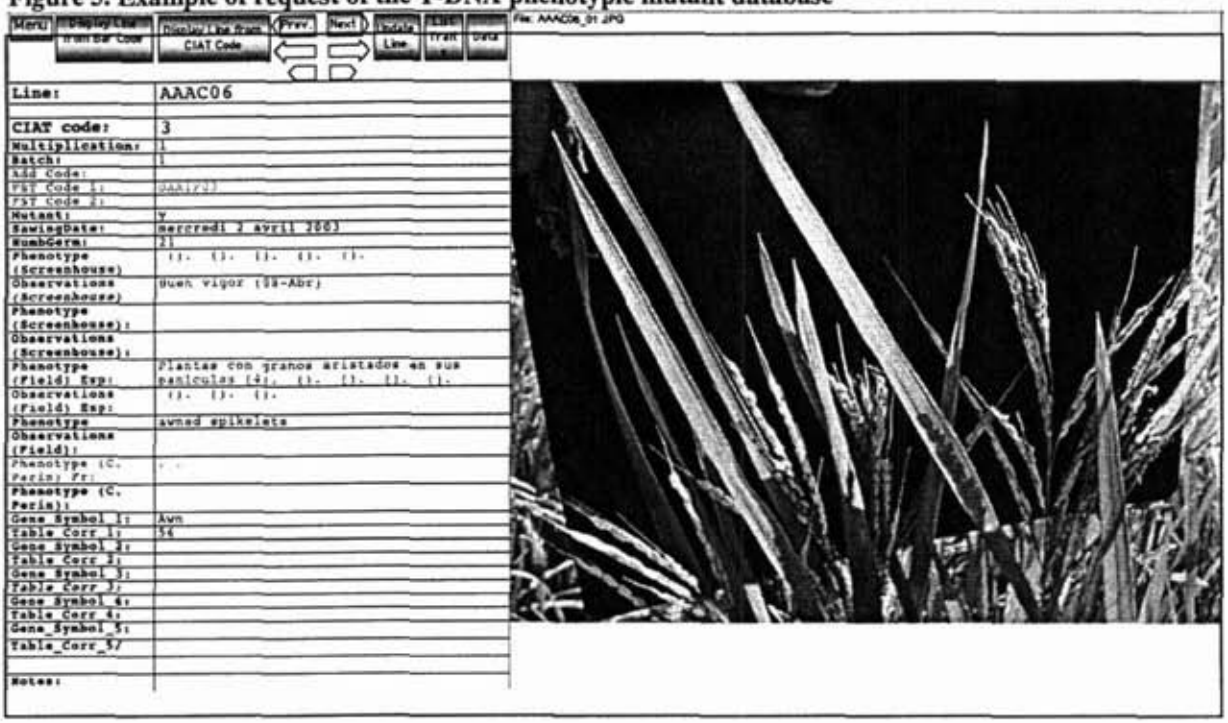
a

b

Figure 4. Examples of mutant phenotypes observed in the rice T-DNA collection. a: growth retardate, b: high tillering

a **b**

Figure 5. Example of request of the T-DNA phenotypic mutant database



1.3.16 Identification of genes induced during the defense response of *Brachiaria* to the Spittlebug

C. Romero, I. F. Acosta & J. Tohme
SB-2 Project

Introduction

The molecular basis of plant defense responses to insects is a challenging area whose understanding should make feasible the use of natural immunity in economically important plants. Although molecular biology has recently been incorporated in the exploration of these defense mechanisms, it has been mainly limited to studying the interaction between dicotyledonous plants and herbivorous chewing insects. Herein we focus in the defense responses of a monocot that exhibits resistance to a xylem-sucking insect, an interaction that is poorly understood at the molecular level given the peculiarity of this feeding habitat. One of the possible approaches to get closer to such a system without previous molecular data is the characterization of transcriptional changes during the plant response to the insect attack.

In this work we show the isolation of differentially expressed sequences in the resistant *Brachiaria* hybrid CIAT 36062 when challenged with *Aeneolamia varia* nymphs. This was achieved by a subtractive hybridization technique and a rigorous sequence analysis to identify putative functions of the isolated transcripts. Sequencing analysis of ~240 clones from the subtractive library revealed that they corresponded to 74 unique expressed genes. Putative functions were assigned to 41 transcripts through sequence similarity searches and the predicted proteins were classified in eight functional groups. These include three biosynthetic pathways of important plant signaling hormones, cell signaling, transcriptional regulation, cell wall modification and the homeostasis of the plant during the water stress caused by the insect. Finally, we found two putative effector proteins that may be contribute to the antibiotic action of the resistant plants on the insect.

Methodology

The subtractive hybridization was performed as described in the 2002 Annual Report, where we also showed the results of two 96-well pilot libraries (Annual Report, 2002). Consecutively, we decided to construct two additional libraries of 384 wells in order to expand the coverage of the subtractive product and to avoid the RNA ribosomal artifacts. Four bands corresponding to rRNA generated in the cDNA synthesis were identified by size when the product of the subtraction was run in an acrylamide gel. The rest of the smear observed in this gel was excised and cloned to create the new libraries.

Similarity searches were performed in the GenBank using the BLASTX algorithm. The matching sequences were searched in annotated databases such as TAIR (The Arabidopsis

Research Institute), GRAMENE, SWISS-PROT, and ENZYME in order to determine their putative functions. More specific information was obtained in secondary databases as InterPro (*Integrated Resource of Protein Families*), Pfam (*Protein Families Database*), PRINTS (*Protein Motif Fingerprint Database*), AraCyc (*AraCyc: Arabidopsis thaliana Biochemical Pathways*), and CDD (*Conserved Domain Database*, at NCBI). In these databases we found functional information such as precise biochemical roles, metabolic pathways and redundant proteins (other proteins with the same function). Furthermore, we found structural information such as protein motifs and domains contained in the predicted *Brachiaria* proteins. Finally, in some cases we performed two pair alignments of amino acid sequences using the pair BLAST algorithm to confirm the structural relationship between elements isolated from *Brachiaria* and the proteins previously reported in resistance studies in other species.

A macroarray experiment was carried out in order to test the differential expression of the isolated transcripts. The clones of the four libraries were arrayed using a 384-well pin replicator on duplicated nylon membranes and grown on LB-agar medium overnight. The bacterial colonies were denaturalized and the free DNA was UV-crosslinked. These filters were hybridized with radioactivity labeled cDNA from infested plants and from non-infested plants and exposed to autoradiograph films.

Results

The cloning strategy to avoid rRNA sequences in the new libraries -which have been partially screened at this point- was successful since the proportion of these artifacts decreased from 40% to 5%. Bringing back together the results of last year's pilot libraries, a total of 240 clones yielded readable sequences, which corresponded to 74 unique transcripts. No match was found for 26 (35%) of them in the GenBank; these may constitute a reservoir of new genes, absents in model species. Seven more sequences were not considered further (e-values over 10^{-7}). Eighteen out of the remaining 41 sequences are similar to genes that have been shown to be part of defense responses in other plant-insect or plant-pathogen interactions (see Table). These sequences can be divided in six main groups according to their presumed roles in defense (see Figure)

Hormone Biosynthesis Pathways. The first group contains sequences coding for putative enzymes that catalyze important steps in the biosynthesis of oxylipins, brassinosteroids and ethylene.

Oxylipins are the most important hormones in the systemic response to wounding and insects. Plants deprived of Phospholipase (Turner *et al.*, 2002), Fatty acid desaturase (Martin *et al.*, 1999) and Lypoxigenase (Bell *et al.*, 1995) are not able to produce Jasmonic Acid (JA) and are incompetent to activate defense mechanisms such as proteinase inhibitors, defensins and thionins.

Brassinosteroids have been mainly studied in cell division and plant development but recently they were assigned a role as systemic defense hormones in responses to virus, bacteria and fungi in both dicots and monocots (Nakashita *et al.*, 2003).

Although the role of Ethylene is widely recognized in plant-pathogen interactions, its effects can differ in different situations. The emission of ethylene has been proved as a mechanism of communication between organisms that attracts natural enemies of herbivores and causes unwounded leaves to initiate ethylene biosynthesis as a positive feedback loop (Arimura *et al.*, 2002)

Table: Putative functions assigned to the transcripts isolated. Sequences related with defense responses in other systems are shown in bold

Putative Function	E value	Score
O-Methyltransferase	3.00E-89	329
Hypothetical Protein 1	1.00E-71	269
Ornithine carbamoyltransferase (OCTase)	**5.00E-67**	**233**
Cysteine Proteinase	**7.00E-63**	**241**
Phospholipase C (A)	**8.00E-58**	**213**
Hypothetical Protein 3	1.00E-52	206
dTDP-glucose 4-6-dehydratase	4.00E-53	206
Chlorophyll a/b-binding protein (CAB)	5.00E-50	196
Phospholipase C (B)	**1.00E-49**	**195**
Omega-3 fatty acid desatura	**4.00E-47**	**187**
CBL-interacting protein kinase (CIPK)	**1.00E-41**	**168**
Tubulin alpha	2.00E-41	168
Caffeoyl Coenzyme A 3-O-Methyltransferase	**4.00E-39**	**161**
MADS Box	**1.00E-37**	**148**
Glutathione S-conjugate ABC transporter	3.00E-35	147
Xyloglucan endotransglycolase	**2.00E-34**	**145**
Lypoxigenase A (LOX)	**3.00E-34**	**145**
Lypoxigenase B (LOX)	**3.00E-34**	**144**
Unknown protein 3 (TMS1d)	2.00E-31	132
Fructose bisphosphate aldolaseÊ	1.00E-27	122
Ca dependent mitochondiral carrier protein	7.00E-27	119
NAD+ dependent isocitrate dehydrogenase subu	2.00E-25	116
Sequence associated to Pi2 (RG64 RFLP mar	3.00E-24	119
Developmental Protein	3.00E-22	104
Elongation factor 1-alpha	6.00E-21	100
Unknown protein 2	2.00E-20	98
Pur-alpha 1Ê	**3.00E-17**	**88**
SCARECROW	**3.00E-17**	**88**
Putative replication proteinÊ	4.00E-16	84
Extracellular lipase 3 (A)	2.00E-15	55
Cold acclimation protein	8.00E-14	77
60s ribosomal protein L13	9.00E-14	76
Hypothetical Protein 6	7.00E-12	71
S-adenosylmethionine synthetaseÊ	**2.00E-11**	**69**
Hypothetical Protein 4	2.00E-10	66
Caffeic acid O-methyltransferase	**1.00E-09**	**62**
Carbonic Anhydrase	3.00E-09	146
Metalloprotease	**1.00E-08**	**57**
Unknown protein 1	1.00E-07	56
Steroid 22-alpha-hydroxylase (cytochrome	**1.00E-07**	**56**
Extracellular lipase 3Ê(B)	2.00E-07	57

Cell signaling. The presence of a putative CIP kinase can be explained by two different processes: a) this protein may interact with CBL, a plant calcium sensor, in the signaling cascade activated under a spittlebug attack. Ca+ signaling in responses to wounding and pathogens has been well documented. b) CIP kinases contain a SNF1 domain, suggesting a possible role in gene regulation controlled by cytoplasmic carbohydrate concentration (sugar sensing), an activity that may be related with the rapid change in metabolism that should occur to supply the energy requirements of the defense responses (Rolland *et al.*, 2002).

Transcriptional Regulation. SCARECROW is a transcription factor that is rapidly induced upon perception of the elicitor N-acetylchitooligosaccharide. Moreover, its mRNA is induced in rice upon fungal infection but not in the presence of bacterial pathogens (Day *et al.*, 2003). Both evidences suggest that this transcription factor may be involved in

responses to enemies that contain chitin, which could be a way to regulate defenses against a broad but specific range of organisms (fungi and arthropods).

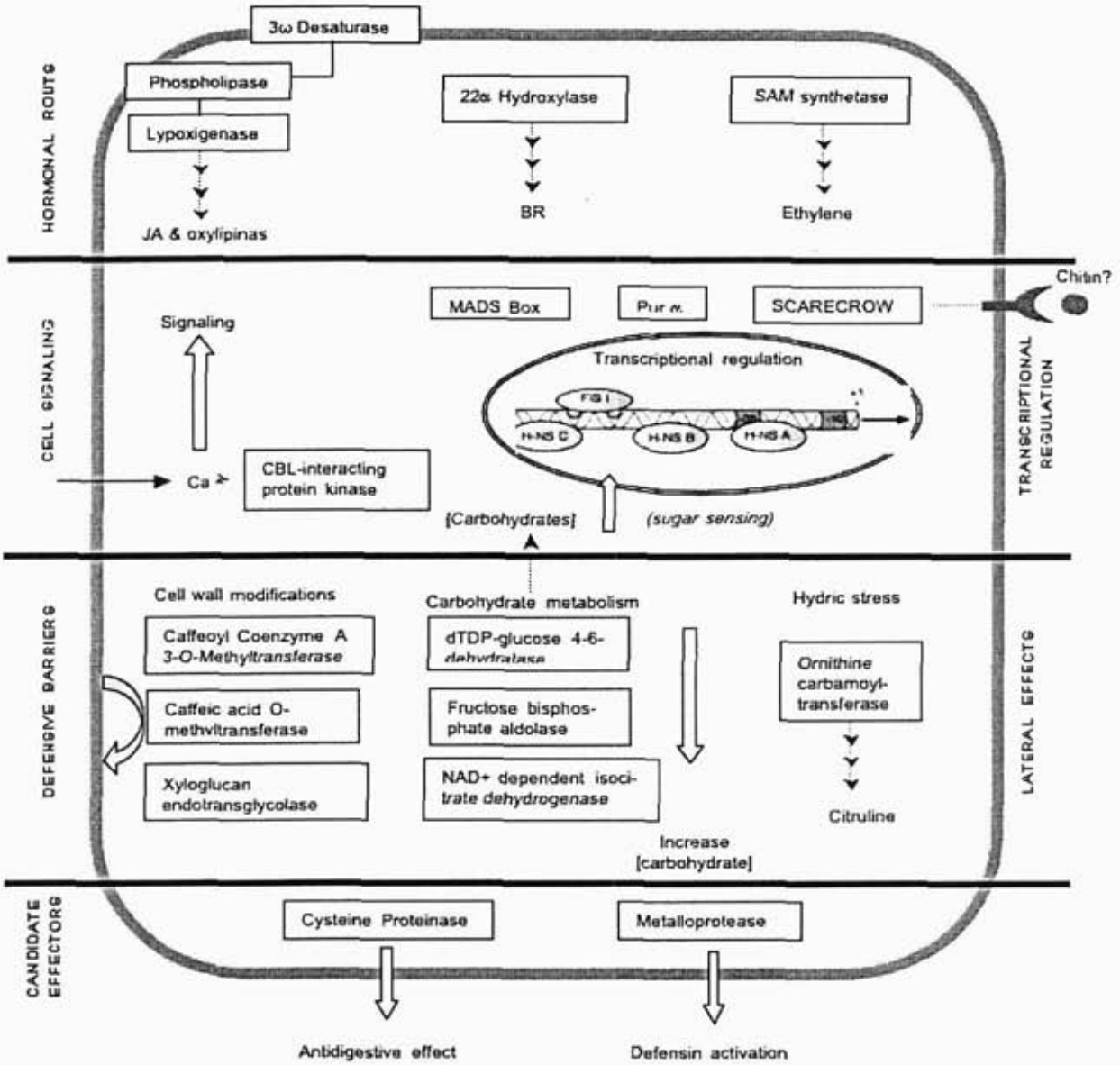


Figure Summary of the candidate defense-related genes and cellular processes found in a *Brachiaria* resistant line in response to the interaction with the spittlebug (*Aeneolamia varia*). They can be divided in 6 main functional groups discussed in the text

Lateral effects (water stress). The ornithine carbamoyltransferase participates in the synthesis of citrulline, a precursor amino acid of arginine. Some plants accumulate citrulline under water stress in order to increase the concentration of compatible solutes (those that do not alter the electrostatic equilibrium and thus do not disrupt the catalytic properties of enzymes). In this way, the plant enhances its capacity to absorb water decreasing its hydric potential. The production of citrulline in *Brachiaria* in its interaction

with the spittlebug may be due to the dramatic alteration of the hydric state caused by this sucking insect on the xylem vessels.

Defensive barriers. Transcripts for 3 putative enzymes implicated in lignin biosynthesis and cell wall modification were detected in this study. This phenomenon is frequently found in response to pathogens to confine them to the site of infection and avoid their dispersion (Ye *et al.*, 2001), an action whose importance in the defense against an insect seem less obvious. The expression patterns of these elements agree with previous evidences because they have shown to be induced by wounding, ethylene and brassinosteroids.

Effector mechanisms. Finally, we found two sequences that encode putative proteins that may participate more directly in the reduced survival of the insect in resistant varieties (antibiosis):
A metalloprotease with a domain similar to that of proteases involved in the activation of defensins (Liu *et al.*, 2001), this, in turn, may have been previously induced by the oxylipins.
A cysteine protease highly homologous to one in maize that induces the disruption of the peritrophic matrix in the gut of a caterpillar. Maize callus transformed with the gene encoding this protein reduce growth of the insect as the resistant plant does. The effect of the cysteine proteinase is probably the perforation of the digestive tube (Pechan *et al.*, 2002), demonstrated by electron microscopy, an event that decreases nutrient absorption and facilitates invasion by pathogens.

The macroarray experiment in order to confirm the differential expression of the transcripts isolated shows a percentage of 5% of false negatives. Consistently the putative functions of these non-differential sequences were unrelated with defense mechanisms, therefore they have not been taken in to account for the discussion.

Conclusions and Perspectives

Our results shed the first lights on the molecular mechanisms that determine resistance of a monocotyledonous plant to a xylem-sucking insect. New and exciting experiments may be designed to complement our findings and to test the assumptions that transcript sequence information has provided. In the short-term these are our goals:

Exogenous applications of jasmonic acid and brassinolide hormone to susceptible varieties in order to determine phenotypic changes in defense capacities
Real Time PCR amplification to quantify expression of these transcripts between plants infested by different spittlebug species in order to detect common central defense mechanisms.

Use of Virus Induced Gene Silencing (VIGS) to evaluate the function in defense responses of the differentially expressed genes.

A new subtractive hybridization to detect constitutive mechanisms of resistance by comparing gene expression of susceptible and resistant plants both exposed to the insect. A more subtle infestation method will be used and the experiment will span the first 24 hours post-infestation.

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Investigating physiological and genetic aspects of a luminum resistance in *Brachiaria*
As part of the restricted core project funded by BMZ-GTZ of Germany, we continued our efforts to investigate physiological and genetic aspects of aluminum resistance in *Brachiaria*.

1.3.17 Exploring surface charge density of root apice as potential factor contributing to aluminun resístanse of *Brachiaria decumbes*

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Introduction

There is a pronounced difference in aluminum (Al) resistance between *B. decumbens* and *B. ruziziensis* (Wenzl et al., 2001). Previous results demonstrated that this difference is not restricted to Al^{3+} ions but is also observed for trivalent lanthanide ions (see IP-5 Annual Report, 2002). We hypothesized that a less negative (or even positive) surface charge of root apices of *B. decumbens* compared to those of *B. ruziziensis* could be the underlying physiological mechanism.

Materials and Methods

Seeds of *B. decumbens* and *B. ruziziensis* were germinated in 200 □M $CaCl_2$ (pH 4.2) for 4 – 5 days. Homogeneous seedlings, with root lengths between 2 and 3 cm, were transferred to continuously aerated solutions containing 200 □M $CaCl_2$ (pH 4.2) and various concentrations of different cationic or anionic toxicants. The seedlings were left to grow in the glasshouse for 3 days. At harvest, root lengths were measured and root elongation was calculated by subtracting the root length at transfer. Relative root elongation (RRE) values were computed by referencing root elongation values against root elongation in the toxicant-free reference treatment.

Results and Discussion

Figure 1 displays RRE values of the two species exposed to various concentrations of a variety of cationic and anionic toxicants. Data points below the diagonal indicate that *B. decumbens* is more resistant than *B. ruziziensis*; data points above the diagonal indicate greater susceptibility of *B. decumbens* compared to *B. ruziziensis*. The left panel clearly shows that *B. decumbens* is more resistant to all the trivalent cations tested. The latter would be consistent with root apices of *B. decumbens* having fewer negative surface charges than those of *B. ruziziensis*. This is because a lower negative surface charge density would entail lower concentrations of cationic toxicants in the vicinity of root surfaces.

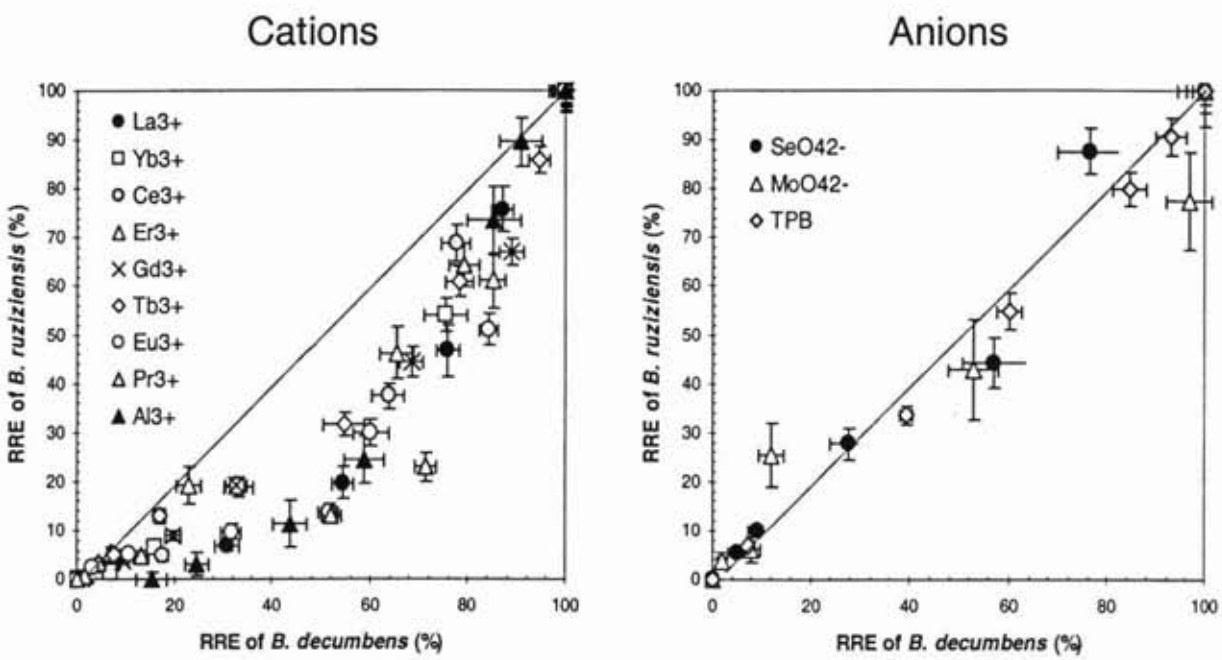

Figure 1. Comparison of relative root elongation (RRE) of *B. decumbens* and *B. ruziziensis* exposed to cationic (left panel) or anionic toxicants (right panel). Cationic toxicants included lanthanum (La^{3+}), ytterbium (Yb^{3+}), cerium (Ce^{3+}), erbium (Er^{3+}), gadolinium (Gd^{3+}), terbium (Tb^{3+}), europium (Eu^{3+}), praseodymium (Pr^{3+}), and aluminum (Al^{3+}). Anionic toxicants included selenate (SeO_4^{2-}), molybdate (MoO_4^{2-}) and tetraphenylborate (TPB). All values are means ± SE of 36 seedlings measured in three independent experiments.

A lower negative surface charge density, however, should also make *B. decumbens* more sensitive to anionic toxicants than *B. ruziziensis*. The right panel of Figure 1, however, does not appear to confirm this prediction because the data points do not cluster above the diagonal. Results from a greater variety of anionic toxicants are required; yet these data do not appear to be consistent with the idea that Al resistance of *B. decumbens* is due to reduced electrostatic attraction of Al ions to the surface of root apices. Instead they appear to point to a mechanism that may be based on binding to cellular ligand(s). Work is in progress to further characterize this generic cation-resistance mechanism.

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1.3.18 Evaluating physiological components of acid soil adaptation in a population of *Brachiaria ruziziensis* x *Brachiaria decumbens* hybrids

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Introduction

Last year we confirmed that a *B. ruziziensis* x *B. decumbens* hybrid population segregates for two physiological components that are important for growth in infertile acid soil: root growth vigor and Al resistance. We therefore continued to evaluate most of the 274 hybrids. The cumulative data from ten harvests are now ready to be combined with molecular marker data to identify QTLs underlying these traits.

Materials and Methods

Stem cuttings of hybrids and parents were rooted in a low ionic strength nutrient solution in the glasshouse during 9 days. Equal numbers of stem cuttings were transferred into a solution containing 200 µM $CaCl_2$ pH 4.2 (reference treatment) and a solution containing 200 µM $CaCl_2$ and 200 $AlCl_3$ pH 4.2 (Al treatment). The solutions were changed every second day to minimize pH drifts. At harvest on day 21 after transfer, the dry weight of stems was measured. Roots were stained and scanned on a flatbed scanner. Image analysis software (WinRHIZO) was used to determine root length, average root diameter and number of root apices. The root growth data from ten harvests were log-transformed because such growth data tend to be log-normally distributed (Causton and Venus, 1981). They were then adjusted for harvest mean (based on the differences between harvest means and overall mean) and the dry weight of the stem cuttings (using linear regression).

Results and Discussion

The B. ruziziensis x *B. decumbens* hybrids showed a broad range of root growth vigor. A considerable number of individuals were superior to well-adapted *B. decumbens*, perhaps as a result of heterosis induced by the interspecific cross (see large number of data points to the right of *B. decumbens* in Figure 1). By contrast there were only few individuals whose roots elongated as poorly as those of *B. ruziziensis*.

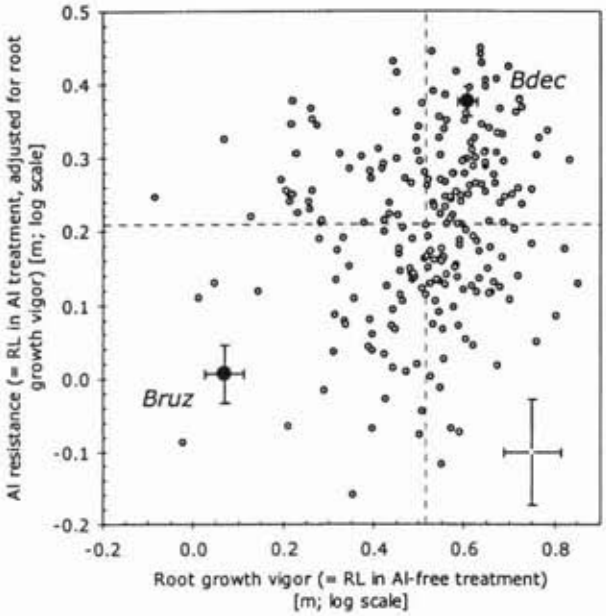

Figure 1. Segregation of root growth vigor (x axis) and Al resistance (y axis), both measured based on root elongation (RL = root length) in a *B. ruziziensis* x *B. decumbens* hybrid population. Al resistance was quantified by adjusting RL of plants in the Al treatment for their RL when grown without Al (see text for further details). Data points representing parents are highlighted with large black symbols. Error bars in the lower right corner denote the average SE of the hybrid population.

Because root growth vigor varies among hybrids, the inhibitory effect of Al on root length (RL) is not easy to measure, and RL in the Al treatment has to be properly referenced. We tested two approaches. First, we calculated relative root length values by comparing – for each pair of stem cuttings distributed among the two treatments – RL in the Al treatment vs. RL in the treatment without Al (the logarithm of the ratio was used for this purpose). The resulting "Al resistance index" was not correlated with root growth vigor. We therefore also tested adjusting RL values in the Al treatment for RL values in the Al-free treatment using linear regression.

The two approaches were compared using the degree of correlation among three parameters describing different effects of Al toxicity as a criterion. These were: (i) inhibition of root elongation (see above), (ii) lateral swelling of roots (resulting in a greater root diameter, RD), and production of a greater number or short laterals (resulting in a higher specific root tip number, sRT = number of root tips per unit dry weight). The RD and sRT parameters had been computed based on the two approaches described for RL. The three parameters were more tightly correlated in the second approach, thus suggesting superior data quality. The latter was also corroborated by the fact that the two parents were closer to the two extremes of the distribution. We therefore used the linear regression approach to present the data in Figure 1.

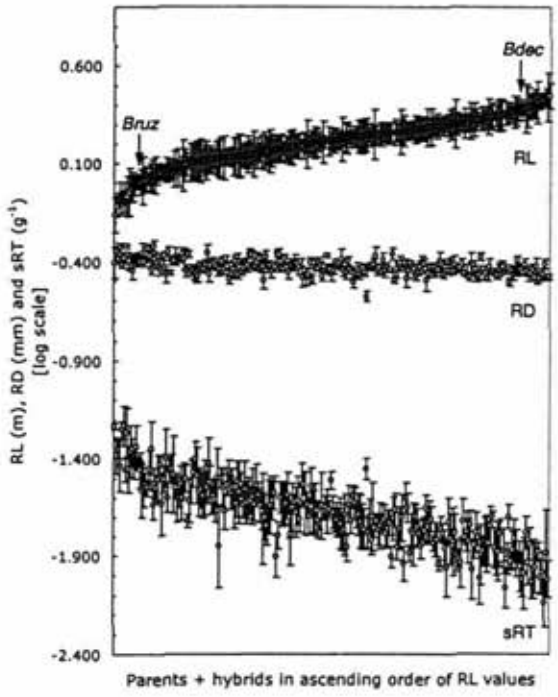

Figure 2. Comparison of three parameters that reflect Al resistance: root length (RL), average root diameter (RD), and number of root tips per gram of root dry weight (sRT = specific number of root apices). The parameters were measured with rooted stem cuttings in an Al-toxic solution. They were adjusted for harvest mean, the effect of the dry weight of stem cuttings used, and root growth vigor measured with another set of stem cuttings in an Al-free reference treatment (see x axis in Figure 1). Data points representing parents are highlighted with large black symbols.

Figure 2 displays the relationship among the three different Al-toxicity parameters used above. Genotypes more severely affected by Al toxicity have smaller root systems (shorter RL) that are made up of thicker roots (greater RD) and are more heavily branched (more root tips per unit dry weight, sRT). It may be possible to increase the robustness of detecting Al-resistant genotypes by computing the principal component of these three parameters to create "a composite Al resistance index" that captures the various Al-induced changes in root growth and architecture in a single parameter.

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1.3.19 Identifying candidates genes whose expression is associated with aluminum resistance in *Brachiaria*

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Introduction

Last year we reported on the identification of individuals of a *Brachiaria ruziziensis* x *Brachiaria decumbens* hybrid population with contrasting degrees of Al resistance. This

suggested the feasibility of isolating candidate Al-resistance genes from root apices based on a comparison of gene expression patterns between an Al-resistant and an Al-sensitive bulk of hybrids. This year we pursued this approach.

Materials and Methods

Rooted stem cuttings of *B. decumbens*, *B. ruziziensis* and a group of hybrids with contrasting Al resistance levels were cultivated as described in "Evaluating traits associated with acid soil adaptation in a population of *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrids". Root apices collected at various harvests were pooled to create 6 samples for RNA extraction: two Al-resistant bulks of hybrids (grown with +/-Al), an Al-sensitive bulk of hybrids (grown with Al), two *B. decumbens* samples (grown with +/-Al) and a *B. ruziziensis* sample (grown with Al). Total RNA was isolated, mRNA was captured with magnetic beads, and ds-cDNA was synthesized using anchored oligo(dT) primer/adapters. To minimize cross-hybridization among genes belonging to the same family, 3'-UTRs were amplified by suppressive PCR after simultaneous digestion of cDNAs with MseI and MspI followed by adapter ligation. The resulting amplicons were then mixed in various combinations and subjected to three rounds of differential subtraction chain (DSC) (Luo et al., 1999).

Results and Discussion

Figure 1 displays the 3'-UTR amplicons obtained from the six mRNA samples. Control experiments confirmed that only 3'-UTR fragments and no internal gene fragments had been amplified (i.e. suppressive PCR was successful). In addition, sequencing of random 3'-UTRs fragments identified homologies to 3'-regions of known genes, including one coding for a root-specific protein. As a result of the double digestion with two 4-bp cutters, the fragments are small. This should have minimized cross hybridization among different genes during the subsequent subtractive hybridization steps.

The 3'UTR amplicons shown in Figure 1 were mixed in various tester-driver combinations to enrich differentially expressed genes using the DCS approach (Table 1).

Table 1. Combinations of tester and driver cDNA samples used for subtractive hybridization.

	Tester		Driver	
Combination	Genotypes	Treatment	Genotypes	Treatment
1	Al-resistant F_1 bulk	200 □M Al^{3+}	Al-sensitive F_1 bulk	200 □M Al^{3+}
2	Al-resistant F_1 bulk	200 □M Al^{3+}	Al-resistant F_1 bulk	no Al^{3+}
3	*B. decumbens*	200 □M Al^{3+}	*B. ruziziensis*	200 □M Al^{3+}
4	*B. decumbens*	200 □M Al^{3+}	*B. decumbens*	no Al^{3+}

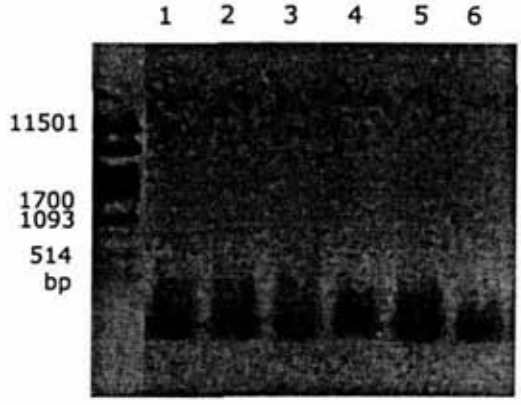

Figure 1. 3'UTR amplicons obtained from six cDNA samples: **1**, Al-resistant F_1 bulk grown at 200 μM Al^{3+}; **2**, Al-resistant F_1 bulk grown without Al^{3+}; **3**, Al-sensitive F_1 bulk grown at 200 μM Al^{3+}; **4**, *B. decumbens* grown at 200 μM Al^{3+}; **5**, *B. decumbens* grown without Al^{3+}; **6**, *B. ruziziensis* grown at 200 μM Al^{3+}.

The 3'-UTR fragments remaining after three rounds of subtractive hybridization were separated on a polyacrylamide gel. Distinct bands were visible in some combinations of tester and driver amplicons (Figure 2). This appears to suggest that the DSC procedure may have been successful.

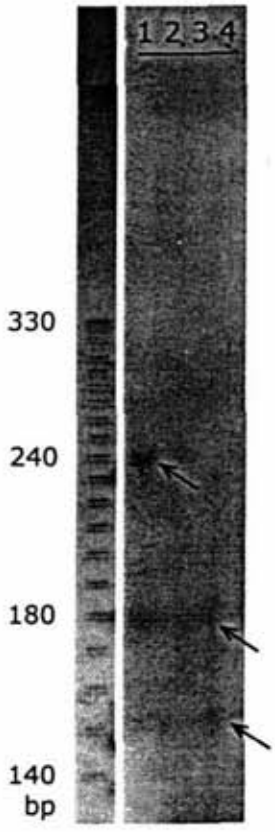

Figure 2. 3-UTR fragments remaining after three rounds of DSC. The numbers refer to the four combinations of tester and driver amplicons listed in Table 1.

Bands will be excised from the gel, cloned and sequenced. In addition, random fragments from the second round of subtraction will be cloned and sequenced. Depending on their redundancy they will be used to fabricate cDNA microarrays for screening for differentially expressed genes.

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1.3.20 Isolating genes from root apices of *Brachiaria decumbens* that enhance Al resistance of yeast

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[1] CAMBIA- Australia; [2]CIAT

Introduction

Some genes that when transformed into plants increase their resistance to Al, were originally identified based on their ability to enhance Al resistance of yeast. We used this approach to identify candidate Al-resistance genes in root apices of *B. decumbens* grown in the presence of Al.

Materials and Methods

Total RNA was extracted from root apices of rooted *B. decumbens* stem cuttings grown in a solution containing 200 □M $CaCl_2$, 200 □M $AlCl_3$ (pH 4.2). After capture of mRNA with magnetic beads, ds-cDNA was synthesized, ligated to adapters, size-fractionated and PCR-amplified. PCR products from the > 2 kb and < 2 kb fractions were ligated separately to linearized pYES2 plasmid and transformed into *E. coli*. The libraries obtained were amplified on plates. Plasmids extracted were mixed at a 1:1 ratio and re-transformed into yeast. Transformants were plated on a medium containing enough Al to arrest growth of yeast cells transformed with empty plasmid. Plasmids from the most quickly growing colonies were isolated, re-transformed into *E. coli*, and extracted for further characterization.

Results and Discussion

Approximately 100 yeast colonies were obtained from two million cfu plated on the Al medium (= 0.005 %). The plasmids isolated from 48 well-growing colonies were digested with a mixture of restriction enzymes to identify clones that had been isolated more than once, thus minimizing the chance of selecting false positives for further analysis. This fingerprinting experiment identified nine clones that had been isolated at least twice from

different colonies. Work is underway to sequence and characterize inserts from these clones.

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OUTPUT 2 Genes and genes combinations made available for broadening the base of mandated and non mandated crops

Activity 2.1 Transfer of gene and gene combinations using cellular and molecular techniques

2.1.1 Development of tepary x common bean interspecific hybrids with improved competence to *Agrobacterium* mediated transformation

Alvaro Mejía Jiménez, Leonardo Galindo, Catalina Oviedo and Joe Tohme
SB-2 Project

Introduction

Among the possible ways for the genetic transformation of plants, the transformation with *Agrobacterium* as vector, offers unique advantages. The common bean *(Phaseolus vulgaris)* has been recalcitrant to *Agrobacterium* mediated transformation. Of the *Phaseolus* species only the tepary bean (*P. acutifolius*) has been transformed through the use of this bacterium.

Two methodologies have been used for achieving it: the inoculation of green nodular callus tissues (Dillen *et al.*, 1997) or of meristems of mature seeds (*Agrobacterium* Mediated Mature Seed Meristem Transformation or AMMSM-Transformation; see Annual Reports 2000-2002). The AMMSM-Transformation method does not require previous induction of callus tissue, and thus allows the screening of large populations of genotypes with less effort than that of Dillen et al.

In the past 3 years we have screened more than 50 cultivated and wild genotypes of common and tepary bean for competence to AMMSM-transformation with no success. Results from other series of transformation experiments, made with tepary bean genotypes and hybrids, suggested that nuclear genes of tepary bean accessions are involved in conferring competence to AMMSM-transformation (see annual report of 2001).

In 2000 we started an interspecific crossing program using as parents a tepary bean genotype with competence to AMMSM-transformation and other common and tepary bean genotypes which show good response to *in vitro* culture and plant regeneration The objective of this crossing program is to breed AMMSM-transformable hybrid lines between these species (or any other *Phaseolus* species which can contribute to the transformation process) that can be used for the fast and easy transfer of transgenes to common bean cultivars by sexual crossing. As strategy for combining different traits involved in tissue culture response, plant regeneration or AMMSM-Transformation in one

genotype, we are using the double congruity backcross (DCBC) methodology (Mejia – Jiménez et al. 2002).

During 2003 we advanced in identifying DCBC hybrids, mainly with the cytoplasm of tepary bean, with improved competence to AMMSM-Transformation.

Methodology

Mature seeds of advanced DCBC hybrids with common and tepary bean cytoplasm were used as explants in the transformation experiments. The DCBC hybrids corresponded to the generations: V-DCBC$_{6A}$, V-DCBC$_{6B}$, V-DCBC$_{7B}$, V-DCBC$_{8C}$, V-DCBC$_{8D}$, V-DCBC$_{8E}$, with *P. vulgaris* cytoplasm and the generations, A-DCBC$_{8D}$, A-DCBC$_{10A}$, A-DCBC$_{10B}$, A-DCBC$_{10C}$, A-DCBC$_{12B}$ with *P. acutifolius* cytoplasm (see table DCBCHybrids: http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf).

The binary *Agrobacterium* strains AGL1/pCambia1305-1 AGL1/pCambia3200 (http://www.cambia.org) or the strain C58C1/ pTARC-B1b (Dillen et al. 1997) were used. The transformation methodology was described in the annual report of 2001.

Results

Screening of hybrid populations for competence to AMMSM-Transformation. A total of 168 DCBC hybrid lines with cytoplasm of *P. vulgaris* (V-DCBC) and 197 lines with cytoplasm of *P. acutifolius* (A-DCBC; an average of 37 explants per line), were screened between October 2002 and September 2003 for AMMSM-transformation. Friable and meristematic calli (m-calli) were recovered after selection in 26 (7.1%) of the screened lines. Initially only the recovered friable calli expressed the GUS-intron gene, which is driven by the p35S promoter.

In several of the selected lines that have been screened for GUS expression the selected m-calli developed other cellular types such as friable calli (for example in several calli selected from the lines GKAX-11B(L)2 F_4 and GKAX-13B F_3).

Due to this and to the fact that during selection conditions *in vitro,* we applied (by using a temporary immersion system) strong and uniform selective conditions, we believe that most of the tissues resisting selection are genetically transformed and that the lack of GUS expression in the m-calli is caused by other factors (for example the inactivity of the 35S promoter in this tissue type).

Since we can only regenerate plants from the m-calli and not from friable calli, we are measuring the formation of m-calli after transformation and selection to detect genotypes with transformation capabilities.

Twenty (10.1%) A-DCBC lines produced m-calli after selection, while only 6 (3.5%) of the V-DCBC lines produced this same tissue type.
However the m-calli of all the V-DCBC lines and of several A-DCBC lines died within 3 months after selection. Thus not only the selection of m-calli seems to be important for the recovery of transgenic plants through the AMMSM-transformation methodology. It is necessary to be able to induce further growth and bud differentiation in the selected tissues. Only 6 (1.6%) of the lines produced m-calli which could be maintained for longer than 3 months and that could be induced to differentiate buds. Within these lines there are two with M-calli survival efficiencies of 21.7 and 35%. That is more than three fold the survival rate achieved with tepary bean hybrids (8.3%).

Although from the m-calli of the V-DCBC lines no bud differentiation or plant regeneration could be achieved, the fact that they could be recovered after selection, is considered already an improvement over the results achieved routinely with common bean cultivars and wild genotypes, which yield no m-callus after selection (Annual report 2001).

Plants are being regenerated from the antibiotic resistant m-calli which differentiated buds (Table 2, last column) through micrografting.

Table 1 Overall results of the AMMSM-transformation experiments of DCBC-hybrid lines with cytoplasm of *P.acutifolius* and *P. vulgaris*, performed between October 2002 and September 2003

Cytoplasmic Background of the Hybrids	Number of Hybrid Lines Screened	Explants	Average number of Explants per Line	Lines Producing M-Calli	% of Lines Producing M- Calli	% of Lines with surviving M-calli
P. vulgaris	168	5018	29.8	6	3.5	0
P. acutfiolius	197	8809	44.7	19	9.6	3.0
TOTAL	365	13827	37.8	25	6.8	1.6

Table 2. DCBC hybrid lines which yielded meristematic (regenerable) calli after AMMSM-transformation and efficiency of recovery and survival of antibiotic resistant meristematic calli.

Hybrid Code/ Generation[1]	Cyto-plasm[2]	Agrob. Strain[3]	Level of Transient GUS Expression[4]	Level of M-Callus Induction[5]	# of Geneticin/ Hygromicin Resistant M-Calli[6]	Recovery Efficiency of M-Calli %[7]	# M-calli Surviving and Differen-tiating[8]	% of M-calli Survival and Differen-tiation[9]
NGPNMNG2-4 F_3	A	I	+++	47/90	6	12.7		
NGPNMNG2-2-1F_4	A	I	+	18/41	1	5.5		
NGPNMNG2-1-3F_4	A	I	+++	32/38	1	3.1		
GKAX-1B(B) F_3	A	I	++	37/47	12	32.4		
GKAX-1B(L) F_3	A	I	++	27/32	6	22.2	1	3.1
GKAX-1A F_3	A	I	+++	45/48	14	31.1		
GKAX-2B(L) F_3	A	I	+++	30/35	10	33.3		
GKAX-2A(L) F_3	A	I	+++	19/20	6	31.5		
GKAX-3B(L) F_3	A	I	+	21/33	6	28.5		
GKAX-13B F_3	A	I	+++	60/88	15	25	1	1.1
GKAX-11A F_3	A	I	+++	40/40	12	30		
GKAX-11B(L)2 F_4	A	I	+++	34/34	15	44.1	12	35.2
XKA-8-1 F_3	A	II/III	+++	28/46	9	19.5		
XKA-8-2 F_3	A	II/III	++	29/32	1	3.1		
ZXTGG-25-1 F_3	A	I	+	25/28	3	12	2	7.1
ZXX-13-2 F_3	A	I	++	45/46	10	21.7	10	21.7
GKX-6-2A F_3	A	II/III	+++	13/13	3	23.1		
GKX-6-3A F_3	A	II/III	+++	28/41	2	7.1		
GKX-11-2A F_3	A	I/II	+	37/43	4	9.3		
BWG-20 F_2	A	I	+++	57/58	5	8.7	4	6.9
AT7-2A F_3	V	I	+++	33/37	5	15.1		
AT7-2B F_3	V	II/III	+++	19/19	1	5.2		
T7KT-90-40A F_3	V	I	+++	18/18	3	16.6		
TZTB-12 F_2	V	I/II	++	32/37	12	37.5		
TZTB-14 F_2	V	I/II	+	18/23	4	22.2		
TZTB-21 F_2	V	I	---	28/28	1	3.5		

1. For understanding the pedigree of the lines see Table DCBC Hybrids http://gene3.ciat.cgiar.org/blast/inicio.htm
2. Cytoplasm of the Hybrids; A = *P. acutifolius*; V = *P. vulgaris*
3. *Agrobacterium* strains: I = C58C1 pTARC; II = AGL1 pC3200; III = AGL1 pC1305-1
4. After three days of co-culture three explants were scored for GUS expression. A + or a – score was given for each of the explants expressing or not GUS in the cotyledonary node or apical meristem.
5. Number of explants forming M-calli/number of explants innoculated.
6. After 1 month (two subcultures) in media containing 50 mg/l geneticin or 30 mg/l hygromycin.
7. Number of M-calli selected x 100 / Number of explants innoculated.
8. M-calli with differentiating buds which survived for more than two months after selection.
9. Selected m-calli surviving and differentiating buds three months after selection.

2.1.2 Progress in the Anti-Sense Mediated Silencing of the Granule Bound Starch Synthase I (GBSS I) for the Production of Waxy Cassava Starch

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Introduction

Higher incomes from cassava in the developing world where the crop is generally found will require the industrialization of the crop and the development of novel industrial products from cassava. There are several novel products that can be produced from cassava, including modified starches such as 100% amylopectin or 100% amylose starches, from the down regulation of the granule-bound starch synthethase (GBSS) gene or the starch-branching enzyme (SBE) gene. Industrial application of either pure amylopectin or pure amylose starches, such as the production of high-value biodegradable polymers from pure amylose starches or the use of 100% amylopectin in thickeners, pastes and glues, have a market with unlimited growth potential. Biotechnology can play a very important role in the production of the above products in cassava.

With funds from the Colombian Ministry or Agriculture and Rural Development, a project was been initiated to genetically engineer industrial cassava varieties for the production of waxy starch using an anti-sense and sense construct of the GBSSI gene. GBSS catalyses the conversion of ADP-glucose to amylose through the linkage of an ADP glucose to a preexisting glucan chain. Antisense disruption of the GBSSI gene has been employed to create potato transformants with 70-100% amylopectin via the down-regulation of the GBSSI gene (Salehuzzaman et al.,1993) and the disruption sense in sweet potato of the gene GBSS (Kimura et al., 2001).

Methodology

Isolation of a cassava GBSS cDNA clone. More than 87,000 clones of a cassava root and leaf cDNA library cloned in the vector pCMV SPORT (GIBCO BRL Inc., USA) was gridded onto high-density filters (Mba et al., 2000 unpublished data). The library was screened using a potato GBSS cDNA clones, a gift from Dr.Christine Gerhardt (Max Planck Institute, Cologne, Germany). The potato GBSS gene was labeled with [^{32}P] dATP by random primer labeling and hybridized overnight to the cDNA filters according to standard protocols for Southern hybridization used in cassava (Fregene et al., 1997). The filters were washed twice with 2X SSC +0.1% SDS at 60°C for 5 min, and autoradiography was al -80 °C using 2 intensifying screens.

Construction of transformation cassettes. Primers were designed from published sequences of a full-length cassava cDNA of the GBSSI gene (Salehuzzaman et al., 1993).

BamHI and XbaI restriction enzyme recognition sites were incorporated in 5'end of the primers to enable sub-cloning of the cDNA clone in the sense and antisense orientation into the multiple -cloning site (MSC) of the vector pRT101. The primers were used to amplify the cDNA clone obtained above, and the PCR product was cleaned using the QIAGEN PCR Clean Up Kit (QIAGEN Inc., Los Angeles, CA) and digested with the appropriate enzymes. A 2.1Kb BamHI /XbaI fragment was subcloned in the sense and antisense between the 35S promoter and the 35S polyadenylated terminator region of vector pRT101, a gift from Dr. Ryohei Terauchi, Iwate Biotechnology Research Center, Kitakami, Japan. The 35S promoter, GBSSI gene in pRT101 was liberated using the restriction enzyme PstI, separated on a agarose gel, eluted and cloned into the PstI site of the binary vector pCAMBIA 1305.2 having the GUSPlus^{R} and HPT reporter genes.

Transformation by Agrobacterium tumefaciens, of varieties. Friable Embryogenic Callus of the cassava genotype TMS60444, Mcol.2215 y CM 3306-4 was transformed via Agrobacterium tumefaciens with the GBSSI gene in antisense-sense orientation, mediated technology CIAT.

Results

Three GBSS cDNA clones obtained form screening the cassava library were sequenced, and one was found to be a complete cDNA clone. The cDNA clone has the ATG start codon 81 bp down stream from the beginning of the cDNA sequence and a stop codon about 100 bp from the poly-A tail. PCR amplification with the designed primers yielded a fragment about 2.1 kb in size that corresponds to the full-length GBSSI cDNA clone (Figure 1).

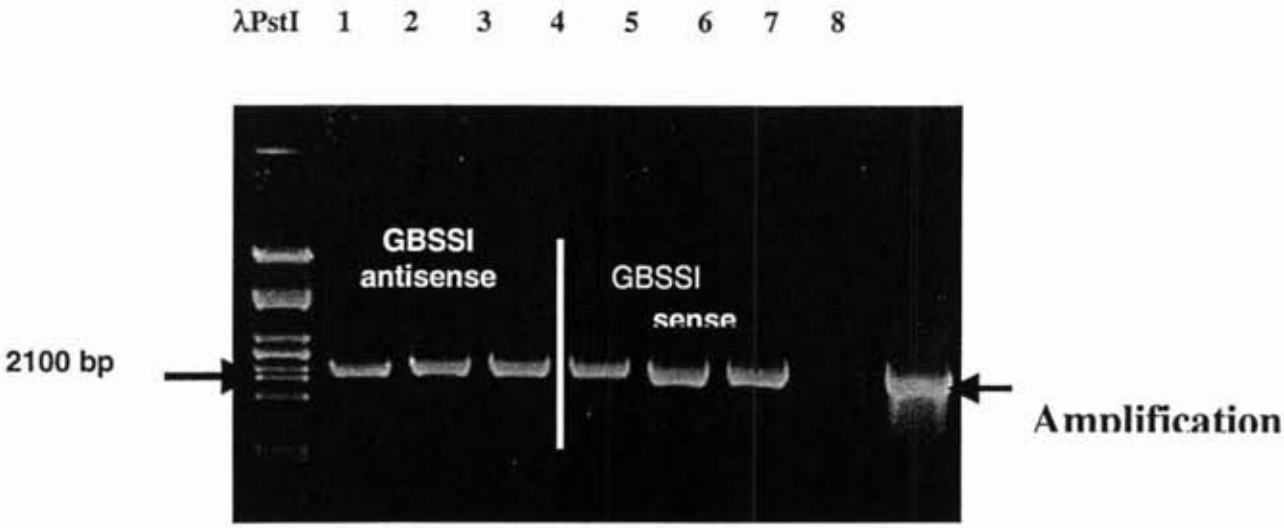

Figure 1. PCR amplification of the GBSSI cDNA clone using primers to introduce restriction enzyme sites at the ends of the gene. The first lane on the right is molecular weight marker Lambda DNA, digested with PstI, the next three lanes are PCR of the gene GBSSI antisense and next are PCR of the gene GBSSI sense.

The resulting PCR fragment, digested with BamHI and XbaI restriction enzyme, was cloned into the MCS of pRT101. Next, the GBSSI gene, promoter and terminator sequences, excised with PstI and the resulting fragments separated from the vector fragment (sizes 2.8 and 2.7 kb) by electrophoresis was cloned into the PstI site of pCAMBIA (Figure 2). These are the constructs that were used in the Agrobacterium-mediated transformation. Two genetic constructions (Figure 3) with the GBSSI gene in anti-sense and sense orientation in the vector pCAMBIA 1305.2 were made to achieve silencing of the gene.

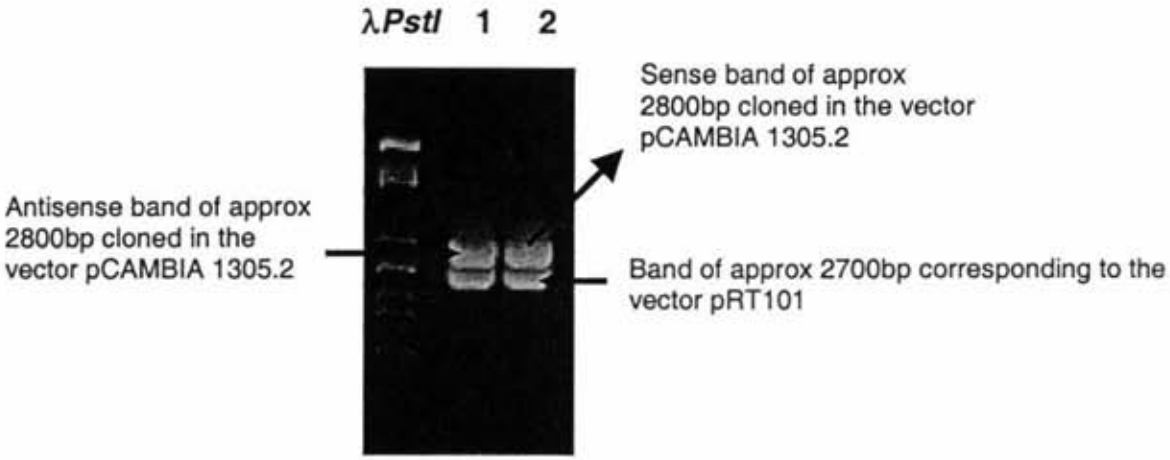

Figure 2. PstI digested of pRT101 plasmid containing the cassava GBSSI gene in anti-sense-sense orientation. The fragments of about 2.8 and 2.7 Kb in size represent the GBSSI gene, flanked by the 35S promoter and the polyadenylation terminator sequence, and the rest of the pRT101 plasmid respectively.

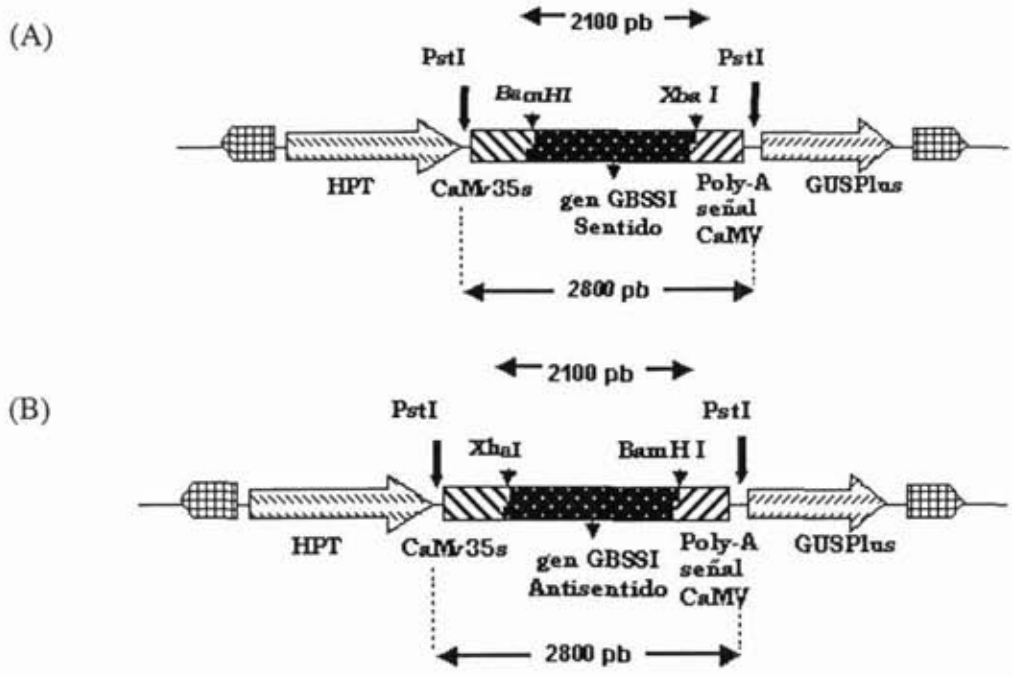

Figure 3. Gene constructs of GBSS in sense (A) and anti-sense (B) in the binary vector pCAMBIA 1305.2

The constructs were transformed into friable embryogenic Callus (FEC) of the model transformation genotype MNG11 via *Agrobacterium tumefaciens*. Results of GUS transitory assay revealed a successful incorporation of the gene (Fig. 4).

Figure 4. Positive test of GUS in Cotyledonary embryos of variety TMS 60444 with the GBSSI sense gene construct in the vector pCAMBIA 1305.2

Conclusion

We have successfully transformed full-length sense and anti-sense constructs of the GBSSI gene into the model cassava transformation variety MNG11. Transformed calli are being regenerated following which molecular and biochemical tests will be conducted to test stable expression of the gene and the eventually the waxy phenotype. The project was carried out as an undergraduate project for the Colombian undergraduate student project, Gina Jazbleidi Puentes P. of the Universidad Nacional de Colombia -Sede Palmira.

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2.1.3 Interspecific hybridization of common and tepary bean for introgression of competence to *Agrobacterium* mediated transformation and insect resistance traits

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[1]SB-2 Project - CIAT; [2]IP-1 Project

Introduction

The tepary bean (*Phaseolus acutifolius* A. gray) possesses several traits that are important to common bean breeding, such as resistance to drought (accessions G40020 and G40023), bruchids (*A. obtectus* and *Z. subfaciatus*; G40199), leaf hopper (*Empoasca kraemeri*; G40019 and G40036) and bacterial blight (*Xanthomonas campestris pv phaseoli*; G40020), among others. Also one genotype of tepary bean has been dentified as competent to *Agrobacterium* mediated transformation (the wild accession NI576; see report on bean transformation).

Crossing of common with tepary bean has been traditionally difficult. Using facilitator genotypes of both species, and applying recurrent and congruity backcrossing (CBC; Haghighi and Ascher, 1988), we could produce a large number of fertile hybrids (Mejía-Jiménez *et al.* 1994), from which common bean lines with high levels of resistance to bacterial blight were developed (Singh and Muñoz, 1999). However using these two backcross strategies, no fertile progeny could be obtained from hybrids involving several of the above mentioned tepary bean genotypes.

Using a modification of the CBC methodology, we called Double Congruity Backcrosses (DCBC), we could develop fertile interspecific hybrids involving several tepary bean accessions that were formerly impossible to cross (Annual reports 2001, and 2002). In 2002 the project IP-1 reported the identification of drought tolerant lines after screening of DCBC hybrids (Beebe et al. 2002).

During 2003 our backcross program through DCBC continued, and from the fertile progenies developed during this or previous years, lines with improved competence to *Agrobacterium* mediated transformation or resistance to *Empoasca* or bruchids were identified.

Methodology

Double congruity backcross hybridization on the cytoplasms of common or tepary bean (V-DCBC and A-DCBC hybrids) were started in 1999 using advanced congruity backcross hybrids, developed in the past, with cytoplasm of common bean (Mejía-Jiménez *et al.*, 1994), as described before (Mejía Jiménez *et al.*, 2000; annual report project SB-II). This backcross strategy consists of backcrossing alternately hybrids developed in a *P. acutifolius* or *P. vulgaris* cytoplasmic background, with the most advanced DCBC hybrids generated in the other backgrounds and viceversa. This is repeated several times to increase recombination between both genomes and pyramid desired traits in the hybrids. Initially only *P. vulgaris* and *P. acutifolius* genotypes were included in the DCBC, but later genotypes of different *Phaseolus* species were also incorporated (see table DCBC hybrids) Embryo rescue was applied when necessary to recover viable hybrids from aborting embryos (Mejía-Jiménez *et al.*, 1994).

Results

Identification of DCBC-hybrid lines with improved response to *in vitro* culture and plant regeneration or *Agrobacterium* mediated genetic transformation

The inter-specific hybridization between common and tepary bean genotypes is being used for the development of hybrids with improved competence to AMMSM-transformation (*Agrobacterium* Mediated Mature Seed Meristem-transformation; see report on genetic transformation of *Phaseolus* beans).

Three hundred and sixty five DCBC hybrid lines were screened during 2003. The production of antibiotic resistant calli of a singular morphological type, from which plants can be regenerated (meristematic calli or m-calli), was used as a parameter for selecting genotypes with superior performance.

Six lines with the cytoplasm of tepary bean yielded antibiotic resistant m-calli which are potentially transgenic. In two of the lines (GKAX-11B(L) 37.2%, and ZXX-13-2 21.7%; see also Table 2 of the transformation report) the recovery efficiency of antibiotic resistant m-callus was higher than in tepary bean intraspecific hybrids G40065 x NI576 (8.3%; Annual report 2001). This indicates that probably alleles involved in the response to the AMMSM?- transformation methodology are being accumulated through the DCBC process which combines the screening for superior genotypes, in both cytoplasmic backgrounds, with the further backcrossing of the selected genotypes found.

Three lines with meristematid calli survival and differentiation efficiencies ranging from 7 to 35%, and two V-DCBC lines which yielded antibiotic resistant m-callus after selection, were chosen as parentals for the next generation of DCBC hybrids (see below).
It will be tested if the lines, which show the highest percentages of m-calli survival and differentiation (Table 2 of the tansformation report), can be used as bridge for the production of transgenic common bean cultivars.

Introgression of *Empoasca* resistance to common bean through DCBC
After a field screening performed by the IP-1 project, at least seven common bean lines derived from DCBC crosses were identified as *Empoasca* resistant (lines $DCBC_{7A}$, $_{7C}$ and $_{8D}$; see table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/inicio.htm).
All these hybrids include in its pedigree the genotype of *P. acutifolius* G40036 and one of them the genotype G40019, which are resistant to *Empoasca* (table 1). These accessions of tepary bean may be the source of the detected resistance. In the past 10 years several common x tepary hybrid lines produced using a different backcross strategy, like recurrent or congruity backcrosses, were evaluated in the field for *Empoasca* resistance with no success in identifying resistant genotypes. This is an additional proof of the usefulness of the DCBC-strategy to allow introgression of traits from alien germplasm (last year the IP-1 project reported the identification of drought tolerant lines derived also from DCBC hybrids).

The resistant common bean lines are being crossed to other *Empoasca* resistant A-DCBC hybrid lines (table 2), to pyramid different, possibly available, resistance alleles and increase the levels of resistance already found.

Resistance to the bean weevil (A. obtectus) found in A-DCBC hybrids. Several populations of A-DCBC and V-DCBC hybrids have been screened by the IP-1 project for resistance to the bruchid *A. obtectus*. Five lines with tepary bean cytoplasm have shown to be resistant (results were confirmed through three individual infestations with the insect; (hybrids A-DCBC with codes GNVAV-2 F_3, GVV-1 F_3, GKX-6 F_2, GKA-12 F_2, ZXX-5 F_2). All the

resistant seeds were planted in the greenhouse and the plants obtained are being used to generate other DCBC hybrids with common and tepary bean cytoplasm (table 2). It is not clear which is the origin of the resistance allele(s) in the hybrids.

Development of new fertile DCBC hybrid progenies of *P. vulgaris* or *P. acutifolius* cytoplasms, with parental hybrid lines selected for its response to AMMSM-transformation or for resistance to insect pests.

During 2003 new DCBCs were started, or completed, using as parental hybrid lines selected for its good response to *in vitro* culture, callus induction or competence to *Agrobacterium* transformation (V-DCBC hybrids with codes TZTB-12 and AT7-2A; and A-DCBC hybrids with codes GKAX-11B(L)2, ZXX-13-2 and BWG-20), resistance to *Empoasca kraemeri* (V-DCBC hybrids with codes A36Y-42, EMPZ-2, -8, -9, TZTE-20B and –71B) or *A. obtectus* (lines with code GKA-12, GKX-6, GVV-1, GNVAV-2). These lines were or are being crossed to the most advanced DCBC hybrid lines available of the same cytoplasm. Morphological traits are being used to verify the hybridization (Table 2) The generated fertile hybrids will further be screened for improved expression of the desired traits.

Table: 1. Pedigree of the interspecific DCBC hybrids with *P. vulgaris* cytoplasm, which have been found to be resistant to *Empoasca* in field experiments (see also IP-1 Annual Report). Highlighted in yellow are the resistant parental lines, and also the A-DCBC hybrid G36NGP-3, derived from the cross G40036 x NGPNMNG, which may be the principal source of the resistance found in the hybrids. To understand the pedigrees of the hybrids, see also the table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/inicio.htm).

Code of the Hybrid	Pedigree			
A36Y-42	T6		x G36NGP-3	
	KBNKN-1 X NGGNV-2		G40036 x NGPNMNG	
EMPZ-2 and EMPZ-3	A99Y-90		x ZXTGS21-9	
	KBNKN1 X G99NGPZ		ZXTG6 x G36NGP-3	
EMPZ-5	A36Y-42		x ZXTGS21-11	
	T6 X G36NGP3		ZXTG 8 x G36NGP-3	
EMPZ-8, EMPZ-9	A19Y-103		x ZXTGS49-8	
	KBNKN3	x G19NGP-3	ZXTG5 x G36NGP-3	
		G40019 x NGPNMNG		
TZTE-20B	TZT-3		x EMPZ-3	
			A99Y-90	ZXTGS21-9
				ZXTG-6 X G36NGP-3
TZTE-71B	TZT-4		x EMPZ-3	
			A99Y-90	ZXTGS21-9
				ZXTG-6 X G36NGP-3

Table 2. New DCBC hybrid progenies developed during 2003. DCBC= Double congruity backcross hybrids (for complete pedigree see table DCBC hybrids:http://gene3.ciat.cgiar.org/blast/inicio.htm); V-DCBC= DCBC with the cytoplasm of common bean (common bean as female parent); A-DCBC= DCBC with the cytoplasm of tepary bean. Highlighted in yellow are the lines selected from *Agrobacterium* mediated transformation experiments, in green are the lines selected as *Empoasca* resistant and in red are the lines resistant to *A. obtectus*.

Cytoplasm of *P. vulgaris*			Verificaiton of the hybrid
Female Parent/ Code	Hybrid/ Code	Male Parent/ Code	
V-DCBC$_{7C}$ TZ	X ↓ V-DCBC$_{8C}$ TZT	V-DCBC$_{6}$ T7K, TK	Female parent self-sterile
V-DCBC$_{8C}$ TZT	X ↓ V-DCBC$_{8D}$ TZTA, TZTE, TZTU, TZTK	V-DCBC$_{6A}$ ACT, AT7, EMPZ, UAC, UQUQ	Female parent self-sterile
V-DCBC$_{7A,-8A,-8D}$ TZTB, AT7, TZTA, TZTE, TZT	X ↓ V-DCBC$_{8E}$ TZTTZ	V-DCBC$_{7A,-8A,-8D}$ TZT, TZTU, TZTA, TZTE,	Hypocotyl pigmentation and flower color
V-DCBC$_{8C}$ TZTA, TZTE, TZTU	x ↓ V-DCBC$_{9A}$ TZTEB, TZTAB	A-DCBC$_{12A,-12B}$ BW, BWG, BWZ	Hypocotyl pigmentation and flower color; hybrids became self-sterile
V-DCBC$_{9}$ TZTEB, TZTAB	x ↓ V-DCBC$_{10}$ R	V-DCBC$_{8C,-8E}$ TZT, TZTTZ	Female parent self-sterile
CYTOPLASM OF *P. acutifolius*			
A-DCBC$_{10A}$ ZXTG	x ↓ A-DCBC$_{10C}$ ZXTGS	A-DCBC$_{6B}$ G36NGP-3	Hypocotyl pigmentation and flower color
A-DCBC$_{10}$ ZXTG	x ↓ A-DCBC$_{11}$ ZXTGT, ZXTGU	V-DCBC$_{6, 8B}$ T7K, TK, UAC	Hypocotyl pigmentation and flower color inherited from the male parent; primary leafs with the large petiole from *P. vulgaris*; hybrids became self-sterile
A-DCBC$_{11}$ ZXTGU, ZXTGT	x ↓ A-DCBC$_{12A}$ BW	A-DCBC$_{10A,10B}$ ZXTG, Z99ZX, ZXTGX, GKXZG	Female parent self-sterile
A-DCBC$_{12A}$ BW	x ↓ A-DCBC$_{12B}$ BWG, BWZ	A-DCBC$_{8C,10A,10B}$ GKA-12, GKAX-11, ZXTG, ZXTGS, ZXTGG	Female parent self-sterile; Hypocotyl pigmentation and flower color
A-DCBC$_{12A}$ BW	x ↓ A-DCBC$_{13A}$ BWT	V-DCBC$_{8C,-8D}$ TZTK, TZTE-20, -71, TZTU, TZTTZ	Hypocotyl pigmentation and flower color inherited from the male parent; primary leafs with the large petiole from *P. vulgaris* in the hybrids; hybrids became self-sterile
A-DCBC$_{13A}$ BWT	x ↓ A-DCBC$_{14A}$ BWTZB	A-DCBC$_{12B}$ BWG-20, GKA-12, GVV-1, GKX-6, GNVAV-2, BWZ	Female parent self-sterile

Conclusions

A-DCBC lines have been identified which show an increased competence to *Agrobacterium* mediated transformation. These lines may have different levels of introgression of common bean and may be used to transfer transgenes to this species through sexual crossing.

DCBCs allowed the introgression, to common bean, of tepary bean genes involved in *Empoasca* resistance.

Also, lines with tepary bean cytoplasm resistant to the bruchid *A. obtectus* were found among the progeny of the screened DCBC hybrids.

Future plans

- To continue with the DCBC strategy to produce more advanced DCBC hybrid generations
- To perform additional DCBCs with hybrids that have already shown competence for *Agrobacterium* mediated transformation, *Empoasca,* or *A. obtectus* resistance.
- To study the introgression in the hybrid populations of DNA fragments from the species involved in the DCBCs using AFLP techniques.

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Recovery of transformed M-callus after transformation with binary Agrobacterium strains. The AMMSM-transformation methodology was developed using the hypervirulent *Agrobacterium*-strain LBA4404pTOK 233 that was developed through cointegration (Hiei et al. 1994). An important achievement of 2003 is the recovery of transgenic (GUS expressing) regenerable M-callus after transformation with the binary *Agrobacterium* strains AGL1 and C58C1 (Table 2). Genes of agronomic interest can be cloned faster and easier in binary plasmids than in the cointegrate ones.

Co-transformation with unlinked transgenes. Co-transformation, the transformation of plant cells with unlinked transgenes (which can be localized in the same or different *Agrobacterium* strains), followed by the separation of the transgenes in the progeny of the transformed plants, is a methodology which has been used for the production of antibiotic- and marker-free transgenic plants. With some of the DCBC lines we have been performing co-transformation experiments by transforming with the GUS-Intron gene and the BAR gene in different plasmids and *Agrobacterium* strains (see Table 2). The fact that GUS expressing calli was obtained after selection with the BAR gene in these experiments, indicates that both transgenes were co-transformed. It should be tested if they segregate independently in the progeny of the transgenic plants recovered from this kind of experiments.

Development of a methodology for large scale sterilization of mature Phaseolus seeds with chlorine vapors. The application of the AMMSM-Transformation methodology requires the preparation of a large number of explants from mature seeds (embryos without one cotyledon). This is a time-consuming and a contamination-prone process, because the embryos have to be excised from the pods and seed coats under sterile conditions. During 2003, and based on a methodology developed for sterilizing seeds of *Arabidopsis thaliana* (Clough and Bent; http://plantpath.wisc.edu/~afb/vapster.html) we designed an apparatus and developed a methodology for a simple and efficient sterilization of high quantities of seeds of *Phaseolus* beans. The methodology uses 5% sodium hypochlorite and 4 N HCL to generate Cl_2 vapors, which in 3 hours completely sterilizes more than 90% of the highly contaminated *Phaeolus* seeds samples (seeds mixed with soil).

Development of new populations of DCBC hybrids involving as parental genotypes (hybrids) with increased competence to AMMSM-Transformation. Three A-DCBC and two V-DCBC hybrid lines that yielded antibiotic resistant meristematic calli after transformation were chosen for developing new hybrid lines (highlighted in yellow, table 2). The selected lines were already crossed to the most advanced DCBC lines of the same cytoplasm, and fertile hybrids have been developed. The screening of the new DCBC-Hybrid populations for AMMSM-Transformation is in progress.

Conclusion

As judged by the recovery of GUS expressing and antibiotic resistant regenerable calli after transformation, it is likely that competence to *Agrobacterium* mediated transformation has been transferred to various advanced *P. acutifolius* x *P. vulgaris* – DCBC hybrids with *P. acutifolius* cytoplasm. Some of these show rather high transformation efficiencies.

These hybrids could be used as bridge to transfer transgenes to common bean cultivars through sexual crossing. However, to reduce the number of backcrosses that may be necessary for achieving this, transformable hybrids with common bean cytoplasm should be developed.

Future Plans

- Develop pure lines with transformation competence, from the already identified AMMSM-transformations competent F3 or F4 populations
- Further screen the hybrid progenies developed this year for competence to AMMSM-transformation.
- Continue with the co-transformation experiments (with marker genes and genes with agronomic importance in different strains or plasmids) in order to develop a methodology to produce transgenic common bean plants free of antibiotic markers.
- Start co-transformation experiments with genes of agronomic importance.

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2.1.4 Bioassays with transgenic Bt-cassava plants, cultivars 60444 and CM3306-4, to test for stem borer and horn worm resistance

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[1]SB-2 Project; CIAT; 2IP-[3] Project, CIAT

Introduction

One alternative method to obtain cassava cultivars resistant to insects is through transgenesis. We developed transgenic cassava plants, from three cultivars, that carry one gene for insect resistance. These plants were produced to test the efficacy of this *cry*1Ab gene (Bt gene) to control insect pests like the cassava stem borer (*Chilomima clarkei*, Lepidoptera) and the cassava horn worm (*Erinnyis ello*, Lepidoptera). We present a summary of the main findings after carrying out bioassays with transgenic plants and these insects.

Materials and Methods

Transgenic plants of cassava cultivars 60444 (MNig11) and CM3306-4 were obtained via *Agrobacterium*-mediated transformation, for the former, and the particle gun for the latter. Bioassays for the stem borer (*Chilomima clarkei*) and the cassava hornworm (*Erinnyis ello*) were set in biosafety greenhouses, or in captivity rooms of the IPM program.

We infested at least six young cassava plants per transgenic line with *Chilomima* larvae of second or third instars, one larvae per plant, and let them penetrate the stems and grow for 15 days inside it. All transgenic lines were derived from cultivar 60444. Controls included non transgenic plants of 60444 and CMC-40, a cultivar used to feed insects in captivity. Larvae were weighted at the beginning and end of the test, and the average weight increase was estimated and statistically analyzed.

To test resistance to cassava horn worm, we fed 20 first instar larvae with transgenic leaves of lines derived from cultivars 60444 and CM3306-4. As controls we used non-transgenic leaves from cultivar 60444. Unfortunately there were not leaves available from non transgenic CM3306-4 that could serve as proper control. We opted for CMC40, a cultivar routinely used to feed the horn worm in captivity. Therefore, the results presented for CM3306-4 are preliminary and need confirmation. We measured larvae weight increase for 11 days and analyzed differences between transgenic lines and their non transgenic controls.

Results

The main findings for the stem borer bioassays can be summarized as follows:

No differences in average weight increase were detected between transgenic and non transgenic lines derived from 60444. In fact, larvae fed on non transgenic 60444 lines also lost weigh, if compared with those fed on control CMC-40 (Table 1; Figure 1). There were no statistically significant differences between the two transgenic lines 55 and 92 and the non transgenic control of same cultivar.

Developmental stage of larvae seemed critical in determining the effect of transgenic plants on their diet. Plants seem to express very little insecticidal CRY1Ab protein (Ladino et al 2002), so larvae on second and third instar are already too strong to be affected by this amount protein expressed in transgenic plants
Some of the larvae fed on transgenic line 80 increased their weigh as much as did those of controls fed on CMC40, suggesting that this line may just represent an escape or a chimera.

The bioassays with the steam borer, although limited by the number and developmental stage of available larvae (the insect is not found in the Valle del Cauca), indicated that larvae loose weight when fed on leaves from transgenic and non transgenic plants of cultivar 60444. If there was an effect of the low expressing cry1Ab gene, it will be surely masked by the varietal effect. Feeding on non transgenic control from cultivar CMC-40 produced much healthier larvae, with higher average weigh increase.

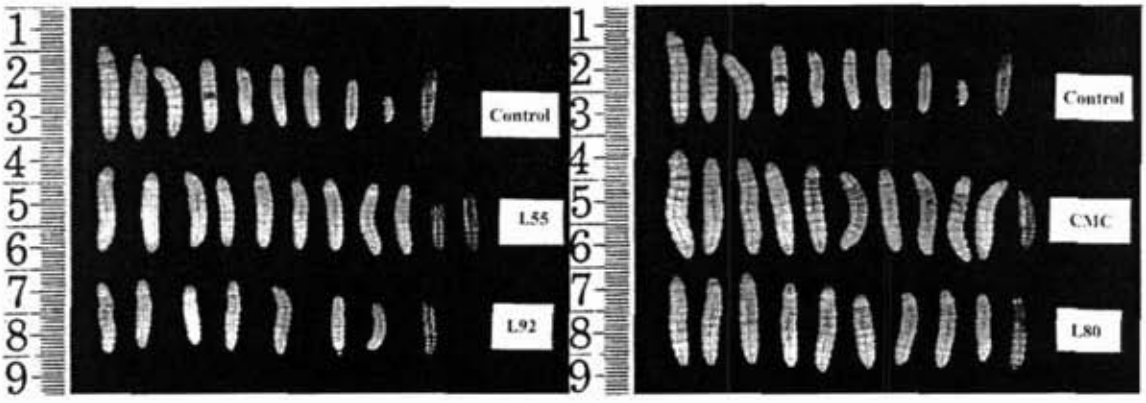

Figure 1. *Chilomima clarkei* larvae fed for 15 days on transgenic (L55, L80, L92) and non transgenic plants (Control) of cassava cultivar 60444. Larvae fed on a non transgenic control of cultivar *CMC-40* (CMC) is also presented.

Table 1. Average weigh increase of two weeks old *Chilomima* larvae fed for 15 days on transgenic and non transgenic cassava plants. Lines labeled with the same letter on the first column do not show statistically significant differences. No difference could be detected between transgenic lines 55 and 92 and their respective non transgenic control.

Qualification	Average weight increase	No of observations	Line
A	0.11280	10	CMC (control)
A B	0.08350	10	L80
B	0.04000	8	L55
B	0.03425	8	TMS (control)
B	0.03183	6	L92

There was a significant difference between larvae fed with non transgenic CMC40 and non transgenic 60444. L55, L80, L92 and TMS (control) are derived from cultivar 60444. CMC (control) is derived from CMC-40 (= 0,05)

For the cassava horn worm bioassays, we evaluated the average daily weight increase of 20 first instar larvae during 11 days. The results are pictured in Figure 2. Up to the 8th day larvae fed with non transgenic CMC40 increased weight beyond 3 g, while those fed with the other lines did not exceed 0,5 g. After day eight, larvae fed on CMC40 entered pre-pupa stage and lost weight, so weighting stopped at day 10. Comparing the behavior of horn worms feeding on CMC40 and Ica Negrita, at day 11 we counted 14 pupa alive and 6 dead larvae fed with CMC40 (30% mortality), while there were no pupa and 18 larvae died (90% mortality) when fed on transgenic Ica Negrita. From the latter's, only three larvae survived to day 11 reaching less than 2 g final weight (Figure 3).

The assays indicated that there was a trend of larvae to develop slower (smaller daily weight increments) and die if they fed on transgenic plants of L55 and Ica Negrita, or non transgenic 60444., while they developed stronger and faster until the majority reached the state of pupa if fed with no-transgenic CMC40.

As stated before, results obtained with the horn worm are preliminary and will be repeated to confirm observations. This time proper Ica Negrita non transgenic controls will be tested to confirm if transgenic lines effectively protect from horn worm attacks.

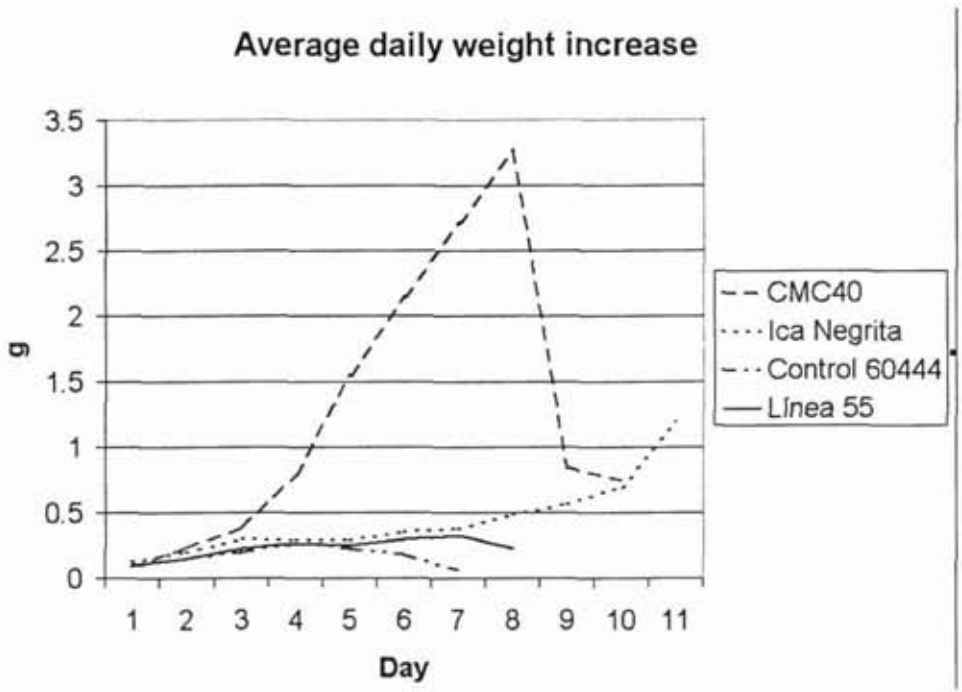

Figure 2. Average daily weight increase of cassava horn worm larvae feeding on *transgenic and non* transgenic lines carrying a *cry*1Ab gene. (CMC40 and Control 60444 are non transgenic; Ica Negrita and L55 are transgenic).

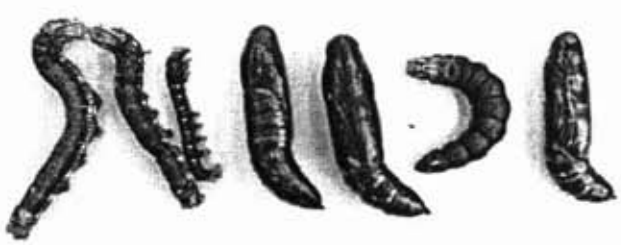

Figure 3. Developmental stages reached by cassava horn worms fed on transgenic and non transgenic lines. The three larvae on the left are the only survivors of 20 larvae fed with leaves of transgenic Ica *Negrita plants.* The four on the right are examples of the 15 out of 20 larvae, changed to pupas, that survived when fed with non transgenic line CMC40.

Conclusion and Future Activities

Transgenic and non transgenic plants derived from cultivar 60444 seem to delay, or even prevent, the normal development of larvae from the stem borer and the horn worm. The effect seemed stronger on the latter where mortality reached 90%. This may be due to higher concentration of the "insecticidal molecule" in the leaves than in stems. It is very unlikely that this effect comes from the *cry*1Ab transgene, at least in L55 where gene expression seems low. This finding opens the door to explore new insect resistance sources in cassava germplasm that contains genes from wild relatives. This is the case of MNig11 (60444), a variety developed in Africa from crosses with *Manihot glaziovii* to obtain resistance to CMD.

Transgenic Ica Negrita also seemed to delay the development and kill the horn worm. However, these results need replication to confirm the observations since the proper control plants were not available in the first assays. A second set of bioassays will be performed again towards the end of 2003.

If transgenic cassava lines that protect against the stem borer and/or the horn worm are identified, then it will be wise to test them under field conditions.

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2.1.5 Herbicide tolerance in cassava: Second generation of transgenic plants carrying the *bar* gene

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[1]CIAT SB-2 Project; ; [2]Cassava Breeding

Introduction

Transgenic cassava plants expressing tolerance to the herbicide Basta were obtained in CIAT in 1995. Since then, those plants have been tested to confirm if the transgene bar is still expressed finding that current expression levels do not confer resistance to the herbicide. It suggests that the transgene is being silenced. We decided to produce new transgenic lines, with different cultivars and the same construct, to follow expression of the gene throught the years and propagation cycles. Besides, resistance to herbicide might be an important character for cassava industrial plantations.

Materials and Methods

The *Agrobacterium* strain LBA4404-pGV1040 was selected to transform 383 clusters (approx. 31 g) of friable embryogenic callus (FEC) of cultivar CM3306-4, and 11 clusters (0,9 g) of cultivar 60444. Embryogenic tissues were then selected on semisolid media with geneticin (20 to 50 mg/l). Those FEC that survived the first round of selection on antibiotic were induced to produce mature embryos (cotyledon stage embryos), and transferred to temporal immersion (RITA) system to continue selection with phosphinotrycin (ppt) 5 to 10 mg/l. Mature embryos in RITAs were induced to produce multiple shoots (organogenesis) to recover plantlets.

Results

We recovered 28 transgenic, gus-positive lines of cultivar CM3306-4 (Figure 1) on media with geneticin. Mature embryos of two lines of CM3306-4, and one line of 60444, were transferred to RITA to develop shoots via organogenesis under selection with ppt 5 mg/l. Embryos from CM3306-4 survived selection and produced shoots (Figure 1), while those of 60444, although they were gus positive, did not pass selection. At this point, transformation efficiency for cultivar CM3306-4 is **0,9 independent transgenic lines per gram of FEC** (28 lines/31g FEC), or 28 transgenic lines out of 383 explants (**7,3%**).

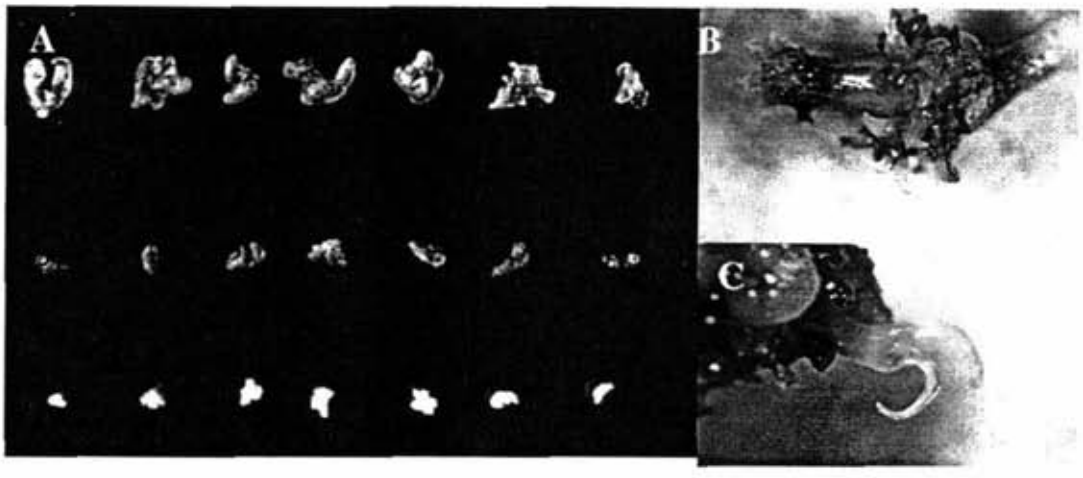

Figure 1. (A) Transgenic, gus+ embryos of cultivar 60444 (first row) and CM3306-4 (second and third row). (B and C) Transgenic embryos from two lines of of CM3306-4 regenerated shoots via organogenesis in RITA, under selection medium with 5 mg/l ppt.

Future activities

- Continue selection and regeneration of transgenic plant sin RITAs with 5 mg/l ppt for the remaining transgenic lines
- Root plants on medium with ppt (5 to 10 mg/l)
- Transfer to greenhouse and test for resistance to spraying with ppt at 100 mg/l minimun concentration

2.1.6 RHBV (Rice Hoja Blanca Virus) Resistance mediated by RNA-Cross Protection in Transgenic Rice

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[1]SB-2 Project, [2]IP-4 Project

Introduction

In the past decade there has been a great progress in genetic transformation methods for the incorporation of genes conferring important agronomic traits in rice using both particle bombardment and *Agrobacterium*. Transgenic plants expressing resistance to herbicide, disease, insects, nematodes and tolerance to drought and salt stress have been obtained (Jiang *et al.*, 2003). At CIAT, our earlier work was mainly using particle bombardment, but current work uses *Agrobacterium* mediated transformation as the method of choice because the simpler integration patterns without or fewer rearrangements, and stable

inheritance and gene expression throughout generations. Our research has resulted in an improved transformation protocol for the production of large number of transgenic fertile rice with different genes including resistance to RHBV mediated by the viral nucleoprotein (N) or the non-structural protein 4 (NS_4) genes, fungal resistance by the PAP gene (Pokeweed Antiviral Protein a plant origin ribosome inhibitor protein) and regulation of expression of lignin synthesis by the OMT gene (O-metiltransferase). The most advanced project had generated transgenic rice with resistance to RHBV encoded by the viral N protein gene. This project has served as a learning experience to scale up the use of genetic transformation and to implement the required biosafety regulations from the laboratory to the field. Transgenic Cica 8 variety carrying the RHBV protein N gene shows resistance to the virus. This resistance is RNA-mediated and some plants show hypersensitive reaction when challenge with the RHBV virus (Lentini *et al.*, 2002). Some of the transgenic lines outperform most commercial rice varieties in the field. Attempts to incorporate this resistance into other materials through regular crossing indicate that the RHBV-N transgene is inherited and expressed independently of the genotype background. Thus this transgenic resistance could be used to complement the breeding resistance that has been deployed so far and does not protect plants when younger than 25 day-old. Last year we reported the studies directed to understand the resistance mechanism underlying the transgenic RHBV resistance. This year we report the evaluation of two events of transformation using microsatellites markers and selection of advanced breeding lines in the field.

Materials and Methods

Evaluation of transformation events in the field. Field evaluations were conducted using 18 progeny plants of the T_3-T_6 generation from self-cross of the Cica 8 transgenic line A3-49-60 and 4 progeny plants of the line A3-49-56, both resistant to RHBV. Twenty-five day-old plants were transplanted in the field using an experimental complete randomized block design with three replications, each of 102 plants per replicate. Agronomic traits were evaluated through the cycle up to maturity. Tiller number, plant height, fertility and plant vigor were evaluated. DNA was extracted from young leaves using leaf discs of 5 mm diameter according to Klimyuk et al. (1993). One or two highly polymorphic microsatellites markers that clearly distinguish Cica 8 from other rice varieties were used to evaluate the molecular profile. A total of eight plants per line were analyzed.

Selection of crossing lines. A total of 82 crosses derived from backcross to Cica 8 or crosses between resistant transgenic Cica 8 plants and the commercial varieties Fedearroz 50, Oryzica 1, and Iniap 12 were evaluated in the field. Each line consisted of 51 plants, planted in three rows per line. Individual plants were selected and seeds harvested. Segregation analysis was done using microsatellites markers.

Results and discussion

Microsatellites marker analysis of advanced generations of the transgenic RHBV resistant lines indicated that there was no polymorphism between the non-transgenic Cica 8 variety and transgenic Cica 8 lines (Figure 1). It appears that the genetic change incorporated by transformation is minor and is not resolved by microsatellite analysis (Gonzalez *et al.*, 2001). Some polymorphism was noted for some lines that correlated with phenotypic segregation. This segregation was noted on progenies derived from seeds collected from un-bagged panicles of field-grown plants of previous evaluations suggesting spontaneous outcross. For some lines in spite of the genetic uniformity as indicated by the molecular marker analysis, segregation was noted for some agronomic traits indicating differential gene expression or the need to use a larger number of molecular markers to detect genetic differences. Based on agronomic performance a total of 202 individual plants were selected and harvested (highlighted in Table 1). These plants were alike the Cica 8 in most agronomic traits but some of them out-yielded Cica 8 since a natural infestation of RHBV was present in the field. Grains from only the inner part of each plant were harvested, to prevent the collection of seeds derived from the outcross with neighbor lines. The total yield per plant of the selected material ranged from 11g/ plant to 123g/ plant whereas the variation among different non-transgenic control Cica 8 plant was from 27g/plant to 35 g/plant (Table 1).

Plants derived from line A3-49-60-12-3 were more resistant to RHBV than Fedearroz 2000 over two field evaluations (Fory et al., 2001). Sterility was noted in about half of the lines derived from this transgenic event (Table 1). Fertility in these lines ranged from 0% to 90% (as in non-transgenic Cica 8). The source of sterility in these plants seems not to be due to male sterility, since pollen viability in these plants was 85% ± 1.5 % respect to 90 ± 1.2 % in Cica 8. No polymorphism was noted between fertile and sterile plants either (Figure 1). However, other genetic self-incompatibility mechanisms cannot be discarded at this point. The recovery of fertile as well as sterile plants from genetic transformation mediated by particle bombardment has been reported in other works. Wakita et al. (1998) reported that about half of the transgenic plants carrying the herbicide resistance *pat* gene, and the 3-fatty acid desaturase gene from tobacco generated by particle bombardment were sterile. In this work, fertility was not correlated with the number of gene inserts. It seems also that the low fertility cannot be attributed either to the insertion of the foreign DNA fragments into genes related to fecundity or key for normal plant growth. Different integration sites, copy numbers and transgenic locus configurations, as well as epigenetic silencing mechanisms can all contribute to the expression of low fertility. Other works have also shown that the complex insertion of the transgene(s) may be responsible for phenotypic abnormalities in transgenic rice, including high sterility (Jiang et al., 2003).

Table 1. Agronomic and molecular evaluation of traits of 22 transgenic lines

Lines	Progeny	G[1]	P[2]	AS[3]	MS[4]	H[5]	DF[6]	F[7]	P[8]	W[9]
A3-49-56	A3-49-56-22-M	T_4	Y	S	NS	85	139	75	13	18-56
	A3-49-56-24-M	T_4	Y	NS	S	92	128	84	26	34-73
	A3-49-56-34-M	T_4	N	NS	NS	112	Nd	73	3	40-45
	A3-49-56-66-M	T_4	**N**	**NS**	**NS**	**85**	**Nd**	**0**	**0**	**Nd**
A3-49-60-4	A3-49-60-4-13-18-M-M	T_6	N	S	S	96	132	80	23	17-123
	A3-49-60-4-5-8-M-M	T_6	Y	NS	NS	109	139	64	16	11-53
A3-49-60-10	A3-49-60-10-31-M	T_4	Y	S	S	81	132	59	22	12-64
A3-49-60-12	A3-49-60-12-3-20-M	T_5	N	S	S	104	143	86	14	5-98
	A3-49-60-12-3-3-21-M	T_6	N	S	NS	92	134	83	15	17-81
	A3-49-60-12-3-3-10-M-M	T_7	**Y**	**NS**	**NS**	**80**	**141**	**0**	**Nd**	**Nd**
	A3-49-60-12-3-3-31-M-M	T_7	Y	S	NS	107	139	90	25	15-83
	A3-49-60-12-3-3-58-M-M	T_7	N	S	NS	92	132	87	16	38-48
	A3-49-60-12-3-3-59-M-M	T_7	N	S	S	102	140	86	16	23-85
	A3-49-60-12-3-3-60-M-M	T_7	**Y**	**NS**	**NS**	**75**	**Nd**	**0**	**0**	**Nd**
	A3-49-60-12-3-3-62-M-M	T_7	**N**	**NS**	**NS**	**78**	**Nd**	**0**	**0**	**Nd**
	A3-49-60-12-3-3-65-M-M	T_7	**Y**	**NS**	**NS**	**73**	**148**	**0**	**0**	**Nd**
	A3-49-60-12-3-3-79-M-M	T_7	**Y**	**NS**	**NS**	**75**	**Nd**	**0**	**0**	**Nd**
	A3-49-60-12-4-5-13-M-M	T_7	**Y**	**NS**	**NS**	**77**	**Nd**	**0**	**0**	**Nd**
A3-49-60-13	**A3-49-60-13-28-M**	T_4	**N**	**NS**	**S**	**78**	**Nd**	**0**	**0**	**Nd**
	A3-49-60-13-40-M	T_4	N	S	S	100	134	85	13	17-62
	A3-49-60-13-7-M-M	T_5	**Y**	**NS**	**NS**	**77**	**Nd**	**0**	**0**	**Nd**
	A3-49-60-13-9-M-M	T_5	**N**	**S**	**NS**	**75**	**Nd**	**0**	**0**	**Nd**
A3-78-24-4	**A3-78-24-4***	T_3	**N**	**NS**	**NS**	**100**	**128**	**Nd**	**0**	**Nd**
Non transgenic Cica 8		**NA**	**N**	**NS**	**NS**	**105**	**132**	**90**	**NA**	**27-35**

[1]G, generation; [2]P, bagged panicle (Y) and un-bagged (N); [3]AS, agronomic segregation; [4]MS, molecular segregation; [5]H, Plant height; [5]DF, days to 100 % plants flowering; [6]F, Mean percentage of fertility per line; [7]P, number of individual plants selected; [8]W, range of grain weight (g)/plant of total selected plants; NA, not applicable. * This transgenic only contains hygromycin resistance gene

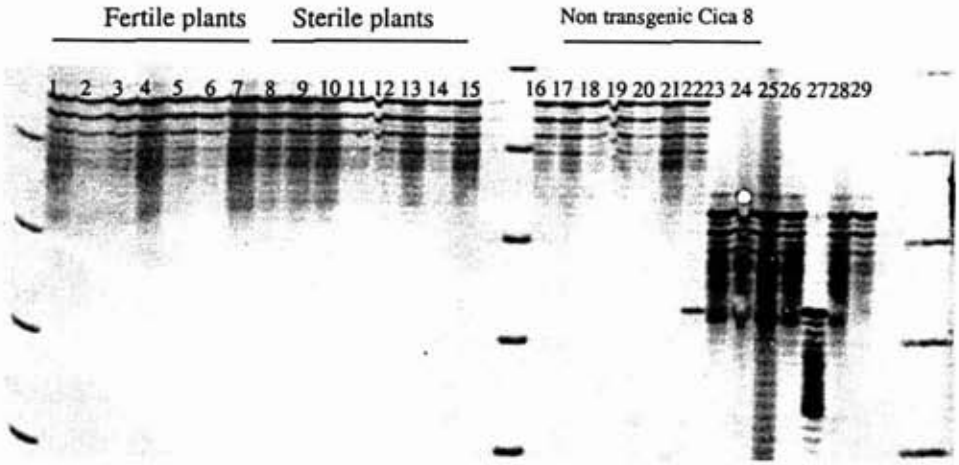

Figure 1. Genetic profile using RM 211 microsatellites marker. Lines 1-7 (fertile plants A3-49-60-12-3-1-31). Lines 8-15 (sterile plants A3-49-60-12-3-3-57). Lines 16-22 (non-transgenic Cica 8,control). Lines 24-29 (correspond to Fedearroz 2000, Coprosem 1, Cimarron, Oryzica 1, Fedearroz Victoria 1, Oryzica Llanos 5 respectably.

Table 2. Agronomic evaluation of F3 lines derived from backcross to Cica 8 or crosses to Oryzica 1, Fedearroz 50, and Iniap 12 of resistant transgenic lines

Transgenic Line (Female parent)	Variety (Male parent)	F3 line-No	P[1]	DF[2]	F[3]	W[4]
60-12-3	Cica 8	-40, -2, -7, -9	30	134-141	40-90	12-77
60-4-13		-6	2	130	90	33-34
60-4-5		-7	3	139	90	38-70
60-12-3	Oryzica 1	-13, -17, -21	20	132-132	80-90	32-73
60-4-13		-4, -13III, -15A, -17	19	131-139	70-90	35-96
60-4-5		-13, -223, -66, -232, -15*	22	125-143	70-80	26-146
101-18-19		-14M, -15, -64	24	130-139	80-90	37-83
60-4-13	Fedearroz 50	-14, -15AM, -18, -18C, -20, -20A	32	127-142	90	24-105
60-4-5		-12A, -19*, -65, -66, -68, -60, -61B, -16A	45	127-139	70-90	33-104
101-18-19		-15, -62,-67B	24	131-129	90	34-84
60-12-3	Iniap 12	-18	5	134	90	62-104
Cica 8	NA	NA	NA	132	90	27-35

[1] Number of individual plants selected; [2] Days to 100% plants flowering; [6]F, Mean percentage of fertility per line; [4]W, range of grain weight (g)/plant of total selected plants. NA, not applicable

Of the 82 crosses derived from backcross to Cica 8 or crosses with the other varieties a total of 11 (13%) were selected based on their resistance to RHBV, high fertility, vigor, and yield potential (Table 2). These crosses included 38 lines with a total of 226 individual plant-selections (Tables 2). The mean yield per plant in these selected lines ranged from 12g/plant to 146g/plant respect to Cica 8 that varied from 27g/plant to 35 g/plant (Table 2). The highest yield potential was noted in lines A3-49-60-4-5/ Oryzica 1-15, A3-49-60-4-5/ Fedearroz 50-19 and A3-49-60-12-3/ Iniap 12-18 with 146 g/plant, 104 g/plant and 104 g/plant, respectively. The lines A3-49-60-4-5/ Oryzica 1-15, A3-49-60-4-5/ Fedearroz 50-19 showed resistance to RHBV (scores 3-5). In addition, the line A3-49-60-4-5/ Fedearroz 50-19 was not only resistant to RHBV but also showed tolerance to *R. solani* under greenhouse conditions. These lines are still segregating since they are in F3 generation. Thus, individual plant selections are being evaluated first for resistance to RHBV for subsequently advance breeding for other traits. The selected plants will be subjected to anther culture for the production of doubled haploid (DH) lines to reduce breeding cycle by rapid fixation of homozygosity.

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2.1.7 Foreign genes as novel sources of resistance for fungal resistance

L. Fory[1], E. Tabares[1], T. Agrono[2], G. Delgado[2], C. Ordóñez [2], F. Correa[2], M.A. Santana[3], N. Tumer[4], and Z. Lentini [1,2].
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Introduction

Rhizoctonia solani (causal agent of sheath blight), *Helmithosporium, Rhincosporium*, and *Sarocladium* are diseases causing important high yield losses of rice in Latin America. All rice varieties are susceptible. CIAT's Rice Project has recently incorporated breeding efforts to develop strategies for controlling these diseases, aiming to reduce the use of pesticides associated with their control. The characterization of different sources of tolerance to *Rhizoctonia solani* is in progress (Correa *et al.*, 2000). The evaluation of wild species as a potential alternative to incorporate resistance is also underway (Martinez *et al.*, 2002). Intermediate tolerance has been found in inter-specific crosses between Oryzica 3 and *O. rufipogon* (Martinez et al., 2002). Efforts are being placed to adapt and improve a reproducible methodology for evaluation of this resistance in the greenhouse (Correa *et al.*, 2000) and more recently in the field. Molecular characterization of field strains of the pathogen has also initiated (Correa *et al.*, 2000). However, not known sources of stable genetic resistance for these rice diseases have been identified yet. Thus alternative approaches are also needed.

The genus *Phytolacca* is the source of a number of proteins whose antiviral and anti-fungal properties have been analyzed. The PAP protein (pokeweed antiviral protein), derived from *Phytolacca americana* a weedy species naturally found throughout the Americas, inhibit the infection of a wide range of RNA and DNA viruses each representing a different plant virus group, such tobacco mosaic virus in plants (Chen *et al.*, 1991), animal viruses, including poliovirus (Ussery *et al.*, 1977), herpes simplex virus in animals (Aron and Irvin, 1980) and human immunodeficiency virus (HIV) (Zarling *et al.*, 1990). Mutated versions of PAP gene have potent antifungal activity (Zoubenko et al., 1997). The work by the Dr Tumer Group Rutgers University, USA shown homozygous progeny of transgenic tobacco and potato plants expressing these PAP genes displayed resistance to the fungal pathogen as *Rhizoctonia solani* and *Phytophtora infestans* (Wang, 1998), and transgenic PAP turf grass are resistant to various fungal pathogens (N. Tumer, personal communication). These results suggest the possibility of designing molecular strategies for incorporating fungal and/or viral resistance into rice.

Of the mutated versions of PAP, the non-toxic version PAPy123 is the one showing the most potent protection against fungal infection. We are interested in constitutively expressing PAPy123 gene in transgenic plants in order to obtain sheath blight resistance in rice. Last year we reported the selection of transgenic rice lines derived from the commercial varieties Palmar (Venezuela) and Cica 8 containing the PAPy123 gene. Last

year we reported the evaluation and selection of transgenic lines with stable integration, inheritance and expression of the PAPy123 gene, as well as with desirable agronomic traits. Here we report the performance of T_2 and T_3 plants derived from these lines when challenge with the pathogen under greenhouse conditions. Also we report the generation of new transgenic lines using the *indica* varieties Cimarron, and Fedearroz 2000, the breeding line CT11275, and the *japonica* variety Nipponbare, widely used in rice functional genomic.

Materials and Methods

Genetic transformation and plant regeneration. Mature embryos-derived callus of the *indica* materials Cimarron, Fedearroz 2000, and CT11275, and the *japonica* variety, Nipponbare, were used. Transgenic plants were generated using the *Agrobacterium tumefaciens* strain Agl1 (Wang *et al.*, 1997) containing the plasmid NT446 that carries the PAPy123 gene (Tabares *et al.*, 2002).

Molecular analyses of PAPy123 transgenic rice plants. Genomic DNA was extracted from 1 g of rice leaves according to McCouch (1988). DNA was digested accordingly to analyze number of gene insertions, copy number, and rearrangements. Gels were denatured and neutralized by standard procedures. Membranes were hybridized using labeled specific- PAPy123 probe at 60°C (Sambrook *et al.*, 1989).

Evaluation for Rhizoctonia resistance in the greenhouse. T_2 and T_3 progeny plants from transgenic lines were inoculated at 50-day-old with two *Rhizoctonia* strains (intermediate and hyper virulent). These strains were collected from rice farmer's fields in Tolima Department (Colombia), and characterized by its virulence using a susceptibility-differential reference. The strain 1953-2 shows intermediate virulence, and the strain 2399-1 is hyper virulent. The inoculated plants were placed in a humid chamber (> 80% of relative humidity and 30°C) to simulate symptom development. Plants were evaluated 15 days and 25 days after inoculation according to IRRI evaluation scale and modified by the CIAT Rice Project (F. Correa, personal communication, Project IP4). The non-transgenic Cica 8 and Palmar varieties were used as susceptible control, and breeding line CT14534-12M-3-4M, derived from the inter-specific cross Oryzica 3/O. *rufipogon* was used as intermediate resistance control. In addition to the PAP-transgenic rice lines, transgenic lines A3-49-60-12-3-1-31, and derived crosses A3-49-60-12-3/Cica 8-2 and A3-49-60-4-5/FB007-19 containing the N-protein gene and with hypersensitive reaction as the mechanism underlined viral resistance were also evaluated. Five plants of each line were analyzed for the presence of the transgene by PCR.

RHBV resistance assays. Independent transgenic events containing the PAPy123 gene were evaluated for resistance to RHBV in the greenhouse. At least, 10 T_2 progeny per each of 22 transgenic events were inoculated with RHBV at 10 days or 15 days after planting using 4 viruliferous insects per plant. Disease evaluations were conducted using

scale from 0 to 9, were 0 refers to no disease symptoms and 9 indicates more than 90% leaf area affected by the RHBV disease.

Results and Discussion. From 83% to 100% of plants analyzed showed integration of the PAPy123 gene according to Southern blot analysis, yielding a total of 70 transgenic plants obtained from 371 callus originally *Agro*-infected (Table 1). The transformation efficiency varied from 11% for Fedearroz 2000 to 54% for Cimarron respectively (Table 1). The molecular analyses showed the integration of single and multiple copies of the PAPy123. All the Fedearroz 2000 plants showed single and non-rearranged gene copies (Figure 1). Most plants from Cimarron and CT11275 also showed simple integration patterns. But Nipponbare showed multiple copies, some of them of higher or lower molecular weight than the expected size indicating rearrangements in some of these copies, including possible gene fragmentation and deletion (Figure 1). These plants will be evaluated for agronomic traits, and selected lines will be advanced to T2-T3 generations and plants with stable inheritance and expression of PAP gene will be screened for sheath blight resistance.

Transgenic plants of Cica 8 and Palmar were evaluated for resistance to *Rhizoctonia* for two consecutive generations (T2 and T3) using intermediate and hyper virulent strains (Table 2). About 50 T2 lines were identified showing intermediate level of resistance, sister plants of these lines were also evaluated for RHBV, and T3 self cross-derived progeny plants from the resistant ones were evaluated for sheath blight in the following year. T2 plants derived from transgenic Cica 8-446 8-1, Cica 8-446-14-6, Palmar-446 4-1, Palmar-446 23-1, and Palmar-446-39-4 showed intermediate resistance to *Rhizoctonia*. This resistance was inherited into T3 plants from the Cica 8-446-14-6-18 and Palmar-446-39-4-7 lines showing intermediate resistance to both strains of fungi (Table 2). These plants showed a significant reduction in disease reaction respect to non-transgenic control. In addition, line Palmar-446-39-4 showed resistance to RHBV and the derived T3 line Palmar-446-39-4-7 showed a reduced leaf area affected by the pathogen respect to the cross Oryzica 3/*O. rufipogon* when inoculated with the hyper virulent strain 2399-1 (Table 2, and Figure 2A). Southern blot analysis of genomic DNA indicated that 100% of these plants contained simple integration pattern of non-rearranged copy of the PAPy123 gene (Figure 2B). These plants showed a fertility of 93-95%.

PAP protein seems to activate the protein expression of host genes associated with hypersensitive response (HR) and defense-related signal transduction pathway in the absence of pathogen infection and elevated levels of salicylic acid (Zoubenko *et al.*, 1997; Wang *et al.*, 1998; Smirnov *et al.*, 1997). Additionally, PAP inhibits local lesion formation to a number of different viruses, including both DNA and RNA viruses (Chen *et al.*, 1993), conferring a resistant mechanism against both mechanically and aphid-transmitted virus. For this reason besides fungal resistance, it is interesting to evaluate the activity of the PAPy123 gene against RHBV and other rice viruses. Because PAP also confers resistance to a broad spectrum of fungal pathogen, it will be interesting to evaluate these transgenic plants for resistance to other pathogens as well. The transgenic Cica 8 line A3-49-60-4-5/FB007-19 containing the RHBV-N gene for RHBV resistance, showed highest level of

tolerance to *Rhizoctonia*. The RHBV-N gene appears to confer resistance to this virus by RNA mediated cross protection where hypersensitive reaction mechanism is involved (Lentini *et al.*, 2002). It could be possible that RNA transcripts from the RHBV-N gene could function as elicitors for the plant defense system before or during pathogen infection. This hypothesis requires more systematic research in order to determine the reproducibility of this phenomena and elucidate the resistance mechanism involve.

Future Plans

- To study the mechanism of action of the gene *PAPy123* in resistant transgenic Cica 8 and Palmar
- To evaluate the possible effect of the gene *PAPy123* on *Tagosodes orizicolus*, the planthopper vector of RHBV
- To explain why some lines are resistant only to one strain of the fungus
- To study protein expression and gene integration in the resistant lines
- To evaluate the tolerance to Rice Stripe Necrosis Virus under greenhouse condition
- To multiply seed (T_3) resistant to *R. solani* and evaluate resistance for *Rhizoctonia,* and other pathogens such as *Sarocladium*, and *Helminthosporium* under field conditions

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Table 1. Transformation efficiency of three *Indica* varieties and one *Japonica* variety using *Agrobacterium tumefaciens* (NT446)

Genotype	[1]R	Callus	Plants	[2]RP (%)	**Plants** Analyzed by Southern	[3]Plants S^+	Plants S+ (%)	[4]TE (%)
Indica								
Cimarron	3	52	28	53.8	27	27	100	53.8
CT11275	2	166	27	16.2	17	17	100	16.3
Fedearroz 2000	2	44	6	13.6	6	5	83	11.4
Japonica								
Nipponbare	3	109	23	21.1	23	21	91	19.3

[1] Number of replicates. [2]Regenerated plants. [3]S^+ = Southern positive. [4]TE = Transformation efficiency

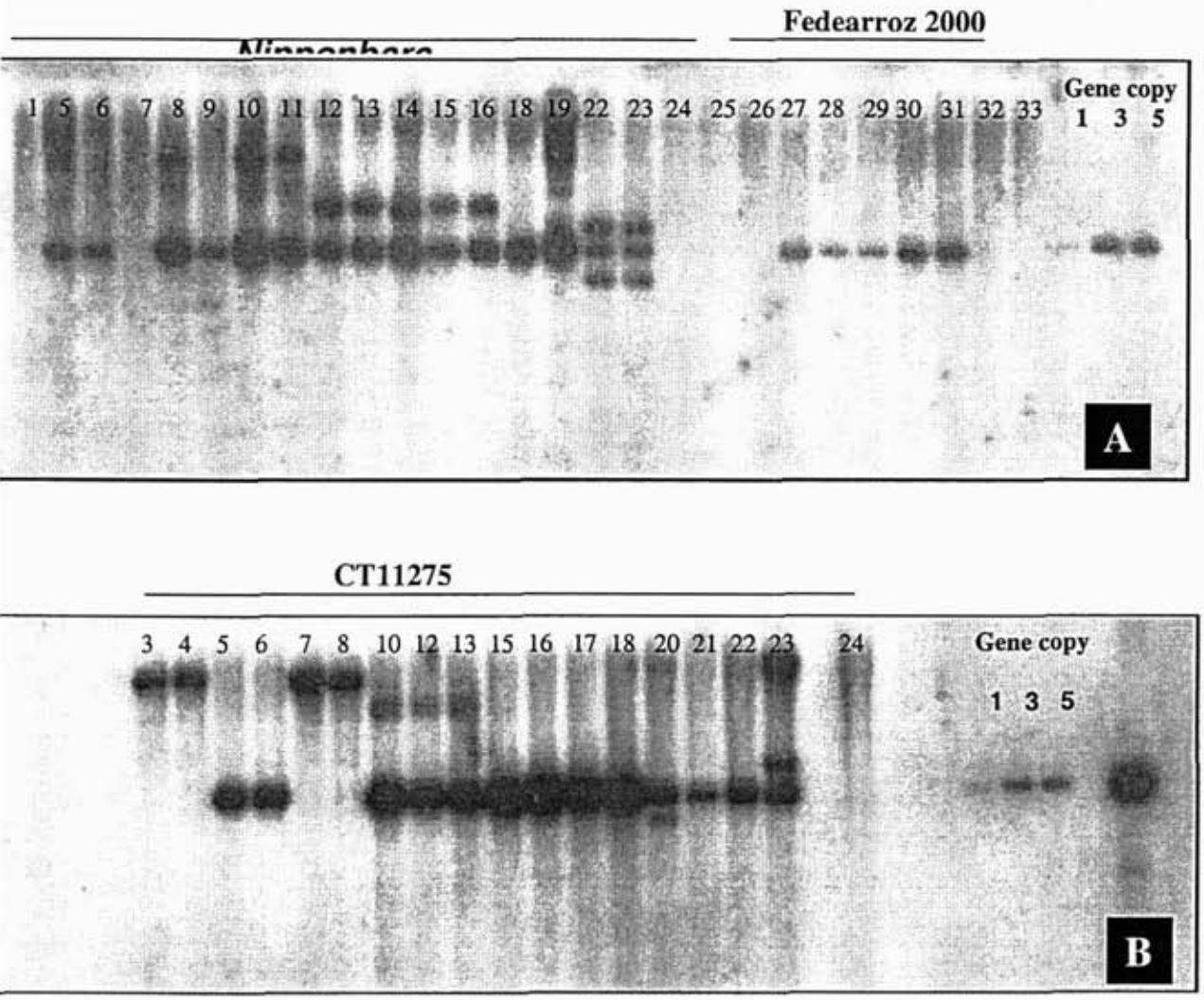

Figure 1. Southern blots analysis of *PAPy123* gene in T_0 plants. (A) Lanes 1-23, DNA from Nipponbare, Line 24 non-transgenic control. Lines 25-31, DNA of Fedearroz 2000. Lines 32-33, non-transgenic control. (B) Lanes 3-23 DNA of CT1275. Line 24 non-transgenic control.

Table 2. Evaluations for resistance to *Rhizoctonia* and RHBV under glasshouse conditions of T_2 and T_3 progeny plants derived from six transgenic lines that carrying PAPy123 gene

				% LAF *Rhizoctonia* (2002)[2]		% LAF *Rhizoctonia* (2003)[3]		
Line	Gene	T_2 Plant	RHBV [1]	1953-2 I<26	2399-1 I<31	T3 Plant	1953-2 I<40	2399-1 I<60
Cica 8-446-	PAPy123		9	17	88	11	52	57
						12		72
		8-1				13	53	55
			Nd	29	22	16	63	55
						17	53	65
		14-6				18	38	52
						19	46	67
Palmar-446-			5	23	60	11	45	50
						12	51	68
		4-1	5	26	26	6	41	73
						12	55	58
		23-2				16	53	58
						17	50	81
						18	38	77
			3	23	46	7	48	48
						14	58	68
		39-4						
Non- transgenic Cica 8	None		9	37	46	NA	57	64
Non-transgenic Palmar			7	26	39		51	92
Non-transgenic Fedearroz 2000		NA	2	41	56		Nd	Nd
Non-transgenic CT14534-12-M-3-4-M			.	26	23		48	60
	RHBV-							
A3-49-60-12-3-57-3	N		1	63	95		Nd	Nd
A3-49-60-12-3-1-31-9			6	29	25		62	73
A3-49-60-12 3/ Cica 8-2		NA	5	Nd	Nd		61	57
A3-49-60-4-5/ Fedearroz 50-19			4	Nd	Nd		33	55

[1] Mean score value of ten plants evaluated for RHBV per each T_2 line.
[2 and 3] Mean values of percentage leaf area affected (LAF). Reactions to two strains of *Rhizoctonia* (1953-2, intermediate virulence; 2999-1, hyper virulent). Two evaluations on two consecutive generations. (I) alues refer to the percentage of LAF indicating intermediate resistance. [2]Means of five plants per each T_2 line and [3]Means of fifteen plants per each T_3

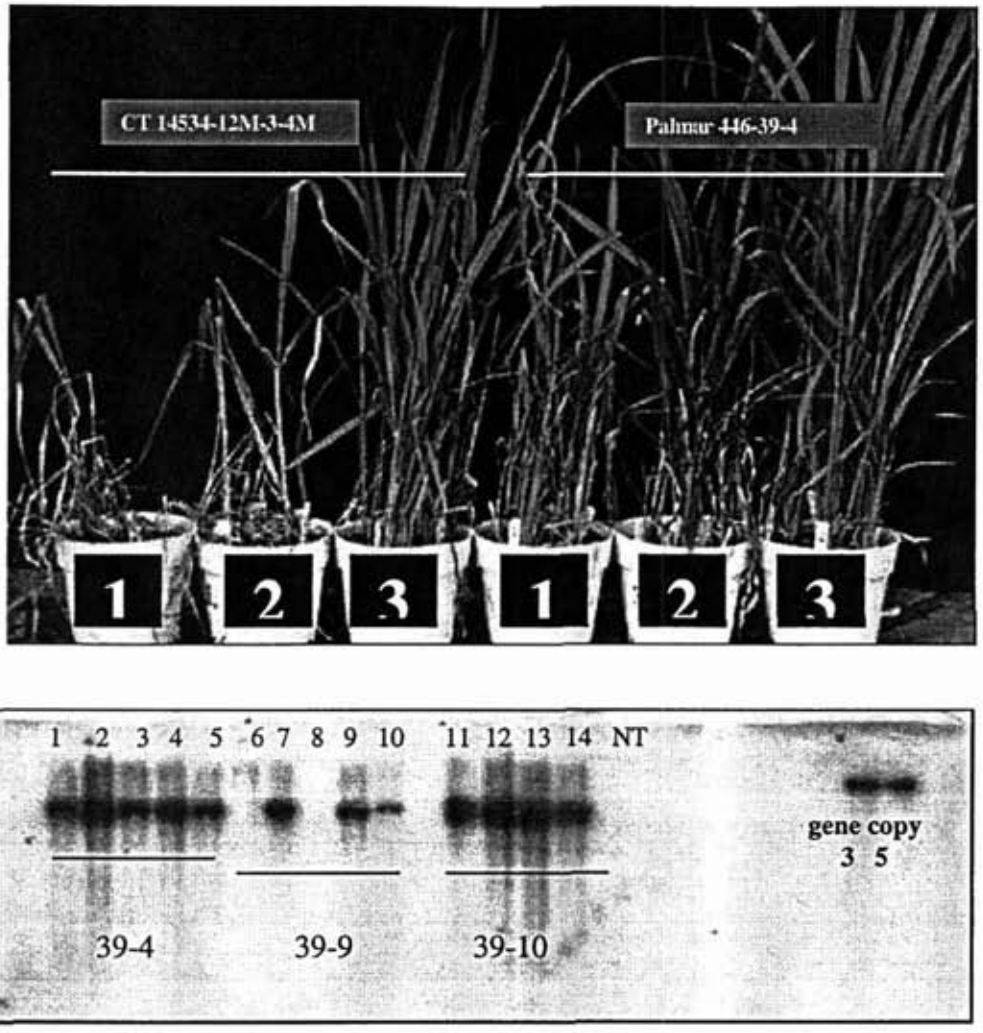

Figure 2. (A) Disease reaction of plants inoculated with hyper virulent *Rhizoctonia* strain 2399-1. Left non-transgenic CT14534-12-M-3-4-M derived from interspecific cross Oryzica 3/ *O. rufipogon*. Right, transgenic Palmar 39-4 plants carrying the PAPy123 gene. Labels refer to disease prossure (1) high (2) medium and (3) low.

(B) Southern blot of transgenic plants carrying the PAP123y gene. Lanes 1-14, transgenic plants from lines 39-4, 39-9 and 39-10 respectively. NT, non-transgenic control.

2.1.8 Use of red rice as potential trait source for commercial rice breeding

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Introduction

Recent studies on genetic diversity of Latin American rice commercial varieties had shown the need for broadening the genetic base of breeding materials. Main targets are increase productivity, stability across environments, increase weed competition, and durable resistance to various diseases and pests. Latin American wild *Oryza* species are new sources of genetic diversity not found in the cultigens (Buso *et al.*, 1998). These species represent a rich, largely untapped source of resistance genes to biotic and abiotic stresses. Work by CIAT rice breeding program has demonstrated increased evidence that *O. rufipogon* and *O. glaberrima* harbor genes of interest for the genetic improvement of cultivated rice (Martinez *et al.*, 2002). However, other studies had shown that inter-specific hybrids in rice might be prompt to both F1 sterility and later-generation hybrid breakdown (Oka, 1988: Burke and Arnold, 2001). Red rice (*Oryza sativa f. spontanea*) is a weedy rice with having similar morphologicals traits as cultivated rice at vegetative phase but generally they have profuse tillering, and vigorous growth and other plant traits make this weed highly competitive respect to rice. According to Langevin *et al.* (1990), the red rice can be grouped in ecotypes with characters alike cultivated rice or wild rice (Oka and Chang, 1961). Other researches indicate that red rice shows intermediate characteristics between wild rice *O. rufipogon* and cultivated *indica* or *japonica* varieties of *Oryza sativa* L. (Oka, 1988 cited by Bres-Patry *et al.* 2001). Another hypothesis is that weedy rice may evolve through the degeneration of domesticated rice, as weedy types of rice, where wild rice is not present (Vaughan *et al.* 2003). Our work using microssateliites markers had indicated that red rice accessions collected from farmer's field in Colombia (Huila and Tolima) showed a genetic diversity not present in either rice commercial varieties, and the accessions of *O. rufipogon*, *O. glumaepatula*, *O. barthii* and *O. galberrima* analyzed (Gonzalez *et al.*, 2003 in this report Output 1). Thus red rice could a potential source to broaden the genetic base of rice varieties.

Materials and Methods

Selection of red rice accessions. Red rice materials were pre-selected based on previous phenotypic, phenological and molecular characterization (Gonzalez *et al.*, 2002). Priority selection was given to accessions with resistance to RHBV, higher tiller number, yield potential and high re-growth capacity upon harvest. Selection criteria also included materials genetically diverse identified using the principal coordinate and component analyses, and microsatellite molecular characterization.

RHBV resistance assays. Preliminary evaluations were conducted on a total of 141 accessions of red rice. Second generation of self-progeny seeds of the original plants collected in farmers fields were planted in a randomized plot design with 3 replications. Thirteen commercial rice varieties with known reaction to RHBV and one accession of *O. rufipogon* were used as control. Materials were inoculated at 15 days old with two insects per plant from a *Togasodes* colony of 80% virulence. Plants were evaluated following the IRRI scale at 15, 30 and 45 days after inoculation.

Results and Discussion

Of the 141 red rice accessions evaluated 83% were susceptible, and 24 red rice materials showed intermediate resistance in the field evaluations, with scores ranging from 5 to 6. Except for Fedearroz 2000 (score 3) and the transgenic line A3-49-60-12-3-3-57-74 (score 1) that were resistant to the virus, all the other varieties and *O. rufipogon* were intermediate (Oryzica 1 and Fedearroz Victoria 1, scores 5-6) or susceptible (scores 7-9) to RHBV. Thirty seven percent of the red rice with intermediate level of resistance was derived from field plots where Oryzica 1 has been cropped for at least 2 years in a row, and the other 37% from plots with Fedearroz 50. The other accessions came from plots cultivated with either Coprosem 1 or Cimarron. At this point, it is not clear the source of resistance to RHBV present in these red rice materials, which needs more analysis.

A total of 54 red rice materials were selected by having a significant higher number of tillers respect the commercial varieties. These red rice materials showed a range in tiller number from 22 to 30 tillers per plant, whereas the varieties showed from 11 to 24 tillers per plant. Similarly the number of tillers that re-grew after harvest in red rice varied up to 29 tillers per plant, while the best variety (Fedearroz 50) showed a maximum of 29 tillers per plant. A total 59 red rice materials was selected based on its yield potential. These plants showed from 2 to 39 gram per plant, while the commercial varieties (Cimarron, Coprosem, Oryzica 1 and Fedearroz 50) showed a production from 10 to 25 g per plant. Other materials were selected to include the diversity found in the red rice population based on the analysis of qualitative and quantitative traits, as well as on the microsatellite markers profile and resemblance with commercial varieties (17 materials) or wild species *O. rufipogon* (15 red rice). Selected materials will be planted in the field this semester for further characterization and selection, to initiate a breeding scheme (Figure 1). In addition to evaluations for agronomic traits, materials will be evaluated for RHBV resistance, milling and cooking quality traits. Selected plants will be crossed with Fedearroz 50, Fedearroz Victoria 1 and three FLAR lines, segregating populations will be evaluated and selected F2 plants will be processed through anther culture to develop fix lines to initiate yield potential and regional evaluation earlier.

Future Plans

To initiate the field characterization of agronomic traits of the selected red rice

To conduct molecular and taxonomic characterization of individuals similar to *O. rufipogon* by means of AFLP
To initiate the crossing program between red rice and selected commercial varieties
To advance backcrossed populations and select best candidates for the generation of doubled haploids through anther culture

To identify quantitative traits loci (QTLs) associated with yield increase and/or out performance in populations derived from crosses with red rice and commercial varieties

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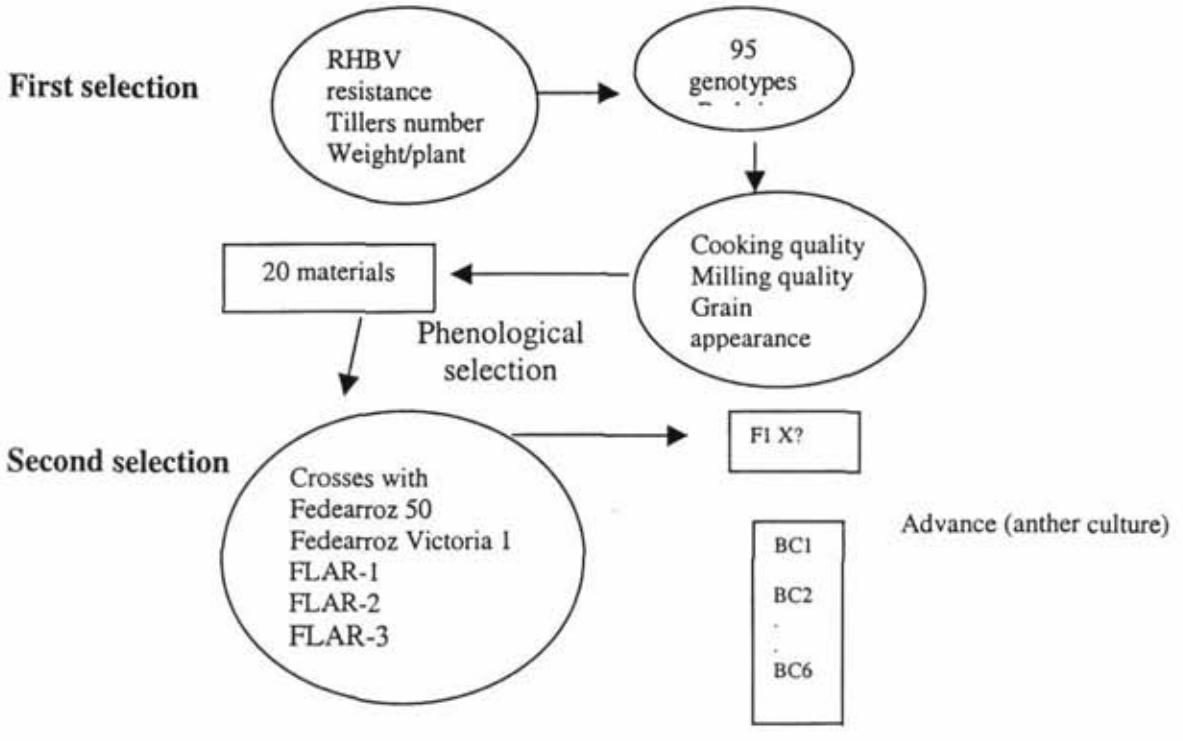

Figure 1. Breeding scheme to incorporate red rice traits into commercial rice varieties

2.1.9 Field performance and fruit quality of *in vitro* propagated plants of *Solanum quitoense* (lulo) and their use as elite clone materials by farmers

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Introduction

A large number of fruits of Andean origin have great potential to become premium products for local and export markets with a high economic return for the farmers. *Solanum quitoense*, locally known as lulo in Colombia and as naranjilla in other countries, is among these fruits. This species is native from Colombia and Ecuador, and it is normally cultivated between 700 and 2000 meters above sea level. Some of the main attributes of this fruit include its high level of vitamin C, and the sub-shrubby perennial growth amenable for cultivation in hillsides and inter-cropping, aiding soil conservation practices. Recently in Colombia, naranjilla changed from being a fruit of local fresh consumption to become an important industrial fruit for juice and yogurt products, increasing its market value. A major constraint for the rapid adoption of naranjilla by the local farmers is the limited availability of elite germplasm free of pathogens from clonal propagation. The high level of heterozygosity of this species is reflected in the high segregation of traits by its multiplication through botanical seeds. Rapid multiplication of high quality planting materials is of paramount importance. One of the main objectives of this project is to develop a protocol for *in vitro* propagation of naranjilla with application for conservation and rapid multiplication of elite clones free of pathogens. This protocol would facilitate the conservation and multiplication of high quality of planting farmer's material aiding to control one of the major constraints of this crop. In the previous two years we reported the development of an efficient protocol for the maintenance and propagation in *vitro* of clones selected from farmers field, for the plant regeneration from tissue cultures, and the preliminary evaluation of those plants in the field. This year we report the agronomic performance of those materials in the field, including quality traits of the fruit, as well the progress made to evaluate jointly with farmers the application of this technique at larger scale.

Materials and Methods

Plant material. High quality and elite clones provided by the Andean Fruit Center (Centro Frutícola Andino – CEFA) were used. This collection includes naranjilla with or without thorns commonly grown by farmers. The plants were propagated in *vitro* or plants were regenerated following procedures as reported by Segovia et al. in SB2 Annual Report 2002.

Plant and fruit evaluations. A small-scale field trial was conducted at 1700 m over sea level and a mean temperature of 22C to compare the growth, development and agronomic performance of regenerated plants respect to *in vitro* propagated clones. Fruits produced

during a period of five months were harvested, counted, weighted, and classified by size according to standard scale. Premium size: > 5.5.cm diameter. Commercial size: from 3.5 cm to 5.5 cm in diameter. No commercial: < 3.5 cm in diameter. The fruit maturation process (days from harvest to ripe to rotten) was evaluated using the Munsell color scale. Fruit quality trait analyses were conducted at the Valle University (Cali). These analyses included Brix grade at 20C, relative humidity, acidity percentage, vitamin C, sugar content, pH. A sensorial panel analysis was conducted to evaluate the fruits for its appearance, color, aroma and flavor as fresh fruit or processed in juice, and compared with those available in the supermarket.

In vitro introduction of plants from greenhouse or field. Greenhouse or field grown plants were tested to select the best protocol for the *in vitro* introduction of elite farmer's clones from the field. In order to establish plants in the greenhouse, the recovery of plants from axillary buds using shoot stakes was tested. Clones stakes of 20 cm in length from adventitious shoots with 2-3 axillary buds were taken from selected plants in the field and disinfected first with 3 ml/l of fungicide propamocarb HCL (*Pervicur)* for 5 min. The basal section of the stake containing the lower two buds were either: a) Cultured in water until root formation; b) Soaked in a solution of 10 mg/l NAA for 3 days and then transferred to water until root formation; or c) Potted directly in sterilized mix of soil, sand and sugar cane plant residues (Franco et al., 2002). Treatments (a) and (b) were aerated with a fish tank pump system. In order to establish plants *in vitro*, apical or axillary meristems from plants grown in the greenhouse or field were tested. Explants were surface sterilized and cultured *in vitro* on medium A instead of medium 17N (Segovia et al., 2002. SB2 Annual Report). Riphampicine (antibiotic) 100 mg/l was added to the medium to control bacterial infection from field explants, since the presence of trichomes in the tissues prevented thoroughly disinfections.

Evaluation of the technology with farmers. A total of 20 farmers from two regions of Colombia (Cauca y Huila) with commercial production of lulo were selected. Farmers were chosen based on the years of experience cultivating lulo, the number of lulo plants currently grown and their interest to participate in this initiative. A strategy was planned jointly with the farmers to evaluate the potential commercial use of *in vitro* generated plants as an alternative of planting material clean of pathogens. This activity was conducted in collaboration with IPRA, the tropical fruit project of CIAT, and Corpoica-Popayán.

Results and Discussion

Last year we reported no differences in plant growth and development between regenerated and *in* vitro propagated plants. Likewise, no significant differences were noted between the regenerated and *in vitro* propagated plants respect to the total number and total weight of fruits produced (Figure 1A). However, the clones with thorns (T) showed higher disease and pest susceptibility that significantly reduced fruit production (Figure 1A). The average productivity in 5 months of harvest indicated a mean number of fruits per plant of 142 for materials without thorns (G) and 28 fruits for T (Figure 1A), and a total weight of fruits per plant of 5.58 kg for G and 1,57 kg for T (Figure 1B). About 80% of the fruits harvested

from these clones showed a commercial size (from 3.5 cm to 5.5 cm in diameter). The rest of the fruits were classified as 15% small fruits (< 3.5 cm in diameter) for the G plants, and 10% premium size (> 5.5 cm in diameter) for the T materials (Figure 2). Commercial production of lulo may yield per year about 135 fruits/plant and 9 kg fruits/plant when cultured at a density of 3,000-plants/ hectare (3 m^2 per plant, CCI). In this experiment plants were also planted at a distance of about 3 m^2 per plant. Of the plants evaluated four were identified because of its high yield potential. These plants produced 9 kg fruits/plant during the ½ year experimental harvest time. These plants were selected; seeds and stakes of these plants were collected, and grown in the greenhouse and *in vitro*.

In relation to fruit quality, no significant differences were noted between the *in vitro* propagated clones, the regenerated plants and the commercial fruits bought in the supermarket for most chemical traits evaluated. The exception was the content of reduced sugar, which was lower in the supermarket fruit possibly indicating a longer post-harvest time respect to the experimental fruits at the moment the analyses were conducted (Table 1). The two sensorial analyses conducted gave different results. The first panel with 5 panelists shows a higher acceptance for the experimental materials for all traits, but the panel with 11 panelists indicated a higher acceptance for the supermarket fruit in relation to flavor and aroma, and higher acceptance for the experimental materials for color and physical appearance. This analysis seemed to be highly subjective, thus it may need to be conducted with a larger number of panelists to elucidate clearer preferences. No differences were noted in shelf life between the different materials.

Attempts to establish plants in the greenhouse from field grown materials indicated that the highest percentage of stakes with new shoots was obtained when the stakes were planted directly into soil in the greenhouse. About 50% of the stakes had healthy looking plantlets 1 month after planting (Figure 3). Explants derived from clones with thorns responded poorly, perhaps due to the weak stage of the donor materials since they were highly affected in the field. Once plants were established in the greenhouse stakes from greenhouse-grown plants produced profuse roots when treated with aerated water, which ease the mass clonal propagation of plants in the greenhouse. A high percentage of contamination was obtained when meristems were introduced *in vitro* directly from field grown plants. Explant survival (elongated shoots with roots) was increased from 40% to 60% when the antibiotic was added to the culture medium. No contamination was noted and at least 80% explant survival was obtained when meristems derived from greenhouse grown materials. Based on these results, successful *in vitro* introductions of elite materials selected in farmers fields can be achieved by establishing first clonally propagated plants in the greenhouse, and use these plants as donors for the *in vitro* culture of meristems. In the case a direct introduction from the field into *in vitro* conditions is needed, a more efficient surface sterilization protocol needs to be revised.

A process was initiated to test with farmers the suitability of using *in vitro* propagated plants as planting materials. The potential advantage on the in *vitro* source is the supply of free pathogens homogenous plants maintaining the selected traits of the elite materials.

Farmers were selected from two sites with commercial production of lulo (Pescador, Cauca, and Tierradentro Cauca-Huila). These sites include small, medium, and large-size production farms. The farmers have between 2 years to 9 year of experience cropping lulo, and 200-5,200 plant-size farms. Farmers attended a workshop at CIAT with the objective to define jointly a strategy to evaluate the potential commercial use of the *in vitro* plants. The advantages and limitations of using *in vitro* grown plants were discussed. The farmers evaluated the plants at the CIAT lulo experimental plot. The criteria were established for the collaboration. Farmers are highly enthusiastic with the project, and a teamwork approach will be implemented. For each site, two nursery plots will be established. Farmers will select the best two plants currently grown in their fields based on productivity, fruit quality, and disease/ pest resistance. These plants will clonally propagated in the field, and explants will be established in the greenhouse at CIAT. Meristems from these plants will be introduced *in vitro*. Pathogen-free *in vitro* propagated plants will be grown in the nursery plots jointly with the field-clonally propagated plants, and seedlings derived from the seeds of the selected plants (standard propagation mode used by the farmers). The *in vitro*, clonal-propagated and seed-propagated plants will be compared throughout the production season. The nursery plots could also be used for the evaluation of new germplasm, one of the main needs identify by the farmers, in addition to assistance for an integrated crop and disease/pest management approaches. Corpoica could play a key role in these aspects, reason why CIAT is trying to strengthen their linkage in the project. Farmers selected their best elite materials and currently the plants are being clonally propagated in the field. Following, plants will be brought to CIAT to initiate their introduction *in vitro*. Field evaluations will be conducted first semester of 2004.

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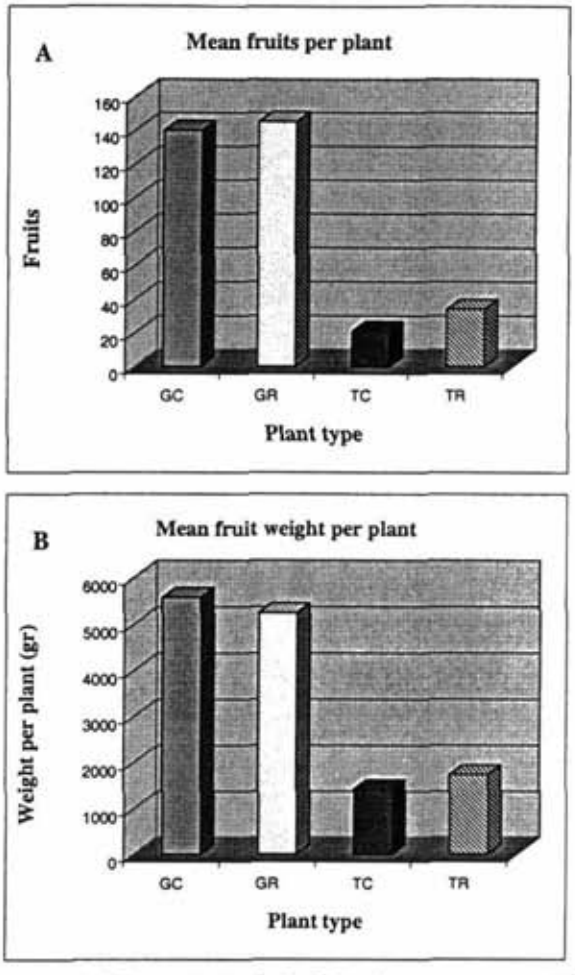

Figure 1. Fruit production in the field of *in vitro* propagated (C) and regenerated (R) plants derived from materials with thorns (T) or without thorns (G)

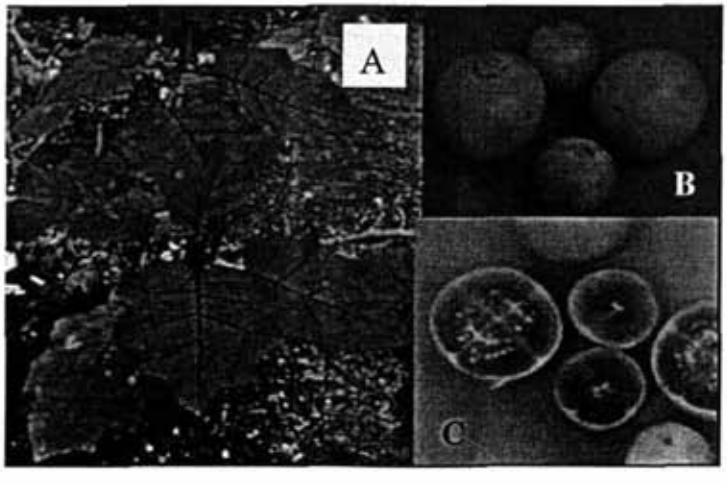

Figure 2. (A) Plant without thorns. (B) Fruits showing commercial and non-commercial sizes. (C) Dark green flesh that showed high acceptance by panelist in the quality trait assay

Table 1. Chemical quality trait analysis of fruits from regenerated plants without thorns (GR), *in vitro* propagated plants without thorns (GC), regenerated plants with thorns (TR), *in vitro* propagated with thorns (TC), and bought in supermarket.

Trait	GR	GC	TR	TC	Supermarket
Brix grade (at 20 °C)	11.5	11.6	11.0	10.0	10.5
Acidity (%)	2.2	2.2	2.7	2.3	2.9
Humidity (%)	86.8	85.2	79.8	87	82.4
Reduced sugar (%)	4.6	5.5	5.0	4.9	0.7
Total sugar (%)	3.1	3.4	2.7	3.1	2.7
PH	3.2	3.2	3.2	3.3	3.2
Vitamin C (mg/100g)	38.2	37.3	48.8	64.2	39.1

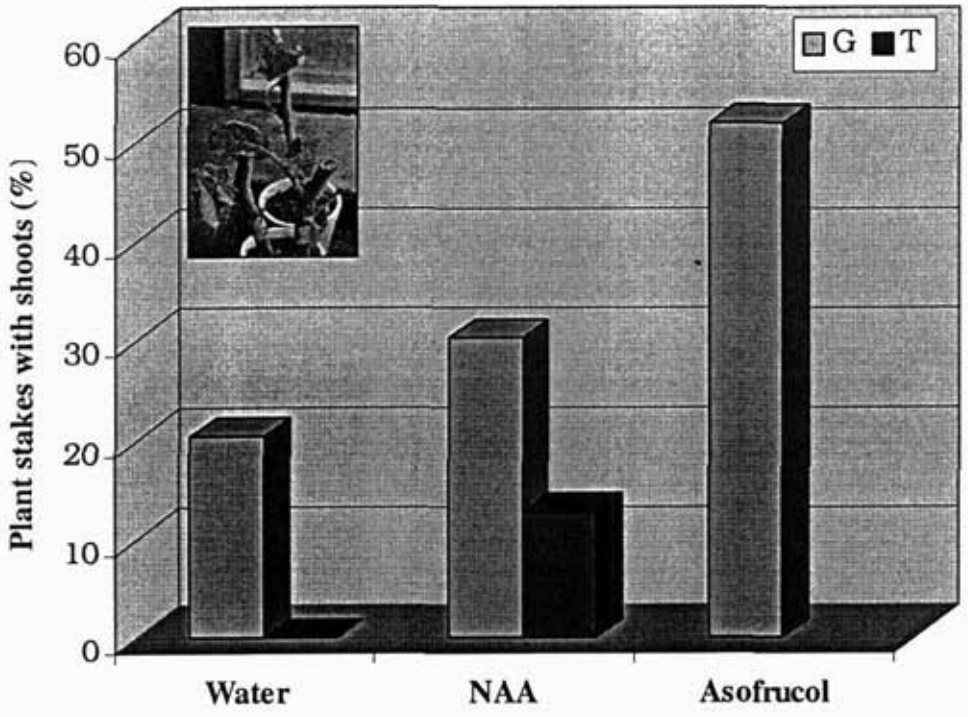

Figure 3. Percentage of stakes with new shoots derived from field-grown plants and treated in the greenhouse. (G) *Plants without thorns*, glabrous. (T) Plants with thorns.

Activity 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base

2.2.1 Development of an *In Vitro* Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding

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[1]SB2, [2]Virology, [3]IP3

Introduction

Cassava is an important crop for marginal agriculture areas in Africa, Latin America and Asia. It produces well under marginal conditions and is identified as a famine reserve in subsistence farming. The globalization of world economy offers cassava new opportunities for becoming an even more important source of raw materials for different industries. To achieve this, cassava productivity must increase steadily and reliably based on an efficient breeding approach. Increased genetic variability will be required to achieve diversified end uses of cassava products. This implies not only access to existing variability but effective tools for proper evaluation and screening, as well as the generation of new genetic variability for several key traits. However, traditional cassava breeding has not changed in the last 30 years and there is a need for a more dynamic breeding system so that cassava can maintain its competitiveness respect to other commodities. Introducing inbreeding in cassava genetic improvement has many advantages, which will facilitate and expedite the generation of diversified improved breeding lines. But developing inbred lines through self-pollinations would require in cassava about 9-12 years. Rapid and complete homozygous can be reached by using *in vitro* haploid technology. The aim of this project is to develop a protocol to induce androgenesis in cassava for the generation of doubled haploids. Doubled haploids of cassava will regenerate from cultured anthers or microspores of elite varieties via embryogenesis. An overview of the results with out-crossing species prompt to inbreeding depression likewise cassava, indicates that an efficient technology for the production of DH in cassava could be developed and spite of inbreeding depression, doubled haploid lines can still be useful for uncovering desirable genetic variability that is masked in highly heterozygous plants (Andersen et al., 1990, Paillard et al., 1996, Zhang et al., 2002). Previous preliminary work conducted by Roca and Iglesias (1993) reported the induction of sporadic callus derived from *in vitro* culture of isolated microspores. Although these results were not reproducible, it is a proof of concept indicating that it is possible to induce cassava cultures of isolated microspores to divide *in vitro*. Thus a more systematic analysis of the factors affecting androgenesis in cassava as proposed in this project, may allow the development of a reproducible protocol for the generation of DH in this species. In this first report we present a preliminary study directed to the identification of optimal microspore across different genotypes and environments, the optimization of environmental conditions for shipment of flower buds

for establishment of collaboration with ARIs, and the cytogenetic analysis of various clones.

Materials and Methods

Clones gown at CIAT experimental Station or Santander de Quilichao of varieties with contrast flowering cycle (early, intermediate and late) were used: AM244-31, MCol-1505, CM4574-7, MCol1468, AM312-103, CM523-7, MPer-183 and AM523-7; preference was given to clones with profuse flowering. Immature flower buds were collected when plants were about 8-12 month-old. Based on previous work on cassava flower biology conducted at Ciat by Roca et al. (1989), flower buds were collected when they were between 0.8 mm and 2.5 mm is diameter and fixed in a solution of 3:1 ethanol: glacial acetic acid with 0.5% ferric chloride for 24 hr for determining the stage of microspore development according to flower bud size. The number of microspores per anther was determined, and cytogenetic analyses were conducted to identify the different stages throughout the meiosis, the number of chromosomes and bivalents (ploidy level), identify chromosomal aberrations, as well as the development from cytokinesis to mature pollen grain. Two treatments were tested to evaluate optimal conditions for flower bud shipment. Flower buds were surface sterilized with ethanol 70% for 3 min followed by chlorox 20%-30% for 10-15 min, and three washes with distilled sterile water. Sterilized flower buds were either transferred in a Falcon 50 ml tube containing sterile water at 24-26 C (room temperature), or in zip-plug plastic bags 4 C (refrigerator). Pollen viability was measured through one week, time expected for the shipment to last.

Results and Discussion

The cassava pollen is generally from 122 to 148 μm in size, which is large compared to other flowering plants. Preliminary work suggests that most varieties show microspore in the tetrad stage (Figure 1B) of development when flower buds are between 0.8 and 1 mm in diameter (Figure 2). Flower buds of 1.2-1.5 mm in diameter contain microspores at the uninucleate stage (Figure 1C), whereas those of 1.6-1.8 mm in diameter are at the binucleate (Figures 1D and 2). Most flower buds contain mature pollen grains when they reach 2.0-2.2 mm in diameter (Figures 1F-G, and 2). This association between flower bud size and microspore developmental stages appears to change with each peak of flowering. Early varieties seem to show a faster development of pollen grains in smaller flower buds (AM 244-31, Figure 2).

Preliminary data suggest that about 100-200 microspores are produced per anther = 1,000-2,000 per flower bud. However, a variation in the number of microspores/ anther was found between anthers from different flowers of the same inflorescence and between different inflorescences. The intermediate flowering genotype CM523-7, produced about twice microspores per anther (200 microspores/ anther) than the late flowering material MPer-183 (100 microspores/ anther). This figure needs to be checked in a broader range of genotypes across different environments. If this low amount of pollen grains is

corroborated, it will be an important bottleneck for isolating large number of microspores required for isolated microspore culture, in such a case the pollen shed system should be considered.

In addition, MPer-183 was more affected by the different environmental conditions between the two sites, producing about half of the microspores/anthers (about 80 microspores/anther) when grown at Ciat (saline soils) respect to Quilichao (acid soils, 166 microspores/anthers). The intermediate clone CM523-7 seemed to be less affected by the contrasting conditions in the two sites, producing 189 microspores/anthers and 224 microspores/anthers at the two field sites CIAT and Quilichao, respectively. Based on agronomic performance, CM523-7 is a clone known to have a broader adaptation to contrasting environments respect to MPer-183. This preliminary data suggests that there is a strong genotype-environmental interaction affecting the production of microspores. This interaction seems not only to affect the yield of microspores/ anthers but also the stage of development. The intermediate clone CM523-7 showed a tendency of a faster development of pollen grains in smaller flower buds when grown at Quilichao respect to CIAT.

The cytogenetic study indicated that about 95% of pollen mother cells analyzed for MCOL 1505 showed 36 small and similarly shaped chromosomes and 18 bivalent pairing at meiosis (Figure 1H). Late chromosome condensation and chromosome adherence was noted in some cases at prophase I. Three nucleolar chromosomes were noted at prophase I and II, and telophase II and I. Cytokinesis showed tetrahedral arrangement (Figure 1A-B). Cassava is a highly heterozygous species. Few complete cytogenetic studies are available in cassava. Studies with molecular markers suggest disomic inheritance for some loci with evidence of gene duplication (Carvahlo et al. 1999 and 2002; Jennings and Iglesias, 2002). Although three nucleolar chromosomes are noticed as for true diploids, duplication of some chromosomes is also present suggesting segmental allotetraploidy.

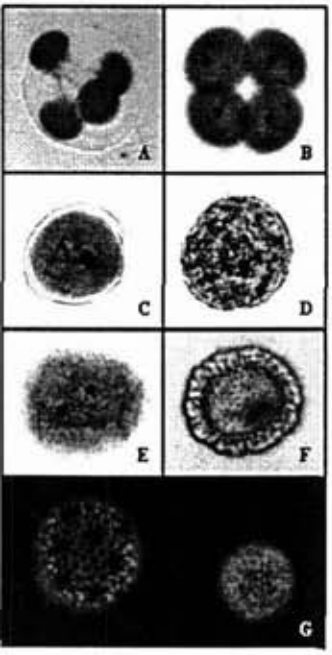


Figure 1. Cassava microspore Delopmental stages within the anther. (A) Cytokinesis. (B) Tetrad, tetrahedral arrangement. (C) Early uninucleate. (D) Early binucleate. (E) Late binucleate. (F) Mature pollen grain. (G) Pollen dimorphism, fluorescent viability assay. (H) Thirty six chromosomes at anaphase I, and 18 bivalents at metaphase I.

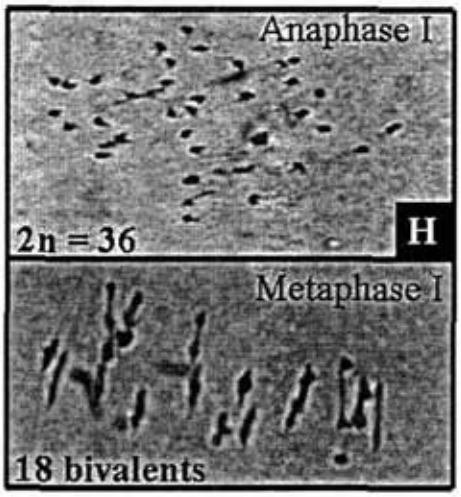
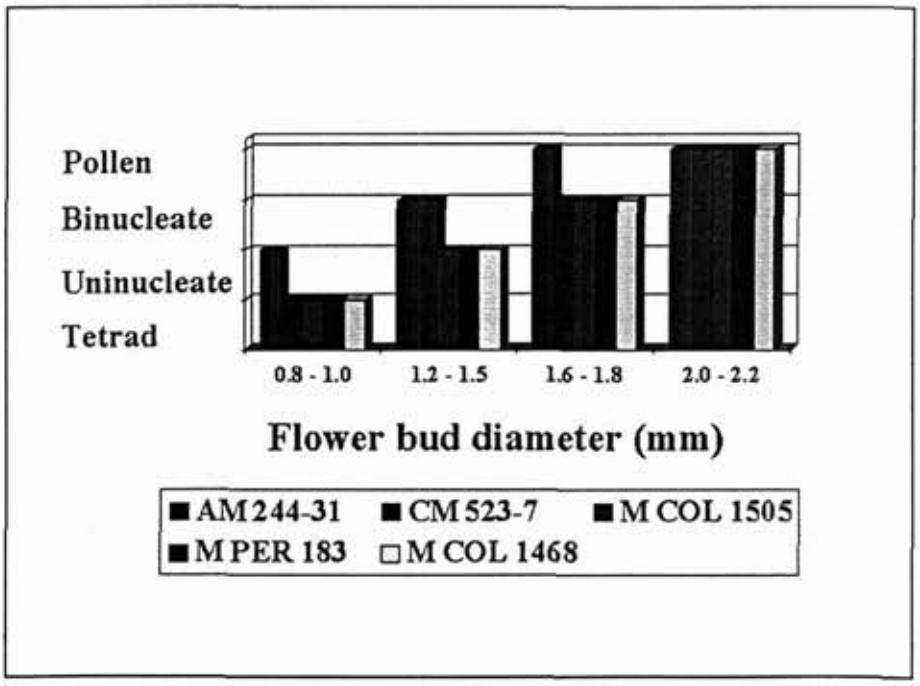


Figure 2. Microspore stages of developmen bud size for different genotypes grown in the field



Future Activities

- Input from breeders will be used to gather information on flowering, environmental adaptation and genetic diversity to select the best candidate experimental genotypes. Genotypes with profuse, continuous flowers, synchrony of microspore development, slow maturation process to pollen grain, ideally with known *in vitro* response, and desirable breeding traits are available, will be used as experimental genotypes to develop a protocol for efficient induction of androgenesis *in vitro*.

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2.2.2 Waxy cassava starch: Transgenic plantlets expressing gus in a vector that contains a GBSSI gene in sense orientation

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Introduction

Cassava starch is a polymer composed of linear (amylose) and ramified (amylopectin) glucose units. Starch is a raw material used to produce glues, textiles, paper, chemicals and

animal feed components among other products. Some of these require a 100% amylopectin-containing starch. One way to obtain it in cassava is by silencing the gene GBSSI (granule bound starch synthase), involved in amylose synthesis via transgenesis. We summarize the latest achievements on producing amylose free cassava.

Materials and Methods

The GBSSI gene was isolated from a cDNA cassava library, using a potato probe, to make vectors containing sense and antisense versions of the gene (pCAMBIA1305.2-S and pCAMBIA1305.2-A, respectively, in Agl1 A. tumefaciens strain), which were transformed into friable embryogenic callus (FEC) of cassava cultivars 60444 and MCol2215. Putative transgenic tissues were subject to selection with hygromycin. The transformation vector contained a gusA gene that allowed for early scoring and follow up of transformation events.

Results

The first transgenic lines of cultivar 60444 are being regenerated (Figure 1A) from in vitro cultures. As expected, 60444 responded faster than Mcol2215 to selection and regeneration, although putative transgenic somatic embryos of MCol2215 are now on embryo maturation medium, without selection, to produce plants (not shown). Some plantlets of 60444 were rooted on media with 10 mg/l hygromycin and expressed gus in what seems to be a chimeric pattern (Figure 1B). It is still too early to say if plants have silenced GBSSI expression.

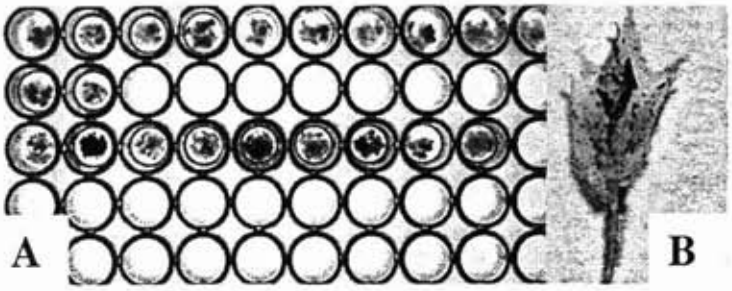

Figure 1. (**A**) Transgenic FEC lines expressing gus, transformed with sense GBSSI, all from cultivar 60444. (**B**) Transgenic leaf of in vitro plant with a chimeric gus expression pattern.

There are two ways to calculate transformation efficiency with cultivar 60444:

- There are 10 independent transgenic plants, seven gus positive and three gus negative, that regenerated plants. The initial amount of FEC that was transformed was 8.2 g of FEC; it gives 1,2 independent trasngenic lines/g of FEC
- The same 10 independent transgenic lines were recovered from 100 FEC clusters. Assuming that each cluster represents an explant, transformation efficiency, in percentage, is 10/100 = **0,10 (10%)**.

The former way of calculating transformation efficiency has been adopted as the standard for transformation in cassava.

Future Activities

- Regenerate plants and molecular confirmation of presence of transgenes
- Test for silencing of GBSSI at molecular level (RT-PCR)
- Move plants to biosafety greenhouse, make them produce storage roots and test for starch composition

2.2.3 Functional analysis of a Caffeic Acid *O*-Metiltransferase gene from *Brachiaria decumbens* (*Bd*COMT) in transgenic rice as a model plant

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Introduction

Brachiaria species are important forage grasses in the tropical lowlands of America, Asia, Africa and Australia. *B. decumbens* cv Basilisk is one of the most extensively cultivated *Brachiaria* species. It shows adaptation to acid soils, rapid growth and provides good soil coverage, and forage yield (Miles and Do Valle, 1996). Tropical grasses have high content of cell wall constituents, and this generally correlates negatively to intake, digestibility and animal performance. The major anti-quality components of grass cell walls are lignin and phenol monomers, which esterifies with cell wall polysaccharides (Smith and He, 2000).

Lignin composition, particularly the relative proportion of syringyl (S) and guaiacyl (G) units in the lignin polymer, and the nature of the covalent linkages between lignin and other polymers are also important determinants of the level of digestibility (Heath *et al.*, 1998). Genetic variation for quality is present in grass species and traditional breeding programs have been using that variability to improve forage quality. Although progress has been made, it has been slow and limited. Developments in genetic engineering now offer additional ways to improve forage quality. Methods are available to down-regulate lignin biosynthesis genes to reduce lignin content and/or change its type to improve forage digestibility. The lignin biosynthesis pathway and several key enzymes are reasonably well understood (Smith and He, 2000).

Caffeic acid *O*-methyltransferase (COMT) is a key enzyme in the lignin biosynthesis pathway. Therefore the modification of lignin content and/or composition by genetic manipulation would be of great economic interest, if consider that a 1% increase in mean

live weight gains per animal (Casler and Vogel, 1999). We had reported the cloning and characterization of a caffeic acid *O*-methyltransferase gene from *Brachiaria decumbens* (*Bd*COMT) (Florez et al., 2003). The objective of this part of the research was to finalize the characterization of this gene by functional analysis of its expression in transgenic rice plants used as model species.

Materials and Methods

Rice genetic transformation. Mature embryos derived embryogenic callus from *indica* (cv Palmar) and *japonica* (Nipponbare) varieties, where used targets. *Agrobacterium* mediated transformation was conducted using the Agl1 strain containing the constructs pC01OMT-2, pC01OMT-4, pC05.2OMT-1 and pC05.2OMT-2 previously generated in the laboratory (Florez *et al.*, 2002, SB2 Annual report p. 254-259). These constructs contain the *Bd*COMT gene in sense or antisense orientations. Transgenic rice was according Tabares *et al.*, 1999.

Southern Blot Analysis: Genomic DNA from rice young leaves was isolated following the methodology McCouch *et al.* (1988). *Brachiaria* DNA (15 µg) were digested with the restriction enzymes Hind III. The fragments were separated on 1% agarose gels and transferred to Hybond N^+ membranes according to the manufacturer's instructions (Amershan). Probe consisted of the open reading frame of *Bd*COMT prepared by PCR. DNA probes were random primer labeled using Megaprime DNA Labeling System kit (Amersham) and [^{32}P] dATP. The hybridization was carried out overnight at 55°C following the manufacturer's recommendations.

Northern Analysis: 15 µg of total RNA from rice leaves was separated on 1% formaldehyde gels and transferred to Hybond N^+ (Amersham) membranes according to the manufacturer's instructions. The probe consisted of the open reading frame of *Bd*COMT prepared by PCR. DNA probes were random primer-labeled using Megaprime DNA Labeling System kit (Amersham) and [^{32}P] dATP. Hybridization and washing were carried out following the manufacturer's recommendations.

Histochemical reactions: Leaves sections were sliced by hand and stained according to Weisner and Maüle reactions. For Maüle staining, leaf sections were immersed for 5 min in 1% $KmnO_4$, rinsed and distained in 30% HCl, washed and mounted in concentrated NH_4OH. For the Weisner reaction, leaf sections were incubated for 2 min in phloroglucinol solution (2% in ethanol/water 95/5 v/v), and then mounted in 50% HCl. Bright field photographs were taken between 20 min after staining procedure.

Plant evaluations: Six agronomic traits of transgenic rice plants were evaluated in the greenhouse. These characteristics included: (1) days to flowering, (2) height, (3) number of tillers, (4) fertility, (5) weight of 100 grains, and (6) total grain weight per plant.

Results and Discussion

Southern analysis indicated that the *Bd*COMT gene was introduced in sense and antisense orientations in the rice genome of cultivars Palmar (*indica*) and Nipponbare (*japonica*). Total of 321 embryogenic hygromycin resistance callus were transferred to regeneration medium containing 50 mg l^{-1} hygromycin. Between 8-75% of the callus regenerated plants. A total of 125 T_0 plants were transferred to the greenhouse and Southern blot analysis confirmed the integration of *Bd*COMT gene in 92 - 100% of the plants analyzed. These results suggest transformation efficiency between 10 – 74% for Palmar and between 15 – 56% for Nipponbare cultivar for different experiments.

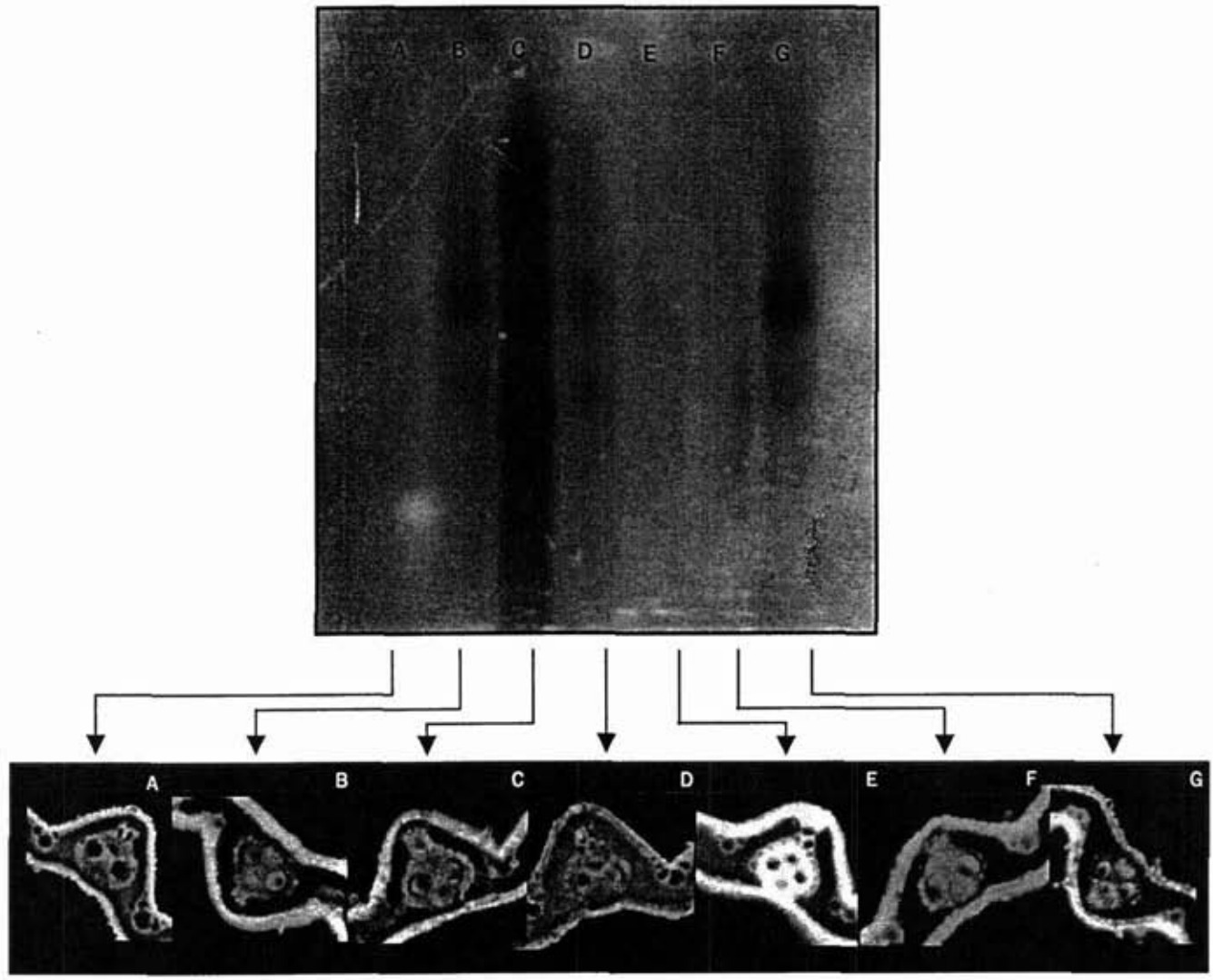
The *Bd*COMT expression was measured using histochemical analysis through Weisner and Maüle reactions. Weisner stain in known to react with cinnamaldehyde residues in lignin and the color intensity reflects the total lignin content. No difference was detected with this stain between control and transgenic plants, indicating no significant variation in lignin quantity. In contrast, the Maüle reaction, which is specific for free S units in lignin, revealed variations in staining intensity from pale yellow (inhibited expression) to red (over expresion). Some plants displayed a bright red color in their vascular tissue suggesting putative over expression of the *Bd*COMT gene in these plants. A good correlation between Northern and histochemical analysis was found. High RNA transcript levels were found in these over expressing plants, while no signal was detected in untransformed controls and a very weak signal or no signal was detected in the most plants showing pale yellow color (Figure 1).

Agronomic characterization of the transgenic plants indicated some variation between transgenic and non-transgenic controls, mainly in the transgenic lines from Nipponbare. High levels of sterility were noted in transgenic Nipponbare plants. This type of phenotypic variation was also noted on non-transgenic regenerated plants, which did not contain the transgenes as indicated by the Southern blots. Transposons and retrotransposons have been found to induce mutation during *in* vitro culture. In Nipponbare, three retrotransposons (*Tos10, Tos17* and *Tos19*) are activated during *in vitro* culture and inactivated during *in planta* growth (Hiroshima et al., 1996). Previous analyses of expression of other COMT genes in other plants have not found effect on plant growth and development in contrast to the O-methyltransferase gene CoA-3, which has been demonstrated to affect plant growth (Picon et al., 2001). Thus the phenotypic variation found in transgenic *Bd*COMT Nipponbare plants seems not to be associated with the presence/ expression of the transgene.

Future Plans

The quantification of the lignin content and composition in transgenic *Bd*COMT rice will be conducted in order to complement these analyses.

Figure 1. (1) Northern analyses, showing different levels of BdCOMT transcript. (2) Maüle Reaction. (A) Non-transgenic Palmar control; (B) Transgenic Palmar line 100; (C) Transgenic Palmar line 82; (D) Transgenic Nipponbare line 10; (E) Transgenic Nipponbare line 110 (inhibited expression); (F) Transgenic Palmar line 37; (G) Transgenic Palmar line 62 (over expression).



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2.2.4 Cassava propagation using low-cost *in vitro* propagation techniques and conservation of native varieties from southwestern Colombia

R.H. Escobar[1], L. Muñoz[1], C.M. Hernández[2], G. Ospina[3], J. Restrepo[3], J. Tohme[1] and W.M. Roca[4]
[1]SB-2- Project, CIAT; [2]Rural Community of Santa Ana, Cauca; [3]Fundación para la Investigación y Desarrollo Agrícola-FIDAR; [4]CIP-Perú

Introduction

Low-cost propagation methods could support decentralized seed systems. Today this project is in a position to distribute clean materials (certified FSD-free material) for small farmers to use in their next planting cycle (2004-A). Our materials could also be used for reestablishing cassava plantations in the Cauca area.

Materials and methods

Materials being grown by farmers were collected from Buenos Aires, Caldono, Morales and Santander de Quilichao (Cauca Province). A total of 27 materials were planted in two local seed banks (Pescador 1500 m alt. and Piendamó 1700 m alt.). Stakes were collected from each site and taken to CIAT HQ in Palmira to initiate meristem culture; and 19 morphological descriptors were evaluated (Fukuda and Guevara, 1998).

Younger leaves, to be used in the AFLP analysis, were collected from the CIAT greenhouse for DNA extraction by the Dellaporta method.

A total of 6061 plants from 6 clones (M Bra 383, CM 523-7, M Col 1522, HMC-1, CM 6740-7 and M Per 183) were evaluated for frog-skin disease (FSD) by ICA officials.

A new plot of cassava plant materials was introduced to the propagation scheme to reactivate a rural in vitro laboratory located in San Rafael.

Results and discussion

The materials collected were Algodona Gigante, Algodona Rápida, Chiroza, Verde, Algodona Grande, Varita, Pate Pava, Bajuna Pequeña, Correita, Independencia Mejorada, Bajuna Armenia, Totoqueña, Falsa Chiroza, Sata, Chiroza Roja, Blanquita, Cáscara Roja, Parroquiana, Amarilla, Algodona Pequeña, Bajuna, Bajuna Grande, Panameña, Sauce, Regional Morada, SM 5080-1 and Yuca Blanca.

Two seed banks were established using materials collected in Pescador (20 materials) and Piendamó (17 materials, some of them replanted). When the materials from Pescador were harvested, the farmers expressed interest in Totoqueña (4.1 kg avg), Algodona rápida (2.76 kg avg) and Algodona grande (2.6 kg avg) (Fig. 1-A). The average per total harvested area ($840m^2$) was 1.8 kg with a total production per area 1650 kg after 14 mo. Materials from Piendamó will be harvested in November.

Good DNA quality was obtained using the Dellaporta method (Fig. 1-B). Quantification and dilutions were initiated in order to start running the AFLP's. We expect to find diversity among the Algodona materials (farmers preferred these materials by its good starch quality). At present they are very interested in obtaining material from Rojita, but it has not been collected yet although some farmers are growing it.

A total of 614 meristems were placed in in vitro conditions and will be used as a source of explants for in vitro thermotherapy.

ICA functionaries certified the San Rafael plot as free of FSD-diseased plants. Those materials were planted last years as part of CBN-PRGA supported activities (Escobar et al., 2002). Some materials (HMC-1, CM 6740-7, M Bra 383, CM 523-7) have good

potential (Fig. 2A-B; Table 1). A rapid propagation scheme will be implemented using these materials as the initial source.

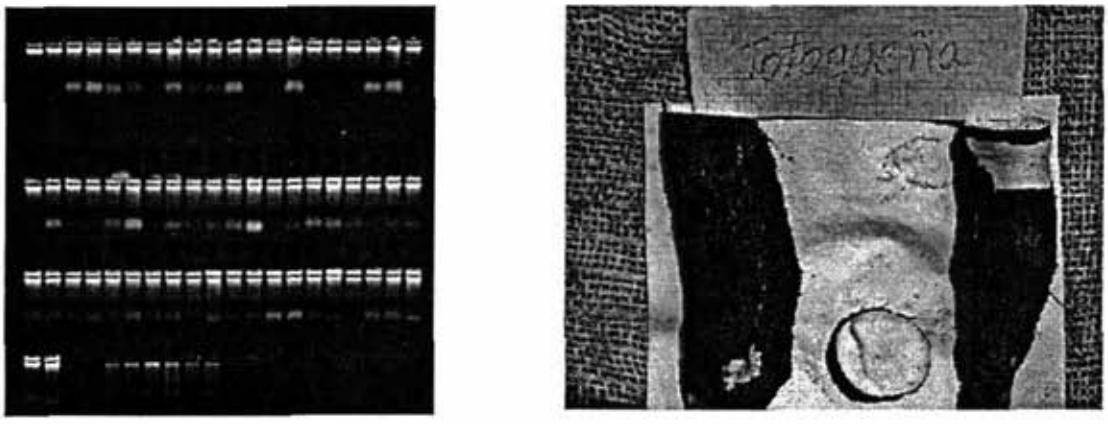

Figure 1. (A) Total DNA using Dellaporta method. (B) Totoqueña material with best average root production after 14 months.

Figure 2. (A) Panorama of plot with clean seed material coming from in vitro conditions located in San Rafael, Cauca. (B) Harvested material certified as free of FSD by ICA officials.

Table 1. Average production of 6 clones produced by farmers using in vitro, low-cost technology

Clone	% Commercial Roots	Average (kg/plant.)	SD	ANOVA Grouping
CM 523-7	52.2	3.67	0.20	a
MC 6740-7	54.6	4.15	0.07	a
HMC-1	67.4	4.57	0.93	a
M Bra 383	60.4	3.42	0.27	a
M Col 1522	25.2	1.25	0.65	b
M Per 183	65.7	4.4	0.049	a

Pr>F (<0.0001); R^2 =0.9256; CV= 12.07; average among clones=3,57 Kg/plant.

Five plants per accession (M Col 1468, M Col 1522, M Per 183, CG 402-11, HMC-1) were give to FIDAR by the GRU after Material Transfer Agreement signature; and 3 plants per accession were maintained by the SB-2 project to increase the number of plants. The remaining plants can be used for farmers' production. After 4-5 propagation cycles, a large part of the plants must be sent to the end-users to be incorporated into in vitro rural activities. A few plants per clone will be maintained as backups.

Conclusions

The use of *in vitro* materials could be used initially as part of a decentralized seed system. This would permit certifying the quality and quantity of material per cycle.

In vitro systems could be use for seed releases or renewing materials that are showing decreases in root production. Materials from San Rafael will give strong support to cassava systems in Cauca.

Future activities

- Running the AFLP fingerprinting.
- Implement two thermo therapy cycles and then material will be tested with Secundina (M Col 2063) for FSD certification
- Clean certified materials will be propagated for the rural laboratory. A second plot will be planted to establish a clean seed bank.
- Implement rapid propagation scheme using certified material planted in San Rafael.

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2.2.5 Implementation of the encapsulation-dehydration cryopreservation method for the cassava core collection

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[1]SB-2 Project, CIAT; [2]SB-1 Project, CIAT

Introduction

Cryopreservation techniques can be divided into classical and new. CIAT has developed alternatives for both kinds of techniques (Escobar et al., 1997, Escobar et al., 2000). New techniques based on encapsulation-dehydration, which are simple and rapid, would be useful for preserving large germplasm collections such as that of cassava. Not too many steps are involved as in the classic methods. Using these techniques with a wider number of clones will give a general idea about how safe this conservation method is in the long term with respect to repeatability and consistency after freezing.

Materials and methods

The encapsulation-dehydration method was implemented using materials from the in vitro bank. These materials were propagated using 4E medium (Roca, 1984) for the bud/node explants. When we tried scaling up, we considered increasing the number of beads per container (recycled baby food jars). Normally each jar with 50 g of silica gel holds 20 beads; we tested 40 beads per jar.

To determine if a clone is amenable to being cryopreserved, it has to form shoots from at least 30% of the beads after freezing. This is considered the threshold (Escobar et al., 2001).

Results and discussion

To date we have received 447 cassava clones, which are being propagated to increase them up to 100-150 plants/clone to produce enough new shoots (2-3 months old and without cuttings) to use in the cryopreservation process.

During the last 6 months we had a problem with dust mites infecting the production and lost 187 clones (42% of the material received). This attack made it necessary to increase propagation activities, reducing the number of clones put under cryopreservation. From the material received, we cryopreserved 348 clones (78% of material received, which corresponds to 55.2% of the core collection).

Of the total clones cryopreserved so far, 68% have surpassed the threshold value (Table 1). Of the 145 clones tested in 2002-2003, 43% reached the threshold (Table 2).

Table 1. Summary of response of 348 cassava clones cryopreserved from the core collection.

Group response*	No. of Clones	% Response
Highest (>70%)	91	26.15
Intermediate (30-70%)	146	41.95
Lowest (<30%)	111	31.90
No. of clones tested	348	

*Represented as shoot formation after freezing in liquid nitrogen

Table 2: Response of cassava clones cryopreserved during 2002-2003.

Group response*	No. of Clones	% Response
Highest (>70%)	16	11.03
Intermediate (30-70%)	46	31.72
Lowest (<30%)	83	57.25
No. of clones tested	145	

*Represented as shoot formation after freezing in liquid nitrogen

The 40 beads in the baby food jar did not dehydrate sufficiently or homogeneously; thus encapsulated shoots did not survive freezing. In contrast, when the 40 beads were placed in a petri dish, they reached similar humidity levels to the 20 beads in a baby food jar (Fig. 1). These results indicate that dehydration, as expected, depends on the number of beads, the type of container and the sucrose pretreatment. Ideally, larger containers should be used to handle more beads; i.e., glass petri dishes with a larger numbers of beads. However, the cost of a glass petri dish is 250 times higher than a baby food jar.

A routine was established to clean the growth room. It permits mite control by combining chemical treatments specific for adults and eggs. Every 15 days we also apply mint or eucalyptus oil scents on the floor as a preventive measure to keep mites away..

Future activities

- Recover clones lost by mite attack
- Continue freeze-testing using core collection
- Analysis of clone behavior and group definitions after freezing

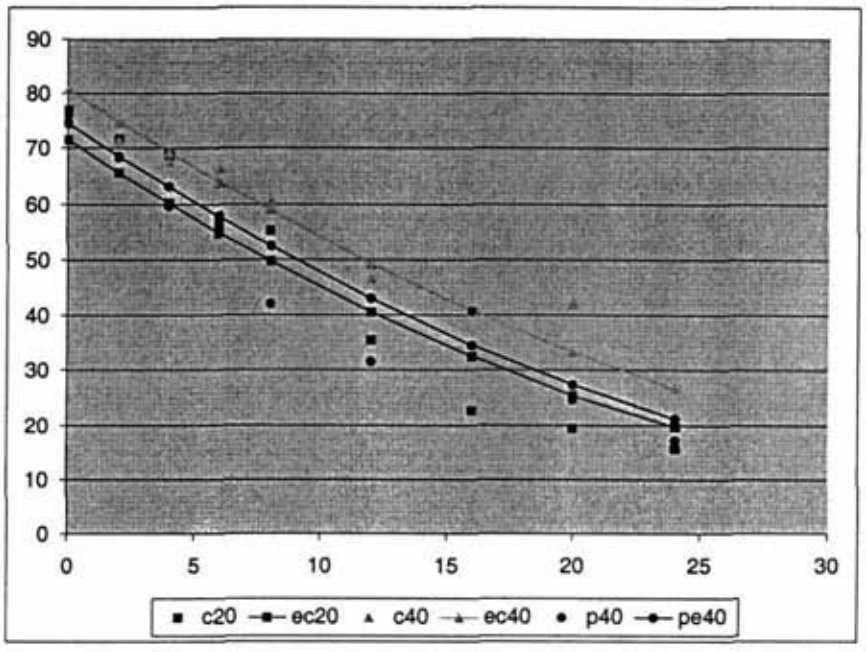

Figure 1. Behavior of beads treated in 0.75 M silica gel for 3 days across different dehydration times. Two types of container were tested: Baby food jars (c) and petri dishes (p)

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2.2.6 *In vitro* systems (conventional tissue culture and RITA®) to support CIAT research agenda

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SB-2 Project

Introduction

Last year the SB-2 project supported others projects using its tissue culture facilities to provide them some interesting materials (Escobar et al., 2002). The massive cassava propagation system using RITA® was developed by CIAT. This system has been validated with a wide range of cassava materials (Escobar et al., 2000), as well as with other crops (Escobar et al., 2002). Using this system makes it possible to increase propagation rates 6-11 times vs 3-4 times under solid conditions. A summary of this activity is presented here.

Material and methods

Conventional *in vitro* propagation using 4E medium was implemented; rooting was doing in a 17 N medium (Roca, 1984). Users were responsible for hardening and greenhouse management, following Roca et al. (1984).

The quality and quantity of new explants recovered by RITA depend on the type and amount of cytokinins used in the media. For cassava best results were observed after treatment with 0.5 mg/l TDZ; it release shoot tips from apical dominance and allow recover more than one shoot per explant. Combining RITA®-Solid-RITA® gave the best combinations and enabled recovery of plants without hyper hidrycity (Escobar et al., 2002).

Technical support was given to Corn Products Inc. (Malambo) with respect to the management of some in vitro problems associated with water quality in their laboratory.

Results

Six different plots were established (Table 1). As only a few plants were requested each time, the solid conditions could be used.

For the Cauca farmers this project could be considered a unique option for obtaining clean materials. As discussed in the article "Cassava propagation using low-cost in vitro propagation techniques and conservation of native varieties from southwestern Colombia" presented elsewhere in this report, material planted in San Rafael, Cauca, last year was certified by ICA functionaries as FSD-free and could be used as a seed source for the rapid propagation system.

Table 1. Material involved in small propagation set and their potential users.

Characteristic of Interest	No. Clones	User or Partner	Material Involved	No. of Plants
Commercial material for regional testing	7	IP-3	CM 523-7, M Col 1468, M Bra 383, CM 1438-2, M Col 3306-4, CM 2772-3, M Tai1 6	484
Wild material for *Bemisia tabaci* testing	5	PE-1	*M. aesculifolia flavelifolia* 444-002, *M. peruviana* 417-003, *M. peruviana* 417-005, M Nga 2, M Nga 11	67
Xanthomonas testing*	22	GDF-61	M Bra 685, M Bol 3, M Bra 12, M Bra 110, M Bra 383, M Bra 881, M Bra 900, M Col 1468, M Col 22, M Col 1522, M Col 2215, M Cub 51, M Mal 2, M Mal 48, M Mex 59, M Pan 51, M Tai 1, M Ven 25, M Col 1505, CM 2177-2, CM 3306-9, M Nga 1	762
FSD testing	1		M Col 2063	90
FSD testing	1	IP-3/ IP-1	M Col 2063	96
Material requested from Cauca farmers	6	ASOPROSA FIDAR	HMC-1, M Bra 383, CM 523-7, CM 6740-7, M Per 183, M Col 1522	600
Propagation and embryogenesis testing	1	SB-2	M Tai 16	1500

* For DNA extraction using *in vitro* tissues.

Whiteflies and FSD are the most limiting problems for cassava in the Cauca regions. In 2002, CORPOICA, MADR and CIAT released a clone with high resistance to the whitefly (*Aleurotrachelus socialis* Bondar) named Nataima-31 (CIAT code CG489-31) (Bonilla et al., 2002). This is a promising clone that could be used under Cauca conditions. Gustavo Jaramillo (pers. com.) has provided us with a list of some materials that he has observed with FSD tolerance. For that reason we included some clones with whitefly and FSD resistance in propagation schemes that could be used for future testing plot under farmers' conditions.

At present, we are running some materials with RITA® in order to cover the needs for whitefly and FSD materials (CG 489-31, SM 7951-5, M Per 183, HMC-1), commercial clones (CM 523-7, CM 6740-7) and some for Cauca farmers (M Col 1522, M Bra 383). For CM 6740 the propagation rate was 1:12.

Conclusions

In vitro facilities could play a critical role in producing clean material to make comparisons with conventional cuttings, releases of new breeding material, renewing materials, clone dissemination, pest and insect testing among others uses.

If CIAT projects are thinking of encouraging the use of these materials they must plan their agenda jointly, making propagation schemes, agreeing upon objectives and supply requirements. Coordination among projects is critical to meet everyone's research agenda.

The *in vitro* system could be used initially for whitefly and FSD management in Cauca by preliminary testing of this material on farm conditions. This scheme could also be implemented in the biofortification program for distributing, testing and promoting high carotene-content materials. Punctual requirements could be covered with RITA® because this system has the highest propagation rate in a short period time. The GRU usually provides 5 plants per clones after signing the Material Transfer Agreement.

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2.2.7 Embryo Rescue of Sexual Seeds from Breeding Populations for Molecular Assisted Selection (MAS) of CMD Resistance

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CIAT

Introduction

CMD breeding at CIAT aims to develop Latin America cassava gene pools adapted to the disease should in case it makes an accidental entry into the region. A second important objective is to facilitate germplasm shipment of CIAT's elite cassava germplasm to regions, such as India and Sub Saharan Africa where CMD is endemic, via the introgression of CMD resistance into CIAT's elite germplasm. To permit marker-assisted selection (MAS) of CMD resistance at CIAT for Latin America and at the same time fulfill plant quarantine conditions for the shipment of the CMD resistant CIAT germplasm to India and Africa, it is necessary to germinate and maintain in vitro breeding populations. This season more than 3000 controlled crosses were made between CMD resistant parents introduced from IITA and elite cassava parents or backcross derivatives of M. esculenta sub spp flabellifolia for resistance to the green mite. A total of 2315 seeds were harvested as mature seeds and have been germinated in vitro from embryo axes. A summary of results obtained from the germination of these seeds is presented below. Once germinated, the plantlets were multiplied, molecular-assisted selection (MAS) performed using the marker NS158 and another SCAR marker RME1, and CMD resistant genotypes will be transferred to the screen house for further hardening and evaluation as well as for shippment to collaborators in India and Africa.

Methodology

The culture of embryo axes from mature or immature seed has become routine in the establishment of cassava CMD breeding populations in vitro. The method being currently used is that developed at CIAT and modified by Okogbenin (2003), in summary, seeds are selected by a floatation test in water, seeds that float (indicating of vain or little developed seeds) are discarded, incubation in 97% sulfuric acid to 97% for 50 minutes, to allow for easy scarification, and washing with water to eliminate the acid. Following, the seeds are disinfected with 70% alcohol to for two minutes then with a solution of sodium hypoclorite (0.5%) and a drop of 20 tween for 12 minutes, finally they are rinsed three times with sterile deionized water. The embryo axes is then removed together with the cotyledon and placed on 17N media and left in the dark for 5 days at temperatures between 28 and 31°C. Next, the embryos are incubated at the same temperature under a photoperiod of 12h light and 12h darkness until they grow into full plants which are then multiplied in 4E media.

Table 1. Sexual seeds of cassava that were embryo rescued and multiplied In vitro in the year 2003.

Code	Mother	Father	Purpose	No. of seeds received	No. of seeds discarded	Total No.of seeds	No.of Plants recovered	% recovery of plants
CR-1	OW183-4	C-127	ALW-ACMD	28	0	28	5	18
CR-2	C-4	AM244-31	ACMD	6	0	6	5	83
CR-3	C-4	CG489-34	ACMD-MSB	56	1	55	21	38
CR-4	C-4	CW66-60	ACMD-ACR	11	0	11	3	27
CR-5	C-4	CW66-73	ACMD-ACR	12	2	10	1	10
CR-6	C-4	CW67-42	ACMD-ACR	18	2	16	9	56
CR-7A	C-4	MCOL 1734	ACMD-YRT	132	8	124	55	44
CR-7B	MCOL 1734	C-4	ACMD-YRT	46	3	43	15	35
CR-8A	C-4	MCOL 2206	ACMD-YRT	168	7	161	31	19
CR-8B	MCOL 2206	C-4	ACMD-YRT	24	6	18	7	39
CR-9A	C-4	MTAI 8	ACMD1	360	0	360	158	44
CR-9B	MTAI 8	C-4	ACMD1	38	6	32	20	63
CR-10A	C-127	MCOL 1734	ACMD-YRT	24	0	24	20	83
CR-10B	MCOL 1734	C-127	ACMD-YRT	37	1	36	27	75
CR-11A	C-127	MCOL 2206	ACMD-YRT	47	1	46	29	63
CR-11B	MCOL 2206	C-127	ACMD-YRT	23	4	19	15	79
CR-12	C-127	MTAI 8	ACMD-Z01	50	3	47	23	49
CR-13	MCOL 1734	C-33	YRT-ACMD	29	3	26	9	35
CR-14A	CM523-7	C-4	ACMD2	11	0	11	8	73
CR-14B	C-4	CM523-7	ACMD2	50	1	49	36	73
CR-15A	CM523-7	C-33	ACMD2	5	2	3	2	67
CR-15B	C-33	CM523-7	ACMD2	1	0	1	1	100
CR-16	CM523-7	C-39	ACMD2	13	1	12	2	17
CR-17	CM523-7	C-243	ACMD2	17	5	12	3	25
CR-18	CM3306-4	C-4	ACMD1	32	3	29	15	52
CR-19	CM3306-4	C-18	ACMD1	3	2	1	0	0
CR-20A	CM3306-4	C-33	ACMD1	12	1	11	6	55
CR-20B	C-33	CM3306-4	ACMD1	3	1	2	2	100
CR-21	CM3306-4	C-243	ACMD1	13	0	13	10	77
CR-22A	CM6754-8	C-33	ACMD1	2	1	1	1	100
CR-22B	C-33	CM6754-8	ACMD1	3	1	2	0	0
CR-23	CM7951-5	C-4	ACMD4	32	4	28	26	93
CR-24	CM7951-5	C-18	ACMD4	12	1	11	10	91
CR-25	CM7951-5	C-33	ACMD4	7	0	7	6	86
CR-26	CM7951-5	C-39	ACMD4	5	3	2	1	50
CR-27	CM7951-5	C-243	ACMD4	35	4	31	25	81
CR-28A	SM909-25	C-4	ACMD2	67	17	50	29	58
CR-28B	C-4	SM909-25	ACMD2	58	4	54	34	63
CR-29A	SM909-25	C-33	ACMD2	3	0	3	2	67
CR-29B	C-33	SM909-25	ACMD2	5	1	4	1	25
CR-30	SM909-25	C-39	ACMD2	20	7	13	9	69
CR-31	SM909-25	C-413	ACMD2	0	0	0	0	0
CR-32	SM1665-2	C-33	ACMD2	54	3	51	13	25
CR-33	SM1741-1	C-4	ACMD4	130	7	123	77	63
CR-34A	SM1741-1	C-18	ACMD4	24	8	16	14	88
CR-34B	C-18	SM1741-1	ACMD4	1	0	1	0	0
CR-35	SM1741-1	C-33	ACMD4	49	7	42	19	45
CR-36	SM1741-1	C-39	ACMD4	15	2	13	8	62

Table 1 (contd)

Code	Mother	Father	Purpose	No. of seeds received	No. of seeds discarded	Total No. of seeds	No. of plants recovered	% recovery of plants
CR-37	C-4	CM4574-7	ACMD2	15	0	15	12	80
CR-38	C-4	OW280-1	ACMD-PTN	31	2	29	27	93
CR-39	C-4	SM1219-9	ACMD2	6	0	6	4	67
CR-40	C-18	CM4574-7	ACMD2	3	1	2	0	0
CR-41	C-18	MCOL 2056	ACMD-YRT	12	0	12	12	100
CR-42	C-18	MCOL 2206	ACMD-YRT	32	5	27	15	56
CR-43	C-33	CM4574-7	ACMD2	28	4	24	15	63
CR-44	C-39	CM3306-4	ACMD1	15	1	14	11	79
CR-45	C-39	CM4574-7	ACMD2	25	3	22	15	68
CR-46	C-39	SM1219-9	ACMD2	5	0	5	4	80
CR-47	C-127	CW66-60	ACMD-ACR	2	0	2	2	100
CR-48	C-127	SM1741-1	ACMD4	3	1	2	1	50
CR-49	C-243	CM4574-7	ACMD2	9	2	7	6	86
CR-50	C-243	CW67-42	ACMD-ACR	5	1	4	3	75
CR-51	C-243	OW280-1	ACMD-PT	34	6	28	21	75
CR-52A	C-243	SM1219-9	ACMD2	94	10	84	43	51
CR-52B	SM1219-9	C-243	ACMD2	2	0	2	0	0*
CR-53	C-243	MCOL 2206	ACMD4	5	1	4	4	100
CR-54A	C-243	MTAI 8	ACMD1	9	0	9	7	78
CR-54B	MTAI 8	C-243	ACMD1	110	20	90	70	78
CR-55	MBRA 1A	C-18	ACMD-YRT	7	0	7	5	71
CR-56	MCOL 2056	C-127	ACMD-YRT	7	1	6	2	33
CR-57	MCOL 2206	C-18	ACMD-YRT	12	5	7	7	100
CR-58	MMAL 66	C-18	ACMD-YRT	11	7	4	4	100
CR-59	MTAI 2	C-18	ACMD-YRT	13	1	12	10	83
CR-60	MTAI 8	C-18	ACMD1	7	2	5	5	100
CR-61	MTAI 8	C-33	ACMD1	4	2	2	0	0*
CR-62	MTAI 8	C-39	ACMD1	18	6	12	10	83
TOTAL				2315	214	2101	1128	54

Results

A total of 2315 mature seeds of cassava originating from 62 F_1 families corresponding to breeding populations for resistance to CMD, denominated "CR" were received from the breeding section. Of this 214 seeds were rejected by the floatation test and 2101 seeds were germinated. Of this number a total of 1128 fully formed plants were recovered and multiplied, a little more 54% recovery. The low % recovery of plants, down from the normal 80% was due to a very severe attack of green mites in the growth room this year. Plants that are observed to be contaminated with green mites are immediately removed and destroyed. A concerted effort of weekly application of acaricide in the growth room, elimination of infected plants, and cleaning of individual tubes with 96% ethanol has been initiated to control the mite infestation. A number of other cassava tissue culture facilities at CIAT also had mite problems this year. The source of the mite problem is not known but it is thought they may have gained entrance via infested in vitro plants transferred from one facility to another.

One mature plant of each of the 1128 genotypes was sent to the cassava molecular marker lab for DNA extraction and marker analysis for MAS. Genotypes that have the CMD2 resistance gene after marker analysis are sent to the green house for hardening and further field evaluation and to partners in Africa and India. At the moment 633 genotypes resistant to ACMD are being transferred to greenhouse, a total off 125 have been established to far, and also being multiplied, at least 10 copies per shipment, to make a shipment to Nigeria, Tanzania and India. Also in the green house are 96 genotypes of 3 S_2 families developed under the S_2 recurrent selection for tolerance to inbreeding and 47 genotypes of inter-specific crosses established last year from immature sexual seeds and propagated in vitro this year. These immature seeds are from plants that had to be removed from the field due to the "zero-cassava" rule of no plants for one month on the experimental station at Palmira.

Other activities in the tissue culture section this year include Embryo rescue of 173 and 123 seeds respectively from 2 S_1 families (AM313 and AM320) of cassava for molecular mapping of cyanogenic potential in collaboration with the Swedish Agricultural University (SLU), Uppsala,

Conclusions

The establishment CMD resistance breeding populations at CIAT for molecular marker-assisted breeding of resistance has become routine. Although a severe attack of mites reduced considerable the % recovery of plants, more than 1000 plants have been processed this year, compared to about less than 500 last year. Future perspectives are to tackle the problems discovered this year with mite infestation in the growth rooms, as well as the current size of the growth room and raise this number to at least 5000 plants every year.

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2.2.8 Dissemination of Improved Cassava Varieties as Tissue Culture plantlets

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Introduction

CIAT cassava project has responsibility to make available improved germplasm to partners in the NARs in Asia, Latin America and Africa. The cassava tissue culture facility was therefore used to propagate clean materials of improved varieties for shipment to NARs in Latin America and Africa on request from partners. The facility has also been used to clean-up and transfer into *in vitro* field plants from the field that have were requested for by partners. An example is the shipment to Vietnam of 57 genotypes selected from a diallel experiment for further evaluation in Vietnam. We present a report of shipments of plants *in vitro* of cassava made to several countries from February to September of the 2003.

Methodology

In vitro plants of the improved varieties were received from the genetic resources unit (GRU) and multiplied using 4E media according to standard protocols established at CIAT (Roca and Mroginski 1991). The method utilized for clean-up and transfer of field plants to tissue culture is that routinely used at CIAT with some modifications, in summary: stems with apices and nodes are obtained from plants in the field or green house, the leaves are removed leaving approximately 5mm of petiole. The stem cuttings are washed with sufficient tap water and cut into nodes and apices, with one node per piece. In the flow hood, the fragments are placed into a 250ml sterile flask and washed with 70% ethanol for 30 seconds with vigorous shaking. The ethanol is discarded and the washed again with 10% sodium hypochlorite (0.5% v/v sodium hypochlorite) and one drop of liquid soap for 5 minutes, with vigorous shaking. The hypochlorite solution is discarded and the cuttings washed 3X with sterile double distilled water. The cuttings are then transferred to a sterile petri dish and, using a sharp sterile scapel, the extremes that have been in contact with the solutions are eliminated. The stem cuttings are then planted in 4E medium and placed in a growth room at 28oC with photo-period of 12h light and 12h hours darkness to develop into full plants.

Table 1. Summary of shipments of in vitro plants of cassava (*Manihot esculenta* Crantz) made to several countries from February to September 2003.

		COUNTRY/ DATE OF SHIPMENT						
	GENOTYPE	HAITI	PERU	CUBA	NIGERIA	NICARAGUA	INDIA	Rep. DOMINICANA
		May 6/03	May 7/03	May 23/03	Jun 11/03	Jul 18/03	Sep 2/03	Sep 10/03
		# Plants	# Plants	# Plants	# Plants	# Plants	# Plants	# Plants
1	BRA 383	30	30	5	-	30	10	20
2	CM 1335-4	-	-	-	8	-	10	-
3	CM 2772-3	15	5	5	8	30	10	20
4	CM 3306-4	30	30	5	-	30	10	20
5	CM 3750-5	-	-	-	-	-	10	-
6	CM 4574-7	30	30	5	-	30	10	20
7	CM 4843-1	30	30	5	8	30	10	20
8	CM 489-1	-	-	-	-	-	10	-
9	CM 4919-1	30	30	5	8	30	10	20
10	CM 507-37	30	30	5	8	30	10	20
11	CM 523-7	30	30	5	-	30	10	20
12	CM 5306-8	30	30	5	8	30	10	20
13	CM 6119-5	30	8	5	8	30	10	20
14	CM 6438-14	30	30	5	8	30	10	20
15	CM 6740-7	30	30	5	8	30	10	20
16	CM 6754-8	-	-	-	-	-	10	-
17	CM 6921-3	30	30	5	8	30	10	20
18	CM 7033-3	30	18	5	8	30	10	20
19	CM 7073-7	30	30	5	8	30	10	20
20	CM 7514-8	30	30	5	8	30	10	20
21	CM 7951-5	30	30	5	8	30	10	20
22	CM 8027-3	30	30	5	8	30	10	20
23	HMC-1	-	-	-	-	-	10	-
24	MCOL 1468	-	-	-	6	-	10	-
25	MCOL 1684	-	-	-	-	-	10	-
26	MCOL 2063 (Secundina)	-	-	-	-	-		5
27	MCOL 1734	-	-	-	-	-	10	-
28	MCOL 2215	-	-	-	8	-	10	-
29	PER 183	30	30	5	-	30	10	20
30	SM 1411-5	-	-	-	8	-	10	-
31	SM 1433-4	30	30	5	8	30	10	20
32	SM 1460-1	30	30	5	8	30	10	20
33	SM 1565-15	30	30	5	8	30	10	20
34	SM 1741-1	-	-	-	8	-	10	-
35	SM 1821-7	30	20	5	8	30	10	20
36	SM 2019-9	30	5	-	-	-	-	-
37	SM 805-15	30	30	5	-	30	10	20
38	SM 909-25	30	30	5	8	30	10	20
39	TAI-8	30	30	5	-	30	10	20
	TOTAL	**795**	**716**	**130**	**190**	**780**	**370**	**525**

Table 2. List of plants rescued from the greenhouse and placed in vitro for shipment to Vietnam (Plants for Ms Cach)

Genotype	Genotype	Genotype	Genotype	Genotype	Genotype
CM 9106-18	CM 9923-30	CM 9954-18	GM 237-22	GM 255-2	GM 281-24
CM 9106-7	CM 9926-17	CM 9954-23	GM 238-29	GM 258-2	GM 281-28
CM 9148-2	CM 9945-22	CM 9957-1	GM 246-25	GM 258-3	GM 287-10
CM 9148-3	CM 9945-27	CM 9957-21	GM 246-3	GM 259-3	GM 287-13
CM 9703-17	CM 9946-11	CM 9958-1	GM 247-27	GM 266-24	GM 289-9
CM 9703-24	CM 9946-12	CM 9958-6	GM 248-26	GM 272-1	GM 291-11
CM 9907-1	CM 9949-1	CM 9966-27	GM 250-24	GM 272-4	GM 291-7
CM 9907-3	CM 9949-25	GM 236-26	GM 250-29	GM 273-13	
CM 9921-25	CM 9952-1	GM 236-7	GM 251-12	GM 280-15	
CM 9923-1	CM 9952-19	GM 237-13	GM 251-9	GM 280-24	

Results

A total of 3506 plants from a list of 39 improved genotypes were shipped to collaborators in Haiti, Peru, Cuba, Nigeria, Nicaragua, India and the Dominican Republic this year (Table 1). Aside from this, 5 plants of 44 genotypes were also shipped to Crop Research Institute (CRI), Kumasi Ghana, for the Ph.D. study of Ms Elizabeth Okai who is looking for heterotic patterns between germplasm from Africa and Latin American based on SSR marker clustering. Ten plants each of a selection of 57 genotypes from a diallel study was also shipped to Vietnam for the Ph.D. study of Ms Cach who was at CIAT in 2002 for 6 months analyzing the same diallel experiment (Table 2). Stakes from the 57 genotypes in the field were planted in the sreen house, disinfected and nodal cutting established *in vitro* in 4E media.

Conclusions

The cassava tissue culture facility is being used to share valuable germplasm with collaborators all over the world within a reasonable period of time, germplasm is shipped within 2 months from when the request is received, smaller requests of 5-10 plants per genotype take a month while larger ones take up to 2 months. Future perspectives include an enlargement of thee growth room to allow for the simultaneous processing of several shipment requests.

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2.2.9 A Simple Method for the Rapid Multiplication of Clean Cassava Planting Material

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Introduction

Good quality and healthy planting material is crucial for high yields in cassava. Cuttings obtained from diseased and/or pest infested plants can reduce yield by 30% to 80% (Guritno1985). Another problem with unhealthy planting material is sprouting. The ability of stakes to sprout is closely related to their starch content at planting and growth during the first 20 days after plating is exclusively at the expense of the nutritive reserve previously accumulated in the stakes (Molina et. al. 1995). Starch content of stakes is reduced with poor soil fertility and disease/pest attack. A 20% drop in sprouting and a yield loss of 10% due to the use of poor quality planting material in a 2000ha farm translates to a loss of 8,400 metric ton of fresh cassava roots, at the national average yield of 14t/hectare. At a conservative estimate of US$50/ton this is a loss of US$0.42 million. Such a loss quickly wipes out the profit margin and puts the entire venture at risk.

As part of its assistance package to the Nigerian commercial cassava production company, the Nigeria Starch Mills (NSM), CIAT and CLAYUCA agreed to provide technical know-how on rapid multiplication of clean planting material. In Nigeria, commercially available cuttings are often taken from plants that are diseased, damaged by insect and pests and inadequately fertilized with a consequent reduction in yield (Okeke 1994, Ospina and Fregene, Personal observation 2003).

Tissue culture of cassava meristematic tissue has been successfully applied to obtain *in vitro* cassava plants that are disease- and pest-free (Roca et al. 1991). The technology also permits the mass production of *in vitro* plants compared to traditional multiplication methods. A multiplication ratio of 100 is possible using new efficient multiplication systems, such as the automated temporary immersion systems (ATIS) or "bioreactors". Apart from the production of healthy planting materials, rapid tissue culture multiplication can be used to mass-produce and deploy a new promising variety over a short period. However, the initial high start-up costs of the automated temporary immersion system and support tissue culture facilities increases the costs of establishing a commercial farming operation and diverts funds from other much need activities, making it less attractive compared to other multiplication schemes.

To reduce costs, a cheaper tissue-culture based rapid multiplication scheme that combines an initial step of tissue culture multiplication, hardening, 4 months of field growth, then a 2-node multiplication scheme in a special nursery, as practiced at CTCRI, Trivandrum, India, followed by another cycle of field growth and multiplication in the special nursery was proposed to NSM. The rapid multiplication scheme is expected to produce 2-3 million plants by July 2004, with enough cuttings to plant a 200ha "seed bank" to be used as a source for good quality healthy planting material. The scheme can also become a commercial source of planting material for sale to other large scale multiplication projects, for example the pre-emptive management of the cassava mosaic disease (CMD) projects that is being initiated in Nigeria by the Government of Nigeria, NDDC, some oil companies, and USAID in collaboration with IITA, Ibadan..

Specific objectives were:

Tissue culture rapid multiplication of 44 improved IITA varieties to produce at least 200 plantlets per genotype
Hardening of the plants in the green house and transfer to the field
Harvest of 2-node cuttings at 4 months after planting from the field and transfer to the rapid multiplication nursery; transfer of plants from the nursery to the field after one month.
Harvest of 2 node cuttings from the new plants at 4 months after planting and transfer to nursery; transfer of plants from the nursery to the field after one month

Methodology

Five stakes from 44 IITA improved cassava genotypes were obtained from Alfred Dixon, IITA, Ibadan, for tissue culture multiplication. A list of the improved lines is shown in Table 1. A request to multiply the materials at the tissue culture facility of the University of Legon, Accra was granted and multiplication began first week of July. The five stakes were planted in plastic bags and after 2 weeks, meristematic or nodal cuttings was harvested, cleaned with sodium hypochlorite and cultured in 1/2 MS media supplemented with BAP and GA (4E media). After 4, 8, and 12 weeks after planting the plantlets were subcloned to obtain the target of 200-250 plants per genotype.

Hardening of the plants will be in the NSM screen house (65% shade), currently under construction, using a soil mixture of 3 parts sand and 1 part top-soil in black plastic bags. Tissue culture plants received will be gently removed from the glass tubes and placed in the plastic bags with soil, a fungicide will be applied to control fungus growth and plants fertilized with a commercial fertilizer rich in phosphorus. A stryofoam cup with holes will be used to provide the plantlets with high humidity. After one month in the screen house, the plants will be transferred to the field and watered regularly. About 500kg of NPK 15:15:15 fertilizer will be added in split applications at 1 and 2 month after transplanting to the field in bands.

After 4 months of growth, 2-node cuttings will be obtained from each plant using a sharp knife and transferred to the special covered nursery. The plants will be watered regularly and at 1 month after planting they will be transferred to the field. The special nursery is simply a canopy of a mesh that allows in only 65% of light while excluding the rest over a nursery bed. The shade of the canopy, combined with constant watering provides an area of high humidity adequate for the fast growth of cassava. A picture of the special nursery is shown in Figure 1. During this period, special care is taken to rouge out diseased plants and to keep the area free of pests by spraying appropriate pesticides.

After the plants multiplied in the special nursery have grown, another round of multiplication is carried out as described above, this is the final round of multiplication. The plants are then allowed to grow until maturity in the field and used as a seed bank of planting material for storage root production

Results

Ten to 20 plantlets of 44 improved varieties from IITA were established from nodal cuttings obtained from 2 week old potted plants in tissue culture (4E media) at the University of Legon. After 4 weeks, the plantlets were subcloned to obtain a 3 to 5 time multiplication of the original plantlets. Two further rounds of multiplication were conducted at 8 and twelve weeks to give between 200 and 250 plants per genotype. The plants are ready for green house hardening and are awaiting the completion of the NSM green house facility under construction at Ihiala, Nigeria. In the mean time an import permit to bring the plants from Ghana into Nigeria has been applied for via the Nigerian Plant Quarantine office and a phyto-sanitary certificate has been requested from the Ghanian authorities to ship the plants to Nigeria.

Table 1. List of 44 improved new cassava varieties that have been multiplied by tissue culture

Genotype	disease	Cassava mosaic blight	Bacterial mealiness	Root 100g fresh root	CNP (mg HCN/ Matter(%)	Root dry yield (t/ha)	Fresh root color	Flesh
1.	92/0057	Resistant	Resistant	Mealy	Medium	30	25-30	White
2.	92B/00068	Resistant	Resistant	Mealy	Medium	32	30-35	White
3.	92/0326	Resistant	Resistant	Mealy	Low	30	30-35	White
4.	93/0098	Resistant	Resistant	Mealy	Medium	30	32-35	White
5.	92/0325	Resistant	Resistant	Mealy	Low	35	20-25	White
6.	97/0162	Resistant	Resistant	Mealy	Low	30	30-35	White
7.	97/4769	Resistant	Resistant	Mealy	Low	30	30-35	White
8.	M98/0028	Resistant	Resistant	Mealy	Medium	30	30-35	White
9.	98/0505	Resistant	Resistant	Mealy	Medium	35	30-40	White
10.	98/0510	Resistant	Resistant	Mod. mealy	Medium	35	40-45	White
11.	99/1590	Resistant	Resistant	Mod. mealy	Low	35	25-32	White
12.	99/6012	Resistant	Resistant	Mod. mealy	Low	35	35-40	White
13.	M98/0040	Resistant	Resistant	Mod. mealy	Low	32	40-45	White
14.	91/02324	Resistant	Resistant	Non-mealy	Medium	35	35-45	White
15.	92/0067	Resistant	Resistant	Non-mealy	Medium	30	25-30	White
16.	92B/00061	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
17.	94/0561	Resistant	Resistant	Non-mealy	Medium	30	30-35	Yellow
18.	94/0026	Resistant	Resistant	Non-mealy	Medium	32	30-35	White
19.	94/0039	Resistant	Resistant	Non-mealy	Medium	32	30-40	White
20.	95/0166	Resistant	Resistant	Non-mealy	Medium	30	35-40	White
21.	95/0379	Resistant	Resistant	Non-mealy	Medium	30	30-35	Yellow
22.	95/0289	Resistant	Resistant	Non-mealy	Medium	32	30-35	White
23.	96/1565	Resistant	Resistant	Non-mealy	Medium	30	35-40	White
24.	96/1089A	Resistant	Resistant	Non-mealy	Medium	32	30-35	White
25.	96/0603	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
26.	96/1642	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
27.	97/3200	Resistant	Resistant	Non-mealy	Medium	32	35-40	White
28.	97/2205	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
29.	97/4763	Resistant	Resistant	Non-mealy	Medium	32	35-40	White
30.	98/2226	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
31.	98/0002	Resistant	Resistant	Non-mealy	Low	35	40-45	White
32.	97/0211	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
33.	96/1569	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
34.	96/1632	Resistant	Resistant	Non-mealy	Medium	40	35-45	White
35.	97/4779	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
36.	Z97/0207	Resistant	Resistant	Non-mealy	Low	30	35-40	White
37.	98/0581	Resistant	Resistant	Non-mealy	Medium	30	30-32	White
38.	98/2101	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
39.	M98/0068	Resistant	Resistant	Non-mealy	Medium	35	40-45	White
40.	99/1903	Resistant	Resistant	Non-mealy	Medium	39	40-45	White
41.	99/2123	Resistant	Resistant	Non-mealy	Low	35	30-32	White
42.	99/3073	Resistant	Resistant	Non-mealy	Medium	30	30-35	White
43.	96/0523	Resistant	Resistant	Non-mealy	Medium	30	35-40	White
44.	96/1317	Resistant	Resistant	Non-mealy	Medium	32	30-35	Yellow

Conclusions

A rapid multiplication of clean improved planting material has been embarked upon for the Nigeria Starch Mill (NSM), if successful it could be a very rapid method for the production of clean planting material for both the medium and large scale cassava production sector in Nigeria as well as small scale rural farming. Although the scheme is still in progress, expected outputs is a 400X multiplication within a single growing season compared to the

traditional 10X multiplication or 100X via tissue culture alone or 200X via 200X via 2-node cutting alone.

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2.2.10 Temporary Immersion System (RITA) for Anther Culture of Rice

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Introduction

Plant *in vitro* culture using temporary immersion (RITA) offers all the advantages of a liquid medium system (automation, large scale production, easy changes of medium, filter sterilization, easy cleaning) without any of its drawbacks (reduce gas exchange, vitrification). Immersion time, i.e. duration or frequency, is the most decisive parameter for system efficiency. The optimization of the nutrient medium volume and the container volume also substantially improves efficacy, especially for shoot proliferation. Several reports confirmed large gains in efficacy from temporary immersion when using liquid medium for micro propagation. The main parameters involved reducing production costs are, firstly the drastic reduction of work labor, followed by a reduction in shelving area requirement and in the number of containers used. Scaling up the use of temporary immersions for embryogenesis and shoot proliferation procedures are currently taking place in order to commercialize this process (Berthouly & Etienne, 2002). This system has proved its efficacy for somatic embryogenesis of banana (Alvard et al, 1993; Escalant et al, 1994), coffee (Berthouly et al, 1995; Etienne et al, 1997), citrus (Cabasson et al, 1997), oil palm and rubber plant (Etienne et al, 1997), and at CIAT for cassava (Escobar and Roca, 1999). High efficiency has also been demonstrated for clonal propagation through micro-

cuttings of coffee, and sugar cane (Lorenzo et al, 1998); for proliferation of meristems of banana, and pineapple, and for micro-tuberization of potato (Teisson and Alvarad, 1998). We have previously reported preliminary results using RITA for the induction of embryogenic callus derived rice from mature zygotic embryos (Tabares et al., CIAT SB2 Report 2000) and from anther culture (Tabares et al., CIAT SB2 Report 2001). This year we report a comparative analysis including various indica and japonica rice genotypes.

Materials and methods

Anther culture of the indica rice Cica 8, PN1, Cimarron, Fedearroz 2000, and CT 11275, and of the japonica breeding line CT 6241-17-1-5-1 were used. Donor plants were grown in the field, panicles harvested, and anthers cultured according to Lentini et al. (1995). Tissues were either culture in liquid medium contained in RITA vessels or in liquid medium in baby food jars (control). Induced callus from each culture system was then transfer onto solid plant regeneration medium according to Lentini et al. (1995).

The effect of different culture media was evaluated. The medium NL commonly used in the rice anther culture laboratory (Lentini et al, 1995) was used as control. This medium was supplemented with 2,4-D 2mg/L; picloram 0.07mg/L; kinetin 1mg/L; maltose 8%; with or without silver nitrate10mg/L. A modified medium was evaluated consisting of NL basal medium but replacing picloram with phenyl acetic acid (PAA) 10 mg/L, with or without silver nitrate (medium M_1). The rest of the culture procedure, including plant regeneration, was according to Lentini et al. (1995).

The optimal immersion frequency was determined by evaluating callus induction and embryogenesis at 3 different immersion frequencies. Treatments were conducted using immersions of 1 min every 4, 6, or 8 hr for a total of 4 weeks of culture. Three RITA vessels were used per genotype with 1,000 anthers per 200 ml medium; and 4 baby food jars (permanent immersion system, PIS) per genotype each with 250 anthers per 10 ml culture medium (control). Cultures were incubated at 24 a 26°C.

Three different treatments were tested to increase plant regeneration. A water stress treatment was induced on callus prior culture by incubation on 1% agarose-containing medium in the dark at 27°C to dehydrate callus. After two weeks of culture, stressed callus from 1% agarose-containing medium were transferred to 0.4% agarose solid medium for regeneration and incubated in light. Another set of callus was not treated with water stress, and was cultured directly on the medium semi-solidified with 0.4% agarose. The effect of osmotic stress was evaluated by sub-cultured on medium containing 3% sorbitol for 24 hr, after this partial desiccation treatment the callus were transferred on regular plant regeneration medium. Control consisted of callus transferred from callus induction medium without treatment to regular MS regeneration medium. A factorial experimental completely randomized design was used. At least 10 replicates of 10 callus each was evaluated per treatment.

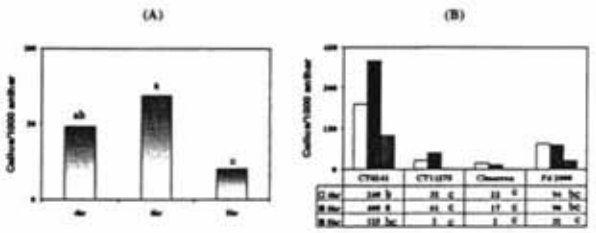
Results and Discussion

A significant higher callus induction was obtained when immersion was conducted every 6 hr than every 4 hr and 8 hr independently of the genotype (Figure 1A). The *japonica* line CT 6241 showed about 7 fold more callus respect to the *indica* varieties (Figure 1B). The maximum callus induction was noted at 40 days after culture on PIS and at 50 days after culture on RITA. But it seems the slower process of callus induction in RITA allows an optimal induction of embryogenesis. A significant higher number of embryogenic callus (95%) were obtained for both *indica* and *japonica* genotypes respect to the permanent immersion system (PIS, 45%) (Figure 2).

There was an interaction between the callus induction medium and the culture vessel used. No significant differences were seen between the different media on RITA, although there was a tendency of higher induction when using media M_1 with or without silver nitrate. However with PIS, the indica genotypes with intermediate to low response such as CT11275, Cimarron and Cica 8 showed significant higher induction on M_1 medium with or without silver nitrate, but Fedearroz 2000 optimal induction was noted on NL medium with silver nitrate. It has been reported that PAA mode of action is similar to that of IAA, although higher levels of PAA are needed and it is more stable in culture inducing a larger number of organized structures. It seems PAA effect is related to the inhibition of ethylene production from the cultured tissues (Ziauddin et al., 1992). Silver nitrate has also been reported to inhibit the action of ethylene of tissues culture *in vitro* (Lentini et al., 1995). The beneficial effects of replacing sucrose by maltose increasing the callus induction from rice anther of recalcitrant genotypes has also been associated with a reduction of ethylene effects. These results jointly with the effects noted when using PAA and RITA in this work suggest that ethylene might be a critical factor determining the induction of androgenesis from microspores in rice.

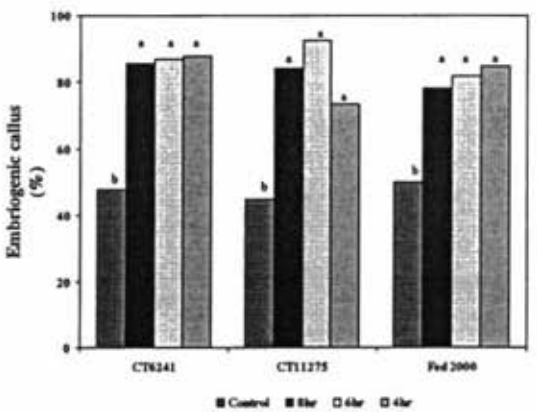
Plant regeneration from embryogenic callus was not affected by the composition of the callus induction medium nor the callus induction culture vessel, indicating that the regeneration capacity depends on the level of embryogenesis. Once embryogenesis is obtained the capacity for plant differentiation is similar. Thus, the optimal callus induction medium and culture vessel should be selected based on the larger number of embryogenic callus produced per anther cultured.

Significant higher green plant regeneration was obtained when water stress treatment was applied using agarose 1% for 1 week followed by agarose 0.4% for the rest of the culture period (Figure 3). Twice as many green plants were obtained with this treatment respect to the control. Osmotic stress with sorbitol inhibited plant regeneration (Figure 2). Independently of the callus induction or plant regeneration treatments, about 50-60% of the green plants were doubled haploids, which is in the range previously reported (Lentini et al., 1995).



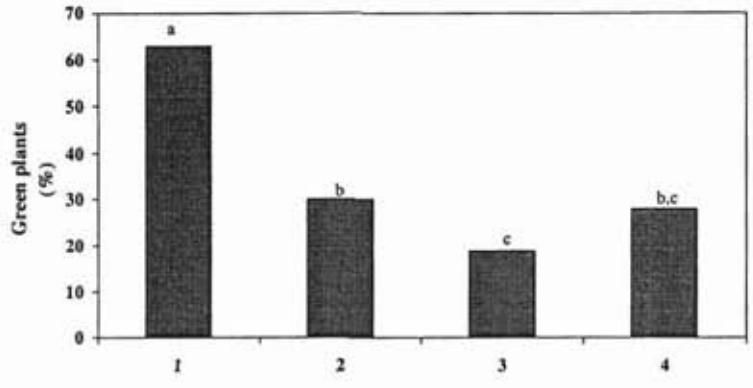
Different letter denote statistical significance (P < 0.05). Duncan's Multiple Range Test

Figure 1.Callus induction in RITA system using 4 hr, 6 hr, and 8 hr temporary immersion frequencies. (A) Mean values of recalcitrant indica varieties. (B) Comparison between different genotypes. Values refer to the mean number of callus induced per 1,000 cultured anthers.



Different letter denote statistical significance (P < 0.05). Ryan-Einot-Gabriel-Welsch Multiple Range Test

Figure 2. Percentage of callus embryogenesis on permanent immersion system (PIS) and RITA at different immersion frequencies (8 hr, 6 hr, and 4 hr, respectively).



Different letter denote statistical significance ($P < 0.05$). Ryan- Einot-Gabriel-Welsch Multiple Range Test

Figure 3. Percentage of green plant regeneration on medium containing (1) agarose 1% for 1 week and then agarose 0.4%; (2) agarose 0.4%; (3) sorbitol; and (4) control

Future activities

- To study systematically different factors affecting the emission and action of ethylene on *in vitro* culture
- To test different PAA concentrations and its interaction with maltose level for optimizing androgenesis in rice
- To evaluate modifications of culture vessel allowing aeration and/or temporary immersion for reducing current cost for the implementation of the RITA system

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Activity 2.3 Identification of points of genetic intervention and mechanism of plant stress interation

2.3.1 Exploring the genetic potential to improve micronutrient content of cassava

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[1]SB-2 Project ; [2]IP-3 Project , [3]Universidad del Valle

Introduction

The overall objective of this project is to improve the nutritional status of people living in marginal environments of the tropics, by selecting and promoting cassava genotypes with high and good bio-availability of micronutrients and vitamins. Related traits are the need for a better understanding of the biochemical and genetic basis of post-harvest physiological deterioration (PPD).

Early work was conducted to demonstrate the existence of genetic variability in cassava for carotene content in roots and foliage and to measure it. Carotene contents in the roots ranged from 0.102 to 1.040 mg/100 g fresh tissue (FT), whereas in the foliage the figures were much larger: 12.05 to 96.42 mg/100 g FT (CIAT 1999; 2000; 2001). PPD is apparently reduced or delayed in roots with high carotene content. Correlations between carotene content in the roots and several agronomic and nutritional traits were estimated, with not relevant information except for the effect on PPD.

The current research focus shifted towards better understanding the nature of the carotenes accumulated in cassava roots and its stability after common processing and storage. This report summarizes relevant information with the introduction of a new methodology (HPLC) for measuring carotene content in cassava roots more precisely.

2.3.2 Stability of carotene content after alternative storage methodologies for overcoming the problem of Post-Harvest Physiological Deterioration (PPD)

Introduction

The short post-harvest storage life of cassava is a characteristic that limits the marketability of the root and requires either consumption or processing shortly after harvesting. Post-

harvest physiological deterioration (PPD) of cassava roots begins within 24 hours of harvest, and results in crop and product quality losses, high marketing margins and risks, and restricted management flexibility for farmers, traders and processors. The reduction of PPD has been identified as a priority target for strategic research. It would also benefit processors in Brazil, who face price discounts for samples processed even 48 hours after harvest, due to grayer and lower quality starch than that derived from fresher roots.

In many respects, PPD resembles wound responses found in other better studied plant systems but cassava appears to lack the wound healing capacity which is normally associated with the inhibition of wounding responses. Normally, such defensive wound responses are inhibited by wound repair. However, this repair process does not occur in the harvested cassava storage root, leading to the hypothesis that unrestrained cascades of wound responses ultimately result in deterioration (Oirschot, Q., 2000). An important component of these wound responses are the oxidative processes. Ascorbic acid and carotene are known to have antioxidant properties and seem to delay the onset of PPD (CIAT, 1999; 2000; 2001).

Because of the rapid deterioration of cassava roots, they cannot be stored until they can be analyzed for carotene content. Therefore, only a few samples (< 40) can be analyzed in one day, with the available facilities at CIAT. A study was conducted to analyze if carotene contents change after different storage conditions for varying periods of time. Two different storage conditions were evaluated: storage at –80 °C and at – 20 °C.

Results

Storage at –80 °C.. Stability of carotene content was measured on root samples from the clon MCOL 2508 by the colorimetric and HPLC methods on fresh roots. This information was used as a check for data obtained after different periods of storage at – 80 °C. Samples of roots were stored for 22, 73, 84, 96, 126, 210 and 230 days and then, carotene contents evaluated using the same measuring methods.

As it was usually the case, total carotene content measured by HPLC was slightly higher than when measured by the colorimetric method (1.07 vs. 0.88 mg / 100 g fresh tissue). Samples of roots analyzed by the colorimetric method averaged 0.88 mg across the different dates (the same value as the original samples) with a standard deviation of 0.038. Values ranged from 0.81 to 0.92 mg / 100 g fresh tissue. Measurements based on HPLC showed larger variation. The average content was 1.01 mg / 100 g fresh tissue, with a standard deviation of 0.156. Individual measurements ranged from 0.81 to 1.26 mg. The larger error associated with HPLC coincides with the findings reported in section 3.1. There was no clear trend suggesting that carotenes measurements will decrease or increase as a consequence of storage. Therefore, it is valid to conclude that root samples can be stored for as long as seven months until carotenes are finally measured. The logistic advantages of this conclusion are very important for different projects involving cassava and carotenoid compounds.

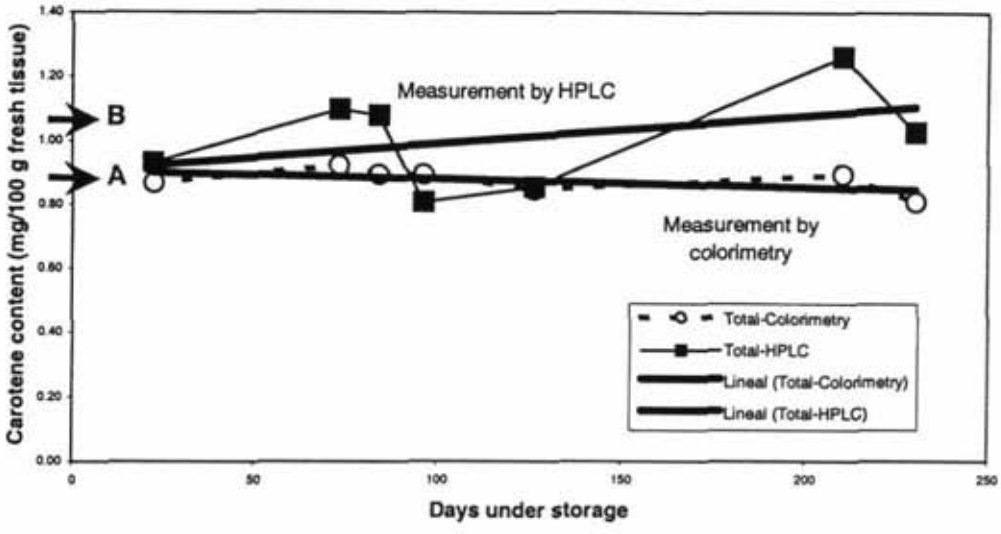

Figure 5. Stability of carotene content measured on root samples from the clon MCOL 2508. Original levels of carotenes were measured by the colorimetric (A) and HPLC (B) methods on fresh roots. Samples of roots stored for 22 to 230 days at – 80 °C were taken and carotene contents evaluated using the same measuring methods.

Storage at –20 °C.

In a separate experiment roots from 20 different clones were analyzed by colorimetry soon after harvest and then stored for six months at –20 °C. The root samples were then recovered and analyzed again for carotene content. Figure 6 illustrates the results from this experiment.

During the storage period the roots were maintained at – 20 °C in open petri dishes. There was a differential dehydration process of root samples from different clones, depending on their position in the storage container. This may explain the obvious lack of correlation between non-stored and stored roots samples of some clones (MCOL2435, MCOL2508, MCOL 2266, AM 273-7 and AM 273-23).

Similar results were obtained when carotenes were evaluated by the HPLC method (Figure 7). In this case, the lack of association between data from non-stored and stored root samples was found in clones MCOL 2580, MCOL 2410, MCOL 1468, and MCOL 2596.

Because of the errors associated after storing roots at - 20 °C using both the colorimetric and the HPLC methods the potential of maintaining root samples at that temperature needs further confirmation under closed containers. The next evaluations should consider the potential of liophylization as well. Until then, roots samples should be maintained at –80 °C.

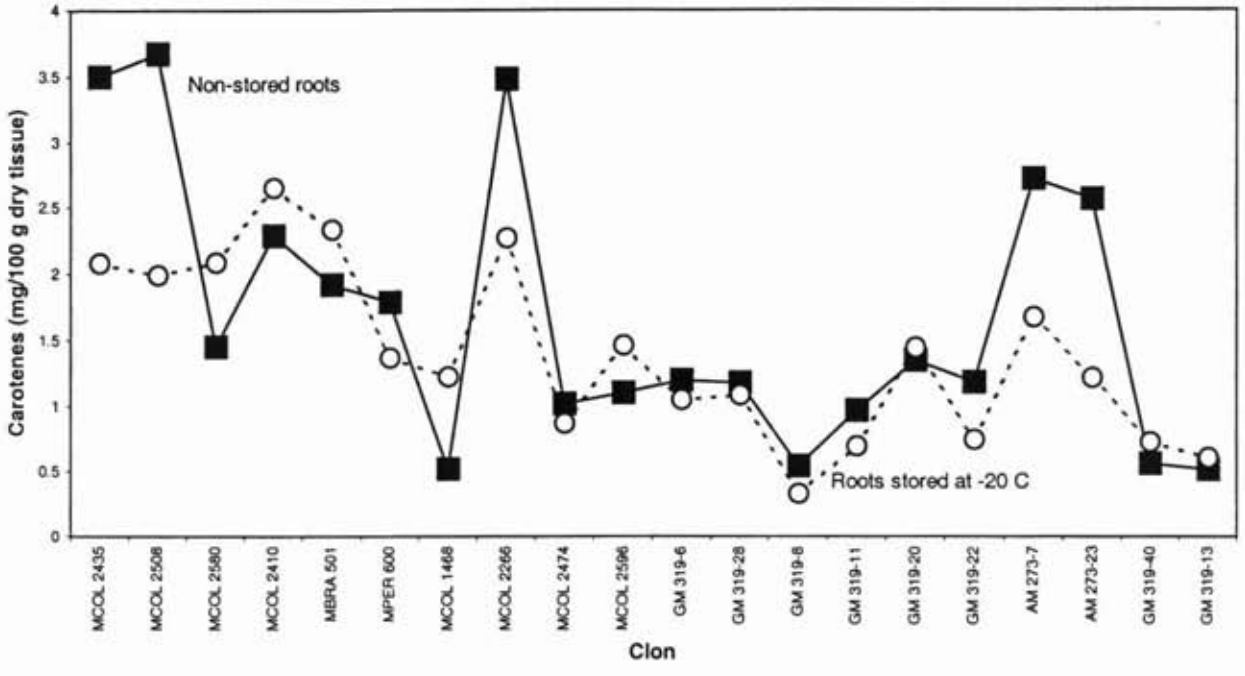

Figure 6. Comparison of carotene content (measured by the colorimetric method) on roots from 20 clones at harvest time and then after 6 months of storage at –20 °C

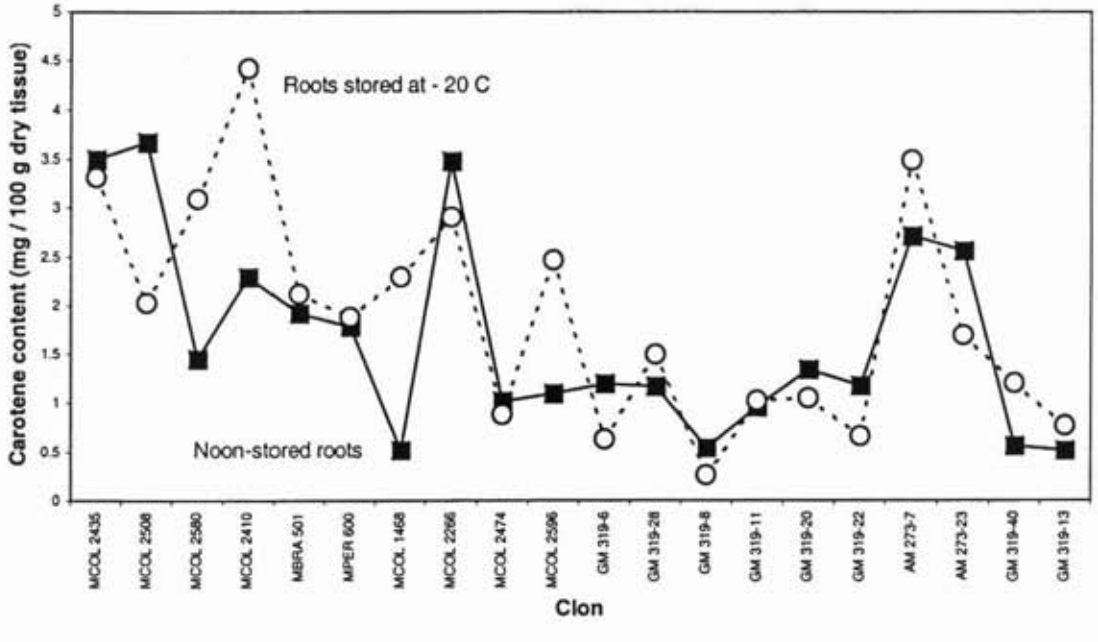

Figure 7. Comparison of carotene content (measured by the HPLC method) on roots from 20 clones at harvest time and then after 6 months of storage at –20 °C

2.3.3 Comparison of colorimetric and HPLC methods for measuring carotene content in cassava roots from 100 clones

Introduction

Cassava roots were harvested and analyzed for carotene content using the colorimetric and the HPLC methods as described above. The purpose of this study was to continue the screening for clones with high-carotene in the roots, but more important, to determine the correlation between results from both methods and to estimate the proportion of different carotenoids (alfa and beta-carotene and lutein) by HPLC. The later information is relevant because the production of vitamin A is twice as effective in β-carotene compared with α-carotene (Combs, 1992).

Methodology

Sampling. Root samples of 100 clones from the germplasm or the cassava breeding project at CIAT were used for this study. Harvest took place at 9-10 months after planting (normal harvesting time for cassava at CIAT) and commercial size, disease-free roots were taken to represent each clone.

Colorimetric method. The extraction procedure outlined by Safo-Katanga et al. (1984) was adjusted by extracting root parenchyma with petroleum ether.. A sample of 5 g was taken out of the root or leaves, taken at random 10 to 11 months after planting. The quantification of total carotenes was done by ultraviolet spectrophotometry using a Shimadzu UV-VIS 160A recording spectrophotometer. UV detection was done at λ = 455nm for root extracts and λ= 490 nm for leave extracts.

HPLC method. Starting from the method used for the spectrophotometric quiantification of total carotenes, aliquots (20 ml) of petroleum extract were completely dried by rotaevapotration. Then the dry extract was dissolved in 1 ml of HPLC mobile phase (methanol:methyl-t-butylether :water, 81:15:4 v/v), centrifugated at 14000 rpm and 10 µl were injected in the HPLC system using a YMC-C30 column (250 mm, ID:4.6mm, Waters). Separation was done by a linear gradient elution from methanol:methyl-t-butylether:water, 81:15:4 v/v to methanol:methyl-t-butylether :water, 20:76:4 v/v during 90 minutes at 1 ml min^{-1} and 23°C. β-carotene was detected by monitoring absortion at 450 nm. Identificaction and quantificaction was done by comparing retention times and uv-vis spectra with a standard of β-carotene (Sigma C-0126).

Results

Based on a sample of roots from 100 clones, (results peviously reported in annual report 2002) the correlation between the two methods of measurements was excellent. If

correlations are measured using fresh tissue data the coefficient was 0.922 (Figure 1). Correlation coefficient was slightly higher (0.935) when taken based on dry tissue data (Figure 2).

Results illustrated in Figures 1 and 2 help to highlight that most cassava clones have low levels of carotene in the roots. As the total carotene concentration increases, so does the disagreement between the two measuring methodologies.

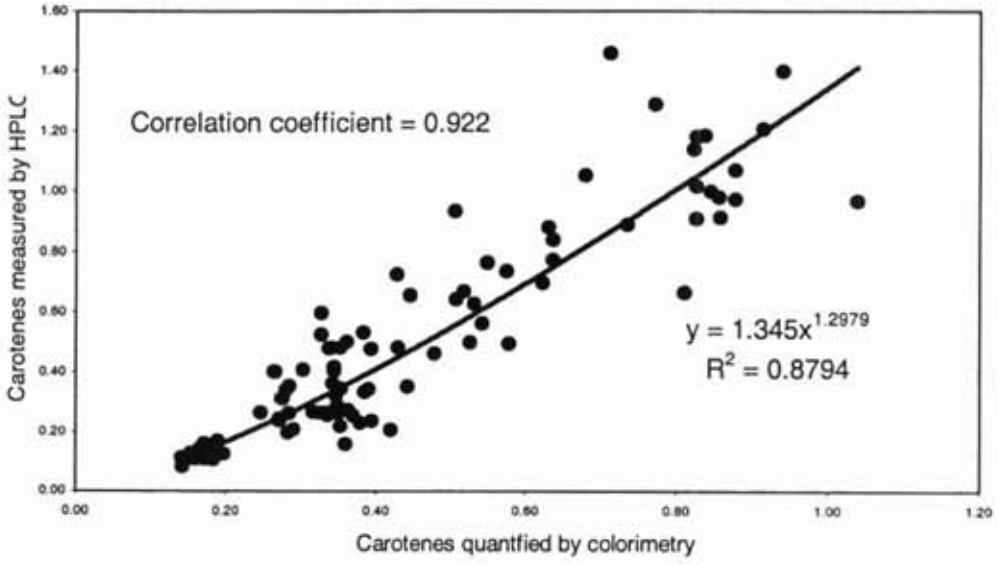

Figure 1. Relationship between carotenes (mg/100 g FT) measured by the colorimetric and HPLC methods (samples from 100 clones, measured in three repetitions).

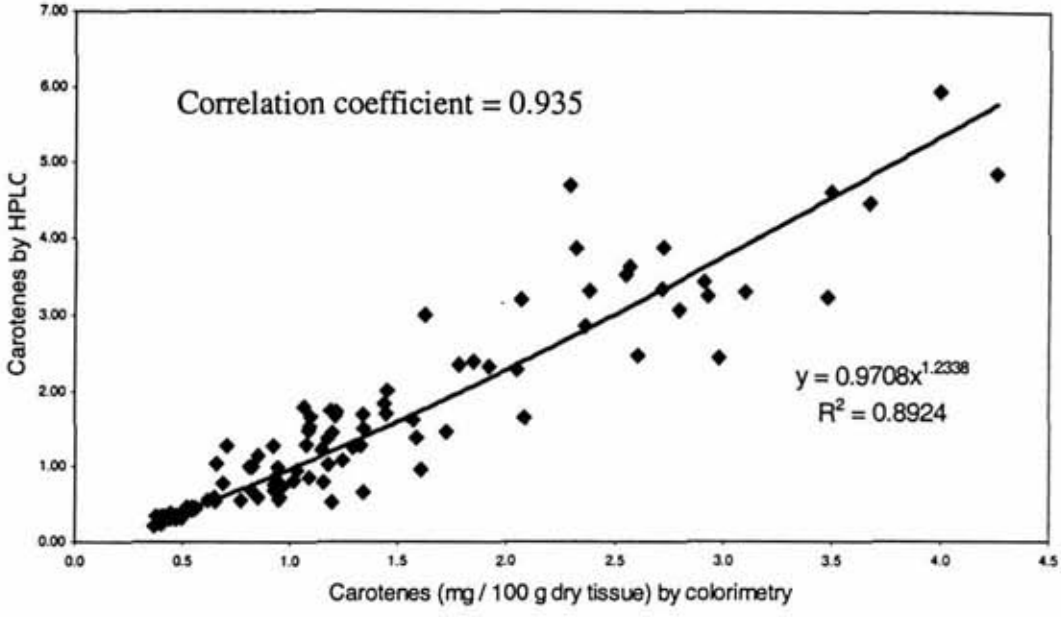

Figure 2. Carotenes (mg/100 g dry tissue) measured by the colorimetric and HPLC methods (based on sample from 100 clones evaluated in three repetitions.

Data in Table 1 present the descriptive statistics to the two measuring methods. The advantage of HPLC is that it allows for the partitioning of total carotenes into its components (α- and β-carotene and lutein). Total carotene was, on average, 13% higher using the HPLC method compared with the colorimetric method.

It was very interesting to observe that most of carotene extracted from cassava roots was β-carotene. On average, about 97% of carotene measured was β-carotene, ranging from 91 to 100% (Table 1). This is advantageous because of the recognized bio-efficacy of this type of carotene to be metabolized into retinol (the active form of vitamin A). The proportion of β-carotene in cassava roots is higher than that found in carrots which was found to have the following proportions (Rodriguez-Amaya, 2001): α-carotene (28.0%); β-carotene (56.6%); γ-carotene(13.2%) and Lutein (2.1%).

Coefficient of variation for colorimetry averaged 7.1% but was 20.9% for measurements based on HPLC. The higher variation for HPLC measurements may be a result of the higher values generally observed for HPLC, but also, and more likely, due to the adjustments of a new methodology in the laboratory. Based on these results some p procedures have been modified to reduce experimental error in HPLC measurements.

Table 1. Statistical description of the measurements of carotene content in root samples from 100 cassava clones using the colorimetric and HPLC methods.

	Colorimetry	HPLC			
	Total	α-carotene	β-carotene	Lutein	Total
Data based on fresh tissue (mg carotene / 100 g of fresh tissue)					
Mean	0.421	0.016	0.455	0.005	0.476
St.Dev.	0.233	0.018	0.331	0.007	0.350
Maximum	1.038	0.077	1.381	0.044	1.459
Minimum	0.141	0.000	0.083	0.000	0.083
Data based on dry tissue (mg carotene / 100 g of dry tissue)					
Mean	1.359	0.052	1.468	0.016	1.536
St.Dev.	0.908	0.064	1.196	0.025	1.267
Maximum	4.255	0.249	5.672	0.165	5.950
Minimum	0.366	0.000	0.213	0.000	0.213

2.3.4 Stability of carotene accumulation across different locations

Introduction

Improving the efficiency with wich cassava acquires micronutrients and accumulates them in the roots and leaves, can have an enormous potential not only in terms of human nutrition, but also in terms of crop production. The purpose of this short study was to evaluate the relative stability of carotene content depending on the locations where the cassava clones have been grown. Results are preliminary given the small number of genotypes grown.

Methodology

Colorimetric method. *(already described above)*
HPLC method *(already described above)*
Sampling *The study was conducted with three clones grown in four locations with three replications/location. Locations are localized in Llanos Orientales: Location 1=La Libertad; Location 2=Santa Cruz; Location 3=Cumaral; Location 4= Cabuyaro.*

Results

Because the clones had to be harvested at about the same age after planting, and taking into consideration that the trials at the different locations were planted at different dates, depending on the availability of rains, the sampling and carotene measurements were

performed on roots that had been harvested at different times. Therefore, there was a confounding effect between batch for carotene measurement and location.
Figure 3 shows the mean carotene content of each clone at each location. The amount of carotenes obtained for clone SM 1859-26 in the third location was unexpectedly high. Because of this only outlying data point, the genotype x environment interaction could reach statistical significance at the 5% probability level. In general, however, the performance at the other 11 data points suggests that genotype x environment interaction is not prevalent for carotene content in cassava roots..

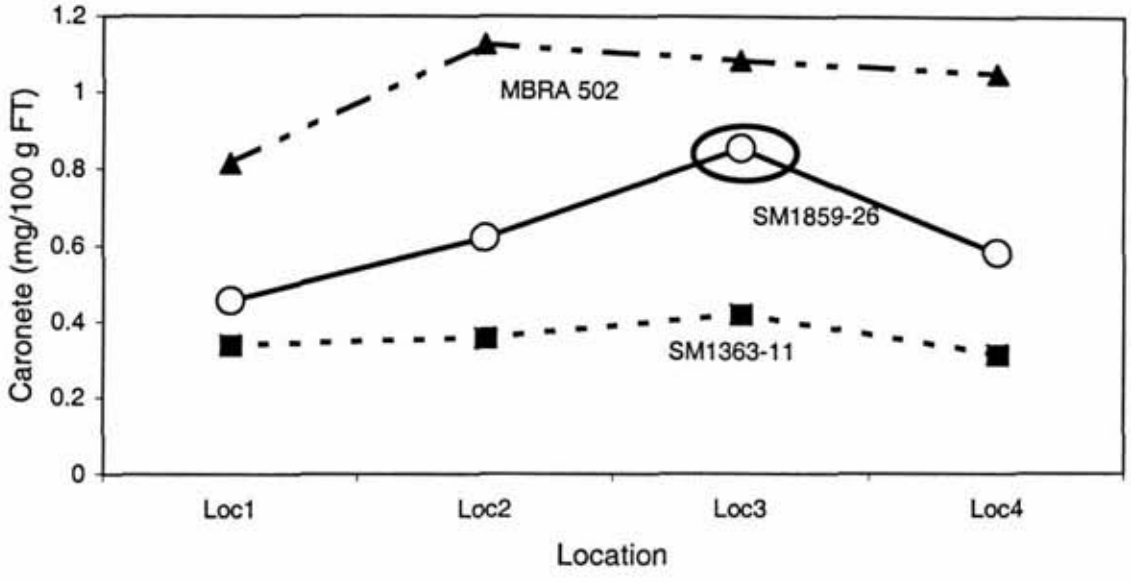

Figure 3. Stability of carotene content (mg/100 g fresh tissue) on roots from three different cassava clones grown in four different locations in Colombia.

Table 2 presents the result of the analysis of variance for this experiment. It is important to highlight that, regardless the significance of the genotype by environment interaction observed, it was not strong enough to induce cross-overs in the performances of the clones evaluated. These analyses were carried out by the HPLC methodology and results are based on the addition of α- carotene, β- carotene and lutein.

Table 2. Analysis of variance for carotene content on roots from three clones evaluated in four different locations with three replications per location.

Source of Variation	Degrees Freedom	Mean Squares
Location	3	0.097644^{NS}
Rep (Location)	8	0.064507
Clones	2	1.325868**
Clones * Location	6	0.024867*
Error	16	0.007845
Total	35	0.106727

NS= Statistically non-significant. ** Significant at 1% probability level. * Significant at 5% probability level.

In conclusion, no significant differences in carotenoids content were found between cassava roots sampled in different areas.

2.3.5 Effect of home processing on carotenes content of cassava roots.

Introduction

Most of the data available on the carotenes content of foods refer to the raw materials. It is evident, however, that data relating to the form by the foods are consumed by the population are needed and the influence of processing on carotenes levels has to be determined. In addition to the quality of carotenes present in cassava their bio-efficacy will depend on the amount lost upon processing the roots.

The purpose of this study is to determine losses of the vitamin A potency during processing of cassava roots.

Methodology

Carotene contents were measured by HPLC on fresh roots, and after boiling, sun-drying and oven-drying the roots from the same three clones (MBRA 502, SM 1859-26 and SM 1363-11) grown and harvest at four different locations in Llanos Orientales: Location 1=La Libertad; Location 2=Santa Cruz; Location 3=Cumaral; Location 4= Cabuyaro.

Processing preparations. *At the laboratory, cassava roots from 2 different plants of the same genotype are peeled and prepared. 3-5 roots are divided into 5 subsamples that are processed in various ways.*

Boiling: The subsample (500 g) was boiled in an aluminium pot with 1 liter tapwater for 20 min.

Sun dry: The subsample (500g) was dryed under sun during a period of 76 hours.
Oven dry: The subsample (500g) was dryed in oven at 60 °C during 24 hours.
Lyophilization : The subsample (500g) was dry by liophylization at –20 °C.
Unprocessed: 5 g of fresh cassava root chopped in small pieces was analized (results in previous report (Stability of carotene accumulation across different locations).

HPLC method *(already described above)*

Results

Figure 4 illustrates the results (averaged across the four locations).

Results demonstrate that relatively small amount of carotenes are lost upon boiling the roots (up to 55% of the original levels recovered), whereas drying the roots by the common sun–drying process or in the oven, resulted in a recovery of 13 and 8%, respectively. These results contrast with previous ones (previously reported, CIAT, annual report 1999) based on the colorimetric method of quantifying carotene contents (with recoveries of 60, 40 and 63% after boiling, sun- or oven-drying, respectively). It is not clear if the differences between these contrasting results are because of the changing in the method for measuring carotenes (colorimetric versus HPLC methods), or else some error in the process.

Losses of the vitamin A potency during processing can be caused by oxidation by wich the total alpha and beta carotene content of the roots is reduced, and by isomerisation, in such case the total carotene content, quantified by colorimetric method, is not altered, but the vitamin A potency (quantified by HPLC as beta carotene content) is reduced because of the transformation of the naturally occurring all-trans isomers into the biologically less active cis-isomers. Further analyses will be conducted to corroborate or correct results so far obtained.

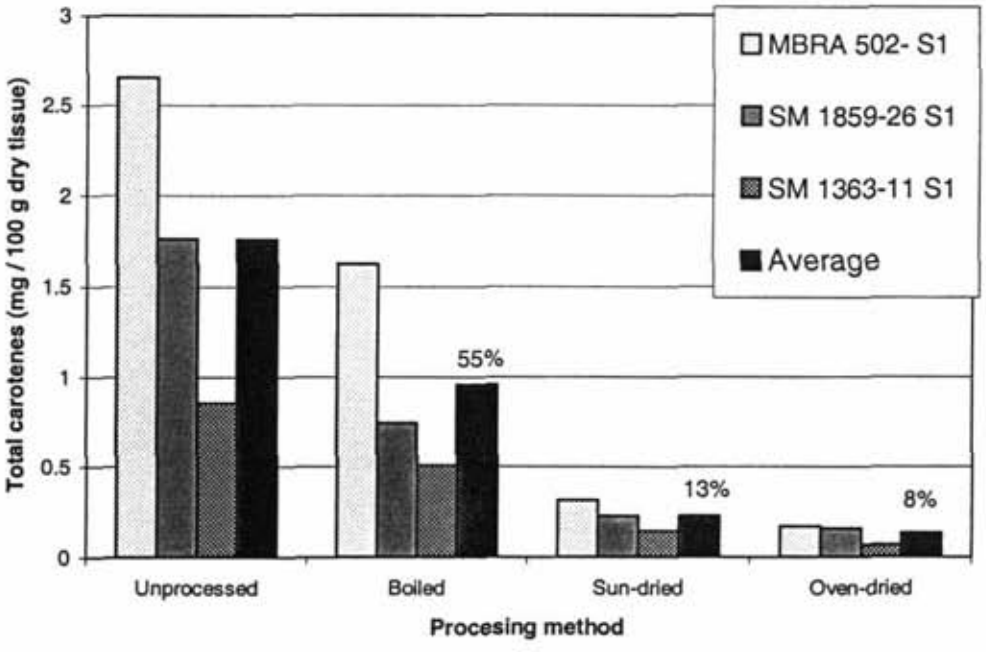

Figure 4. Total carotenes (mg/100 g of dry tissue) adjusted for dry matter content in fresh roots and after different processing methods. Measurements were made using the HPLC methodology.

2.3.6 Inheritance of carotene content based on S1 lines.

Inheritance of carotene content in cassava has been described as relatively simple (Iglesias, et al. Euphytica 94:367-73), but not definite mode of inheritance has been clearly specified. 16 S1 lines obtained from the elite clone MTAI 8 (Rayong 60, derived from the cross between MCOL 1684 and Rayong 1) were evaluated. MTAI 8 (= Rayong 60) has roots that are almost white in color. The S1 progenies, surprisingly, exposed a large range of variation for carotene content. Measurements with HPLC were higher than those with the colorimetric method. Also, the coefficient of variation was higher in HPLC than in colorimetry.

Moreover, as illustrated in Figure 8, the overestimation of HPLC (or underestimation by the colorimetric method) seems to be much higher at high values of carotene content. It is possible that the increased difference between the colorimetric and HPLC methods at high levels of carotene is due to lack of adequate fit of the standardization curves for one or both methods. This explanation makes sense because the curves were originally devised for a range of carotene content lower than 1 mg / 100 g tissue. It is not clear yet if it is a matter of overestimation by HPLC or underestimation by colorimetry.

Results presented in Table 3 clearly suggest that carotene content can indeed be increased over the natural ranges so far obtained. It is important to emphasize that the highest concentration of carotenes based on fresh tissue so far found had been 1.06 mg / 100 g fresh tissue. Four of the sixteen S1 clonal lines evaluated showed much higher levels of carotene concentration (around 1.70 mg / 100 g fresh tissue). Also promising is the fact that these high values were obtained from a clon (MTAI 8 or Rayong 60) whose actual concentration of carotene was rather low (0.31 mg / 100 g fresh tissue).

Future activities

- To standarize the new HPLC methodology for carotenoid compound analysis, adjusting the parameters that decrease reproducibility in the results.

- To measure sampling variation for β-carotene in cassava roots from differets plants, between roots of the same plants and among different sectios of the same root, to obtain more uniform, comparable and reliable data.

- To determine the effect of different preparation or processing methods on ß - carotene and total carotenes content of cassava roots.

Table 3. Dry matter and carotene content in roots from 16 S1 lines derived from MTAI 8 (Rayong 60) measured by the colorimetric and HPLC methods on fresh and dry tissue.

	Dry matter (%)	Colorimetric method		HPLC method	
		Carotene content (mg/100 g)		Carotene content (mg/100 g)	
		Fresh tissue	Dry tissue	Fresh tissue	Dry tissue
AM 320-140	37.47	1.01	2.71	1.76	4.71
AM 320-136	33.62	0.83	2.48	1.66	4.93
AM 320-135	34.47	0.87	2.54	1.78	5.17
AM 320-133	32.61	0.81	2.49	1.49	4.58
AM 320-123	37.36	0.44	1.19	0.66	1.78
AM 320-143	36.89	0.36	0.98	0.25	0.68
AM 320-147	42.69	0.37	0.86	0.40	0.93
AM 320-127	37.20	0.41	1.11	0.49	1.32
AM 320-124	38.86	0.31	0.80	0.40	1.04
AM 320-146	40.74	0.32	0.78	0.42	1.04
AM 320-139	33.78	0.26	0.77	0.21	0.63
AM 320-144	33.91	0.26	0.77	0.26	0.77
AM 320-142	36.41	0.15	0.41	0.10	0.27
AM 320-138	37.63	0.14	0.37	0.10	0.27
AM 320-130	31.53	0.14	0.44	0.11	0.34
AM 320-121	40.82	0.14	0.35	0.10	0.24
MTAI 8	39.98	0.27	0.69	0.31	0.78
Average	36.63	0.43	1.19	0.64	1.79
St.Dev	3.17	0.29	0.85	0.64	1.87
Min.	31.53	0.14	0.35	0.10	0.24
Max.	42.69	1.01	2.71	1.78	5.17

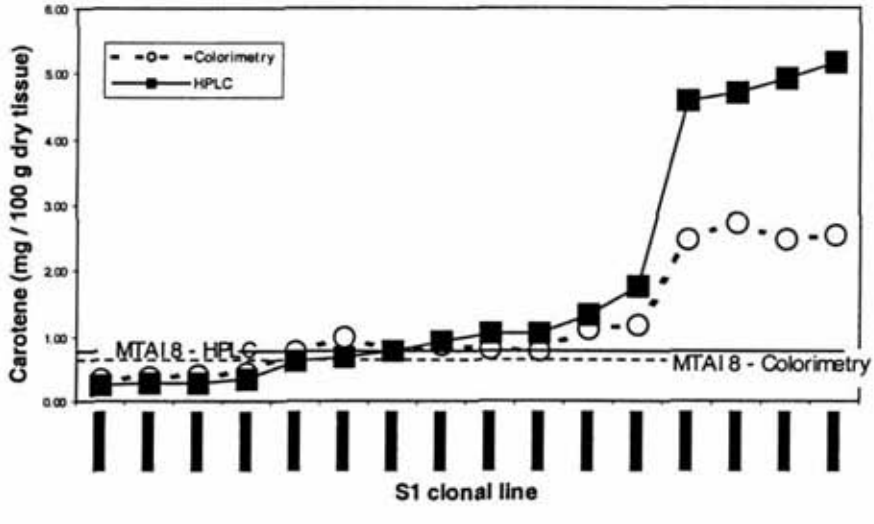

Figure 8. Carotene content in roots from 16 S1 lines derived from the elite clone MTAI 8 (or Rayong 60) measured by the colorimetric and HPLC method. Data based on dry tissue.

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2.3.7 Evaluation of S_1 Families for Waxy Mutants

Allison Smith[1], Chris Hylton[1], Nelson Morante[2], Teresa Sanchez[2], Hernan Ceballos[2], Martin Fregene[2]

[1]JIC, Norwich, [2] CIAT

Introduction

There is a growing interest from both the private and public sectors to develop a waxy starch phenotype in cassava. Three approaches have been embarked upon, via genetic transformation, anti-sense, and sense silencing, irradiation of cassava seeds, and screening the germplasm bank for waxy starch phenotypes. The heterozygous nature of cassava however makes the second and third option difficult, given the low chances that a natural mutant of the waxy gene will be found in the homozygous state. A decision was made to screen many S_1 families developed under a population development effort to for tolerance to inbreeding. About 14 S_1 families were available for evaluation. To further increase the precision of the chemical assay for percent amylase in the above evaluations, Prof Allison Smith`s group at the JIC was contacted for assistance in preparing pure cassava amylose and amylopectin for making an amylose determination standard curve. Current assays use commercially available pure potato amylose and amylopectin in preparing the standard curve, but the chain lengths of amylose in cassava and potato differ and this may introduce errors in the percent amylose measured. Pure amylose and amylopectin was isolated from

root starch of the cassava variety MCol 2216 and used in developing an amylose determination standard curve.

Methodology

14 S_1 families, developed under an S_2 recurrent selection program to develop populations tolerant to inbreeding were the plant materials for the above experimene. They were planted last year at the CIAT station in Santander the Quilichao and harvested July this year. At harvest starch samples was collected from 3 roots of all progenies, a total of 514 individuals, and taken to the laboratory for analysis. Pure cassava amylose and amylopection preparations were made using a sepharose separation column at the John Innes center, Norwich following methods described by Zeeman et. al. (2003). Mixtures of amylose and amylopectin with varying proportions of amylose from 0% to 100% were prepared, the samples were then dispersed in ethanol, hydrolyzed by acid, iodine added to a final concentration o 2% (v/v) and the absorbance measured at wavelengths of 700nm and 525nm in a spectrophotometer. Measuring the sample at this two wavelengths and using a ratio of 700:525 rather than the traditional method of measurement of a single wavelength , 620nm, have been found to be more accurate in determining amylose content (Zeeman et al. 2003). The absorbance and of the samples with varying proportions off amylose was used in generating an amylose determination curve.

Results

Pure cassava amylose and amylopection were obtained from fractions of a sepharose column at maximum absorption at wavelength of 595nm (Fig 1). The preparations were used in making mixtures of different proportions of amylose and the absorbance measured as describd above. Figure 2 and table 1 shows results of the development of an amylose content determination curve. The curve will be used for determining amylose content in the starch samples obtained from the S_1 families. Analysis of the starch samples are ongoing and should be completed by November. Any samples that shows less than 5% amylose will be sent for further analysis, including a quantitative purification of the amylose and amylopectin, and analysis of the molecular structure, to ensure that the low amylose content is not due to extremely long amylose molecules whose absorbance in solution closely mirrors amylopectin molecules

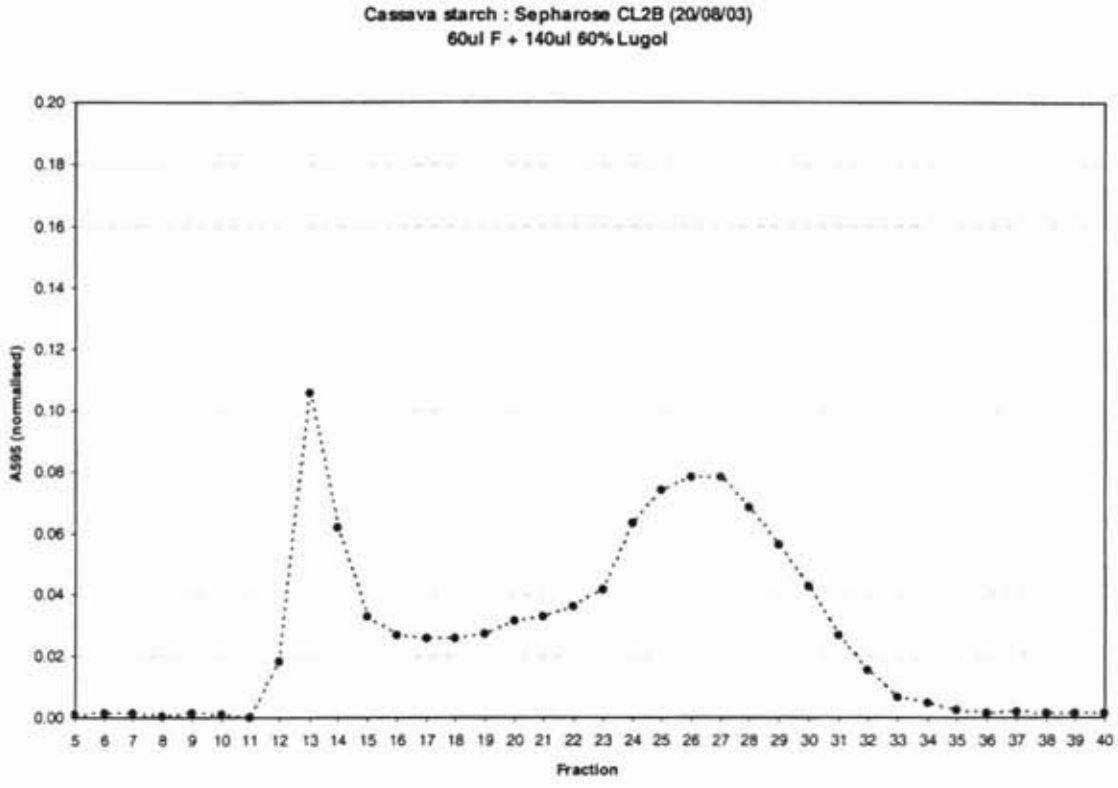

Figure 1. Absorbance of a sepharose column fractions in the preparation of pure cassava amylose and amylopectin from the cassava variety MCol2216

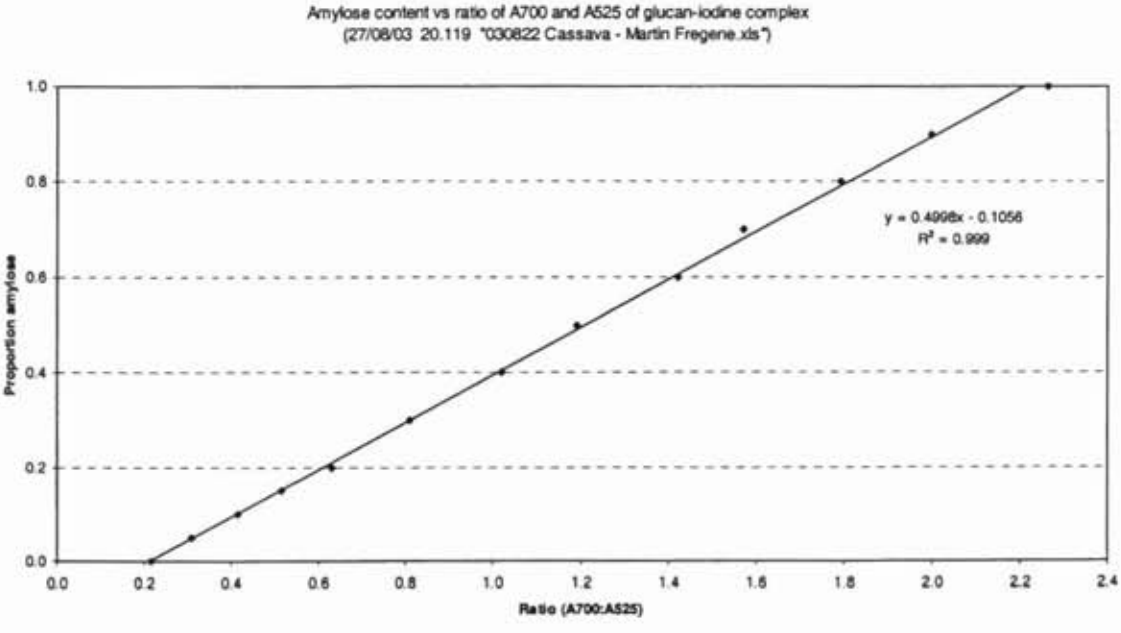

Figure 2. An amylose determination standard curve prepared using pure cassava amylose and amylopectin

Table 1. Data for the preparation of the amylose content determination curve

Amylose		AP		Proportion amylose	A525			A700			Ratio A700:A525
ul	ug	ul	ug		I	II	Mean	I	II	Mean	
0	0.000	103.1	17.816	**0.00**	0.2249	0.2391	0.2320	0.0506	0.0497	0.0502	**0.2162**
3	0.891	98.0	16.926	**0.05**	0.2427	0.2414	0.2421	0.0748	0.0747	0.0748	**0.3088**
6	1.782	92.8	16.035	**0.10**	0.2403	0.2369	0.2386	0.0993	0.0983	0.0988	**0.4141**
9	2.672	87.7	15.144	**0.15**	0.2422	0.2353	0.2388	0.1210	0.1253	0.1232	**0.5158**
12	3.563	82.5	14.253	**0.20**	0.2515	0.2533	0.2524	0.1584	0.1593	0.1589	**0.6294**
18	5.345	72.2	12.471	**0.30**	0.2537	0.2524	0.2531	0.2046	0.2048	0.2047	**0.8089**
24	7.127	61.9	10.690	**0.40**	0.2520	0.2562	0.2541	0.2510	0.2673	0.2592	**1.0199**
30	8.908	51.6	8.908	**0.50**	0.2483	0.2512	0.2498	0.2971	0.2974	0.2973	**1.1902**
36	10.690	41.3	7.127	**0.60**	0.2489	0.2652	0.2571	0.3526	0.3781	0.3654	**1.4213**
42	12.471	30.9	5.345	**0.70**	0.2557	0.2671	0.2614	0.4016	0.4207	0.4112	**1.5729**
48	14.253	20.6	3.563	**0.80**	0.2645	0.2558	0.2602	0.4804	0.4519	0.4662	**1.7919**
54	16.035	10.3	1.782	**0.90**	0.2666	0.2663	0.2665	0.5313	0.5336	0.5325	**1.9983**
60	17.816	0.0	0.000	**1.00**	0.2504	0.2617	0.2561	0.5577	0.6009	0.5793	**2.2624**

Conclusions

A more accurate method for the determination of amylose and amylopection proportions in cassava has been developed in collaboration with the John Innes center. It consists of using an amylose determination standard curve that was prepared using cassava amylose and amylopectin rather than those from potato. The method is currently being used to evaluate starch samples from S_1 families

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2.3.8 Irradiation of Sexual Seeds for the Production of Waxy Cassava and other Mutants

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Introduction

The globalization of economies has meant a search for local crops that are competitive and will preserve local agriculture. Economic surveys in the past ten years in cassava growing regions of Africa, Asia and Latin America have revealed that cassava is an important factor in the improved livelihoods of the rural population of these regions (Nweke et al 2001; Kawano 2001; Ceballos 2002). A change in starch quality, for example the elimination of amylose (waxy starch), via a knockout of the GBSS I gene, implies access to new markets for cassava growers. For most rural communities, a better standard of living depends on increasing income from their crop harvest. An important disadvantage of cassava is the short shelf life of its roots, which have to be consumed or processed within a few days after harvest. This trait, post-harvest physiological deterioration (PPD), results in losses and higher marketing costs and its elimination will lead to higher profit margins for the small producer. PPD is thought to be a wound response cascade that goes out of control, and the knockout of certain key genes in the cascade should lead to a reduction in PPD.

Cassava also possesses a wide range of cyanogenic glucoside content that is often cited as a health risk and a stigma for its acceptance as raw material in certain food and feed industry. Cyanogenic glucosides are produced by a biosynthetic pathway that has a cytochrome P450 as catalyst in the rate-limiting step (Andersen et. al. 2000). Removal of the gene that expresses the cytochrome P450 gene should lead to low cyanogenic potential (CNP) cassava plants. Increased productivity or increased value of cassava roots such as novel starch types and improved marketing through elimination of losses from post-harvest physiological deterioration and the removal of the stigma of cyanogenic glucosides, stands to improve livelihoods of cassava farmers. Mutagenesis has been applied extensively in the production of novel phenotypes in crop species (Van Harten 1998). The project will take advantage of simultaneous research, currently under way, that will facilitate to routine production of inbred materials, for the first time in a cassava breeding project. Once mutants have been identified, molecular genetic analysis will be used to track down genes responsible for the novel traits. The use of these genes as markers or in genetic transformation will permit an increase in the efficiency of transferring these traits to other cassava gene pools through conventional breeding.

Specific Objectives were:

Irradiate, using gamma rays (a Cobalt-60 source) and fast neutrons, of sexual seeds from elite cassava genotypes under improvement for tolerance to inbreeding depression
Establish plants from the irradiated seeds and non-mutated parental genotypes and evaluate them for useful traits such as delayed post harvest physiological deterioration (PPD), low cyanogenic potential (CNP), high dry matter content (DMC), and novel starch types.

Develop selfing of the mutated lines to obtain S_0 progenies and their evaluation for the above root quality traits and any other potentially useful trait
Carry out DNA analysis for genes known to be involved in biosynthetic pathways of the above traits to identify mutants.

Methodology

Cassava has seldom been inbred, and the large "genetic load" hidden in its heterozygous background will likely hinder the production of viable homozygous plants, a phenomenon known as "inbreeding depression". Selection for tolerance to inbreeding depression has therefore been initiated to make cassava populations amenable to the production of inbred lines. Sexual seeds from cassava genotypes tolerant to inbreeding were the source of genetic material for mutagenesis. About 2000 sexual seeds were shipped to IAEA for irradiation, using gamma rays (a Cobalt-60 source), 1000 seeds, and fast neutrons, 1000 seeds. The level of irradiation with gamma rays was 200Gy.

The irradiated seeds were sent back to CIAT for germination and establishment of the plants in the field. The heterozygous nature of cassava implies that mutations in a recessive gene will not be observed in the M_0 phenotype. There is therefore a need to self the M_0 plants to permit identification of the recessive mutants. However, the task of selfing thousands of plants is beyond capacity at CIAT and a selection of mutants for genes of interest will first be carried out to identify mutants. DNA analysis that can identify single nucleotide polymorphisms (SNPs) or insertions/deletions (INDELs) in genes of interest will be employed. At 10 months after planting, the plants from the irradiated seeds selected above will be evaluated for ability to produce flowers. Plants that flower will be cloned and planted the following year in a clonal observation trial fashion of 10 plants per genotype.

Plants in the clonal observation trial above will be selfed to obtain the M_1 (S_0) generation. The seeds from the M1 (S_0 generation) will again be established in the field at CIAT and other key target environments and thoroughly evaluated for the traits mentioned in the previous section. Progeny identified with useful root traits will be introduced into the cassavabreeding program.

Results

A total of 2000 full-sib seeds from full- and half- families of MCol1505, HMC-1, C4 (CMD resistant parent) were sent to IAEA for irradiation with gamma rays and fast neutrons. About 1000 seeds were irradiated with a dose of 200Gy of gamma rays. They were moisture equilibrated over a 69% glycerol solution in a dessicator prior to radiation. These seeds have been sent back to CIAT where they have been planted in a seedling nursery. Seeds irradiated with fast neutron experiment, about1000 seeds, are still being expected back.

Conclusions

This project seeks to use novel methods of mutagenesis, conventional plant breeding and molecular genetic analysis to identify cassava genotypes with value added traits. It will take advantage of the recently initiated research to produce inbred cassava germplasm. The project will also use tools of genomics to track down genes responsible for the above traits, markers associated to these genes can be used to efficiently move these genes around the different cassava gene pools defined by agro-ecologies. The new methods will not only accelerate the production of improved germplasm but also be a model for the development of other traits of interest to the market and farmer.

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2.3.9 Development of Populations Tolerant to Inbreeding Depression in Cassava

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CIAT

Introduction

A S_2 recurrent selection program was set-up for the development of cassava populations tolerant to inbreeding. The principal reason for the program is the development of populations tolerant to inbreeding and for the development of pure cassava lines via the doubled haploid technology. A second reason is the identification of genetic stocks for gene mapping studies, for example excellent segregation of beta-carotene content and cyanogenic potential (CNP) was observed in 2 of the S_1 families from MCol 72 (beta-carotene) and MTAI8 (beta-carotene and CNP). A preliminary selfing of an initial 14 genotypes produced 300 S_1 lines that were evaluated in a clonal observation trial last year (CIAT2002). More than 30 of those S_1 lines that flowered profusely were selfed to produce S_2 families. We describe their production and establishment in the field. Furthermore additional larger sized S_1 families have been developed from 20 varieties and have been established in a seedling trial this year.

Table 1. Summary of S_2 families developed from embryo rescue of selfed S_1 individuals

Date of transplant	Grand Parent	Parent	Code	No. of Plants
Jun-03	MCol1505	AM244-10	AM 244-10-5	2
Jun-03	MCol1505	AM244-16	AM 244-16-1	1
Jun-03	MCol1505	AM244-16	2 AM 244-16	1
Jun-03	MCol1505	AM244-31	AM 244-31	5
Jun-03	MCol1505	AM244-31	1 AM 244-31	5
Jun-03	MCol1505	AM244-31	2 AM 244-31	5
Jun-03	MCol1505	AM244-35	AM 244-35	2
Jun-03	MCol1505	AM244-35	1 AM 244-35	4
Jun-03	MCol1505	AM244-35	2 AM 244-35	7
Jun-03	MCol1505	AM244-35	3 AM 244-35	3
Jun-03	MCol1505	AM244-38	AM 244-38	1
Jun-03	MCol1505	AM244-40	1 AM 244-40	2
Jun-03	MCol1505	AM244-101	1 AM 244-101	3
Jun-03	MCol1505	AM244-109	1 AM 244-109	2
Jun-03	MCol1505	AM244-109	4 AM 244-109	5
Jun-03	MCol1505	AM244-109	5 AM 244-109	5
Jun-03	MCol1505	AM244-109	6 AM 244-109	2
Jun-03	MCol1505	AM244-109	7 AM 244-109	5
Jun-03	MCol1505	AM244-109	8 AM 244-109	2
Jun-03	MCol1505	AM244-109	10 AM 244-109	1
Jun-03	MCol1505	AM244-109	12 AM 244-109	4
Jun-03	MCol1505	AM24-135	1 AM 244-135	1
Jun-03	MCol1505	AM24-135	6 AM 244 135	2
Jun-03	MCol1505	AM24-135	7 AM 244-135	3
Jul-03	HMC 1	AM266-21	4 AM 266-21	2
Jul-03	HMC 1	AM266-41	1 AM 266-41	1
Jul-03	HMC 1	AM266-41	5 AM 266-41	2
Jul-03	HMC 1	AM266-41	6 AM 266-41	1
Jul-03	HMC 1	AM266-45	2 AM 266-45	7
Jul-03	HMC 1	AM266-50	4 AM 266-50	2
Jul-03	HMC 1	AM266-76	1 AM 266-76	1
Jul-03	HMC 1	AM266-76	6 AM 266-76	1
Jul-03	CM849-1	AM312-42	1 AM 312-42	5
Total				95

Methodology

About 30 S_1 progenies were selfed and seeds harvested at 40-60 days after pollination, the early harvest of the seeds was due to the mandatory removal of all cassava plants in CIAT before the beginning of the "zero cassava" month at CIAT, one of the measures adopted to control the incidence of white flies at CIAT. The S_2 families were established from embryo rescue as described in above in the section on the development of mapping populations in cassava. After one round of multiplication *in vitro* they were transferred to the screen house for hardening and to the field.

At 10 months after planting, the progenies will be evaluated for fresh root yield, dry matter content, foliage weight, harvest index, culinary quality, starch content/quality, and frog skin disease according to standard CIAT procedures.

Results

An initial 14 cassava genotypes were chosen for the development of populations tolerant to inbreeding. The genotypes were chosen due to their good general combining ability performance for yield, dry matter yield or root quality. They include the following lines: MCOl22, CM523-7, MCOL1684, MBRA12, MCOL2060, MVEN77, MCOL1522, MTAI1, MPAN51, MECU169, MCOL1468, MCOL72, CM849-1, HMC1. More than 300 pollinations were made per genotype and between 30-150 seeds were obtained per genotype. During a clonal observation of the above families, selfings were carried out and 15 small sized S_2 families, of 2-10 progenies were developed (Table 1). These seeds were germinated from embryo axes. Between 5 and 10 plants per S_2 progeny was hardened in the green house and transferred to the field in July this year.

Conclusions

The development of populations tolerant to inbreeding has continued with the development of 15 small sized S_2 families. These families will be evaluated at 10 months after planting.

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OUTPUT 3 Collaboration with public and private partners enhanced

Activity 3.1 New Collaborative Arrangements and Networks

3.1.1 Update on HarvestPlus (Biofortification Challenge Program)

Project team members continued to played a major role in the formulation, organization and fund raising of the Biofortification Challenge program convened by CIAT and IFPRI. The Biofortification Challenge Program (now called HarvestPlus) was approved in October, 2002. World Bank funding of $3 million for 2003 was made available in December, 2002.

A first Project Advisory Committee (PAC) meeting was held in March to initiate/approve the selection of the Program Director and to set up/approve initial operating procedures and initial project activities for 2003. A second PAC meeting will be held in November 2003 to discuss/approve the workplans developed for 2004, coming out of the various planning meetings.

Renewed contacts with the Gates Foundation were initiated by CIAT and IFPRI in January, 2003. A formal proposal for 25 millions over four years was submitted on behalf of the CGIAR lead consortium to the Gates Foundations including the submission of the external reviews commissioned by the interim Science Council (iSC) and iSC. While the proposal was approved in Aug 1, 2003 a formal announcement and the launch of Harvest Plus took place in Washington on October 14, 2003. A press release was issued announcing the grant, and a press conference was held in Washington, DC. Discussions continue with a number of additional donors in the hope of meeting the proposal target budget of $12.5 million per year over the first four years.

Full start-up of the project is envisioned in January, 2004. Several planning meetings were organized during the year. The most relevant ones were:

I- Planning meeting of core collaborators and selected stakeholders, June 2-6, CIAT headquarters: 75 persons attended the five-day meeting. The planning committee of 15 persons met for the first time as a group for one full day on May 30. The following objectives were achieved:

To introduce and discuss the different broad components of the project to those who will be participating in the project and other stakeholders; there was an initial unevenness among the collaborators/participants in their familiarity with the project.
To flesh out and revise the conceptual framework laid out in the proposal to ensure that the different components are designed to complement each other technically -- thus, it was essential to engage in interdisciplinary learning and exchange at the meeting;
To develop an operational plan to implement coordinated activities over the next 12 months; a workplan and budget allocation for 2003 was agreed upon.

To open and set up lines of communication across the components -- collaborators got to know each other much better, and crop leaders became better familiarized with what would be involved in building and managing their crop teams.
To hear, at a very broad, global level, what might be stakeholder or collaborator concerns on the concept of the project as a whole and to get their buy-in.

II- Private sector meeting, IFPRI headquarters, July 20: Representatives from DuPont/Pioneer and Monsanto corporations, ILSI, and USAID met with Howarth Bouis and Joe Tohme of the BCP to discuss collaboration. The meeting was organized by Bill Neibur of the CGIAR Private Sector Committee. There was strong interest expressed on both sides in cooperating on development of micronutrient-dense, transgenic varieties. Private sector representatives were encouraged to attend the upcoming technical meetings and crop meetings during September-October. . Further meetings are planned for early 2004.

III- Nutrional breeding objectives and Crops meetings: Team members attended the Nutritional Breeding Objectives, and the maize, sweet potato and rice crop meeting and organized the bean and cassava meetings.

3.1.2 Biofortification : Linking The Strategic Breeding Work To Downstream Impact: Reaching And Engaging End-Users

L. Sperling
CIAT, Rome - Italy

Considerable progress was made this year in identifying strategies to ensure that the *biofortified products developed reach users*—and meet their needs. Analysis showed that priority client groups are diverse (rural producers and consumers; urban producers and consumers; intermediary processors; micro-nutrient deficient population; women and children) Further, in three separate sets of meetings (Copenhagen Oct/2002, Cali, June 2003; and subsequent crop-specific workshops; September-October 2004), participants identified the varied activities needed address this diversity, and to encourage widespread utilization of products. As summarized in Table 1, some of these more downstream activities might be programmed several years from prototype development (eg seed multiplication) while others need to start relatively quickly (eg basic community-level *nutritional R&D).*

Table: 1. Broad overview of activities implied in the thrust in :Reaching and engaging end-users

Activity Set	Description
Variety Development and Adaptation with Partners (community level and beyond).	Identification of range of end-users needs/preferences to guide initial selection criteria Adaptive testing schemes which reach clients (decentralized and/or participatory)—and which give good feedback Varietal testing models which can work with the poor and build on farmer skills Preferably testing models which can be replicated over many sites Adaptive agronomic research which follows use of biofortified materials under realistic management conditions
Community-level Nutritional Research and Development	Range of activities for understanding the possible constraints/opportunities for use of biofortified materials inter and intra-household, community and region Determination of potential nutritional benefits for different population groups , given existing dietary patterns Determination of possibilities for expanding the use of biofortified products, e.g. by incorporating the crop into culturally acceptable recipes, developing new recipes, etc. Identification and use of links to global initiatives to improve infant and young child feed, being spearheaded by UNICEF and WHO
Communication Outreach Strategies ---- at regional level (includes 'Social marketing' to guide behavioral change)	Development of strategies to reach diverse major audience/partner groups: Decision-makers/policy makers Potential producers and consumers, traders, processors Micro-nutrient deficient population Tailoring of: * content of messages to specific audience * format of messages (policy brief, radio, drama, extension bulletins) * work in multiple languages
Production and delivery of seed/products (along with its associated knowledge base)	Production and delivery of seed /food *plus* substantial information on use and nutritional consequences/benefits of biofortified materials Activities effected through normal seed channels, nutritional channels and unconventional ones (eg 'coca cola trucks)
Program monitoring and feedback at the community/regional level	Encouraging of co-learning and feedback among varied stakeholders Confirmation /adaptation CP program directions Identification novel mechanisms for enhanced impact Development of specific tools for program implementers to track progress on a regular basis—yearly at a minimum. Most closely look at the uptake by producers and the uptake by consumers
Creation/Fostering of Enabling Policy/Stakeholder environment	Sensitization/feedback from decision-makers as to magnitude of micronutrient problem Stimulation of interest in 'paradigm' shifts to link agricultural research sector to health research sector (new institutional environments) Establishment of first 'buy in' from key collaborators—early Shaping environment for favorable policies on: variety release, seed certification Getting systematic feedback from higher-level producer and consumer groups on: biofortified solution and use of GMOs as one possible thrust

One of the opportunities presented by working across crops in this biofortification program is that it allows research and development workers to address the different dietary needs of the poor, and to work in integrated farming systems, rather than focus on one crop or another. For instance, the four major staples given focus in the Africa program--: maize, beans, sweet potato and cassava-- are often grown in single homesteads, and sometimes even on the same plot. Because of the intercrop complementarity within this Challenge Program, activities of this 'reaching and Engaging User" group should be programmed across crops, when possible. Table 2 suggests broad strategies for across crop coordination. Such intercrop work will demand considerable facilitation, but ultimately could lead to more comprehensive impacts at the field level—as well as increased CP program efficiency.

Table 2: Coordination of thrusts for reaching and engaging users with the Biofortification Challenge Program

Within Crops * Variety Development and Adaptation with users
Across Crops * Community-level Nutritional R&D * Communication Outreach * Program Monitoring and Feedback * Creation/Fostering of Enabling Policy/Stakeholder Environment
Within and Across Crops * Seed production and Multiplication * Marketing of Products

3.1.3 Models of Food Safety Assessment of Transgenic Crops, Workshop funded by USAID and the Rockefeller Foundation, Washington DC, May 6-8, 2003

Joe Tohme[1] and Hector Quemada[2]
[1]SB-2 Project; [2]Crop Technology, USA

The safety assessment of transgenic plants is a major hurdle to their deployment in agriculture. Researchers and regulatory authorities in developing countries find such hurdle especially difficult to overcome. On the one hand, researchers lack the guidelines and experience in the types of tests that need to be conducted to assess safety. On the other hand, regulatory authorities are not experienced in assessing the safety of crops in terms of their general release. These difficulties will be felt more acutely when crops that have been developed within these countries reach the stage of general release. In these cases, developing country researchers and regulatory authorities will not be able to depend upon the previous decisions of other regulatory bodies, as is presently the case with transgenic crops released in all countries except the United States, Canada, China. Instead, they will have to determine the appropriate questions to ask, and appropriate data to be gathered, in order to arrive at a judgment of safety for these indigenously developed transgenic crops.

For many regulatory authorities faced with the problem just described, there is likely to be the inclination to mimic the requirements imposed by regulatory authorities in countries such as the United States, where commercialization of transgenic crops has taken place and is relatively routine. However, there is little understanding of the reasoning behind the requirements that have been imposed upon specific transgenic crops in countries such as the U.S., where regulatory data requirements have arisen out of regulatory frameworks and philosophies that might be quite different from those of other countries. Furthermore,

there is little understanding of the cost and types of expertise required to complete the safety assessments that have already been done. Consequently, the imposition by regulatory authorities of requirements for safety testing that are beyond the means of their countries, either in terms of money or human capacity, will effectively prevent the deployment of the results of biotechnology research in those countries.

In order to assist developing country and the CGIAR researchers in gaining an understanding of the types of food safety assessments needed for the deployment of transgenic crops, and to determine which requirements are appropriate for developing countries a workshop was held in May,2003, with funding from the USAID and the Rockefeller Foundation. The meeting was organized by Joe Tohme and Hector Quemada from Crop Technology.

The goal of the workshop were to provide researchers and regulators with the means to examine 1) the protocols used in the safety assessments of current commercialized transgenic crops, and 2) the rationale for the requirements imposed (or are likely to be imposed) on crops representative of different transgenic technologies. The workshop focused only on food safety assessment.

The participants were from NARS representatives from Brazil, Colombia, Mexico, Egypt, India, Kenya, South Africa, Philippines, Thailand and Uganda; from CG centers: CIAT, CIP, CIMMYT.ICRISAT, ICARDA and IITA; from international Institutions and donors: AATF, AgBios, USAID, Rockefeller Foundation, ILSI and USDA and from the private sector.

Three representative case studies were commissioned to leading expert and were presented at the workshop: 1) Bt potato, 2) virus resistant papaya, 3) mustard oil with enhanced levels of vitamin A.

The outputs of the workshop were the following
Examination of the protocols used in the food safety assessments of current commercialized transgenic crops

Understanding of the rationale for the requirements imposed (or likely to be imposed) on crops representative of different transgenic technologies
Awareness of the regulatory steps to enable researchers to integrate regulatory issues and experiments related to regulatory approval in their overall research and development strategy.

Definition of a roadmap for food safety assessment

3.1.4 Updating CassavaDB, a ACEDB-type data base for Results of Genome Mapping

C. Buitrago; F. Rojas; J.Tohme, M. Fregene (CIAT)

Introduction

The cassavaDB is an AceDB-type genome database designed specifically for handling bioinformatic data flexibly. It includes tools designed to manipulate genomic data that is graphic, flexible and portable. It can be operated on various Unix workstations (SUN, *DEC, NEXT, SGI ...*), The first principle of the program is that any piece of data stored in AceDB can very easily be exported in flat ascii files to be used by other programs.
The second principle of the program is that write acces to the database is organised in macro transactions that is called sessions. All the information is stored in *objects*, which fall into a number of *classes*. The classes are standard units such as genes, alleles, strains, clones, papers, authors, journals, etc., and the names are in most cases the standard names. The CassavaDB was initially hosted at the plant databases at the National Agricultural Library at http://probe.nal.usda.gov. This was later moved to the ARS Genome Database Resource (GDR) server formerly at http://ars-genome.cornell.edu which is now permanently off-line. At the moment it is hosted at http://ukcrop.net/, the website of the UK Crop Plant Bioinformatics Network (UK CropNet) established in 1996 as part of the BBSRC's Plant and Animal Genome Analysis special initiative, the focus is the development, management, and distribution of information relating to comparative mapping and genome research in crop plants.

Since CassavaDB was developed in 1998, hundreds of additional markers, predominantly SSR markers have been developed and mapped in cassava. This information is currently not in the database. Also not in the database are additional genetic maps of cassava made since the first map was published in 1997. Data from several QTL mapping studies have also since become available since then. There is also a need to have the CassavaDB situated on a local server at CIAT to enable more frequent updates and also make it more visible to the international cassava community.

Activities to update CassavaDB. A compilation of all cassava SSR marker mapping data and map location data from work done by Mba (et al. 2001), Zarate (2002) Garcia (2002) and unpublished or undocumented work was initiated in August 2003. This information was formatted according to requirements for AceDB. Similarly, graphics of parental surveys, progeny data and maps were developed from data from the three mapping studies and prepared for CassavDB. All the above information will be up-loaded into CassavaDB in collaboration with the CIAT bioinformatics unit and maintained locally at CIAT. A link to the above will be made on the cassava web page of the CIAT website.

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3.1.5 Report of the Second Tri-Annual Workshop of the Molecular Genetic Diversity Network of Cassava (MOLCAS)

H. Rosling[1] ; M. Akerbolm[2] ; E. Kizito[3], L. Chiwona-Karltun[3], U. Gullberg[3] ; J. Jiggins[4] G. Muhlen[5]; E. Okai[6] ; M.Fregene[7]

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Introduction

Understanding the existing genetic diversity and its distribution within and among individuals, populations, species and gene pools is crucial for an efficient management and use of germplasm collections. The large amounts of cassava genetic resources held by farmers have been demonstrated to represent a critical resource for the future productivity and stability of production of cassava. A highly successful breeding program at the International Center for Tropical Agriculture (CIAT) in the early 1970s began with a great initial germplasm variation, some 2,218 local clones collected from Colombia, Brazil, Venezuela, and Peru (KAWANO 2003). That program achieved more than 100% increase in fresh root yield and more than 20% in dry matter content by the early 1980s. The improved varieties from Latin America combined with local varieties in South East Asia formed the basis of a very successful breeding program for the South East Asia Sub-continent, particularly Thailand. The new Thai cultivars are now planted on more than one million hectares in Thailand alone and the economic benefits from the increased productivity is in the order of one billion US dollars and the rural communities in some of the poorest parts of Asia captured a large proportion of these economic benefits (Kawano 2003)..

The molecular diversity network of cassava comprises of scientists drawn from institutes in Africa, Latin America, Europe and the USA. In its four years of existence, MOLCAS

has studied diversity of local varieties in Tanzania, Nigeria, Ghana, Uganda, Guatemala, , Peru, Sierra Leone and a subset of the World germplasm bank held at CIAT (MOLCAS 2003). Studies are ongoing of diversity in Cuba and Brazil. The ultimate aim of these studies is to identify and exploit useful variability for increased crop productivity and value addition. Between September 3 and 5 this year, members of the workshop from Brazil, Ghana, Uganda, Netherlands, and Colombia got together in Uppsala with network members based in Sweden and the director of the IPICs donor for the 2nd MOLCAS workshop to discuss progress and draw priorities for the next application to IPICs for the period 2004 – 2006. The meeting was held at the Plant biology department of SLU, and was well attended by members of the department including the head of department, Prof Per Bergmann.

Report of the 2nd MOLCAS meeting. The first day of the meeting saw presentations on SSR assessment of local cassava varieties from Ghana, Brazil, Nigeria, Sierra Leone, Guatemala, and Uganda. During second day, members of the plant biology department at SLU working on cassava presented a summary of their findings. They include cloning and transformation of starch biosynthetic genes, molecular biology of the cassava mosaic virus, and breeding cassava for small holders. A discussion was held in the afternoon of the second day on how an application for the third phase of BIOEARN, the Sida funded East African training network could be streamlined with activities being carried out in Africa. Of particular interest was how the wide gap between upstream biotechnology could be applied to secure the cassava crop as a food security crop and also provide improved livelihood through value addition. It was decided that BIOEARN members draw up a draft of their application and circulate it to MOLCAS members for comments and their inputs.

The third day, Prof Rosling presented results of an epidemiology of the Konzo disease in Mozambique conducted in the 1970s as an example of a proper approach to sampling genetic diversity. Two presentations were also made by Prof Janice Jiggins of WAU, Netherlands and Dr L. Chiwona Karltun on a farmer participatory research project taking place at the moment in Malawi. The afternoon of the third day was spent in a priority setting exercise for projects to be conducted in the next phase of MOLCAS. From a total of 10 project, the following were prioritized for the next phase beginning 2004 until:

Evaluation of highly differentiated gene pools in cassava for heterosis (Ghana)
Highly differentiated cassava gene pools may represent heterotic pools. The study seeks to evaluate these accessions for heterosis or hybrid vigor by making genetic crosses between and within representative members of the clusters. The study will be conducted in CRI, Kumasi, Ghana. Latin American germplasms were shipped to Ghana as tissue culture plants, hardened, and established in a crossing block along with African genotypes.
Tracing the lineages of local African Cassava varieties: towards a better understanding of sub-structures in African cassava gene pools (Uganda and Sweden)
To better understand the diversity sub structures in African local varieties, records and germplasm from breeding programs that existed in East Africa in the early part of the 19th

century will be examined and genotypes compared to modern day local varieties. This study will be carried out by SLU in collaboration with researchers from NARO, Uganda.
A search for useful variability in local cassava land races based upon the structure of SSR marker diversity analysis (Nigeria)

Further evaluation of some genotypes that have been observed during the SSR marker study to have novel characteristics, for example novel starch quality, will be carried out to confirm the earlier observations. Should they be confirmed, genetic crosses will be made to attempt a transfer into improved and other local varieties and also for inheritance studies
A comparison of clustering of cassava germplasm from Brazil and Malawi based upon bitter or sweet taste of the roots. (Brazil, Malawi, Sweden)
Studies of genetic diversity in Brazil revealed a clustering along the lines of taste. A similar result was also obtained for cassava germplasm from Malawi. The objective of this study is to combine both data sets and how they cluster. Dr Gilda Muhlen of University of Rondonia, Brazil will be responsible for the study in collaboration with SLU and Malawian researchers.

The meeting was attended by Prof H. Rosling of the Karolinska Institute, Stockholm; Dr M. Akerbolm, Ms L. Sjobolm of IPICs, Uppsala; Dr JaniceMs E. Kizito, Dr L. Chiwona-Karltun, Prof U. Gullberg, Prof P. Bergmann (head of department), Prof Chris Jassen, Dr Roger Anderson, Mr Yona Baguma, of the plant biology department, SLU, Uppsala; Dr G. Muhlen of the Universidad de Rondonia, Brazil; Ms E. Okai of CRI, Ghana, and M.Fregene of CIAT.

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3.1.6 Progress in the Development of a Web Accessible Data Base for SSR Marker Assessment of Diversity of Local Cassava Varieties

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Introduction

The web accessible database of the molecular diversity network of cassava (MOLCAS) was set up to make the results of its SSR marker assessment of local cassava diversity in selected countries of Africa and Latin America widely available. The database is updated as completed country studies become available. A total of 8 country studies, 5 in Africa and 3 in Latin America, have now been completed by the network. The database has been

updated with 2 additional country studies, bringing the total on the website now to 5, namely Tanzania, Peru, Nigeria, Guatemala and Ghana. A very rewarding outcome of the development of the MOLCAS web is the high number of visits the web has recorded since its inception last year. We describe here progress made this year.

Results

The MOLCAS web-based data base (http://www.ciat.cgiar.org/molcas and Fig 1) was conceived as a mechanism to document and make freely available to the cassava community information being generated by the network. It is hosted at CIAT. Results of the SSR analysis of local cassava land races from Ghana and Guatemala, including names and characteristics of local cassava varieties, allele size data by SSR marker locus, genetic diversity analysis, principal component of genetic distance etc, are now available for viewing on the MOLCAS web data base. Results from 3 other country studies namely Uganda, Sierra Leone and Brazil are being compiled and will be available on the web before the end of the year. The MOLCAS web-based data base has since its launch last year received a total of 34,477 visits with an average of 2700 visits per month. Table 1 shows the summary of all visits and visits in the last 3 months

Figure 1. MOLCAS home page on the web showing links to the data base of country studies

Table 1. A record of visits to the MOLCAS web-based data base on the country studies of SSR assessment of local cassava varieties

Request	Total Hits	Sep-03	Aug 2003	Jul 2003
/molcas/imagen.jsp	8,003	130	1,303	496
/molcas/locus.jsp	7,605	247	1,228	517
/molcas/alelosp.jsp	5,515	374	1,002	252
/molcas/estudios.jsp	3,250	125	266	370
/molcas/	2,162	88	138	130
/molcas/markers-det.jsp	1,892	37	101	187
/molcas/imagenbioquim.jsp	1,401	37	72	156
/molcas/appendix1.jsp	1,381	35	69	179
/molcas/intrap_data2.jsp	1,377	37	86	149
/molcas/appendix2.jsp	858	16	40	145
/molcas/studies.jsp	482	11	16	38
/molcas/pcr_cond.jsp	448	10	48	41
/webapps/molcas/	103	2	5	4
Total	34,477	1,149	4,374	2,664

Conclusions and Perspectives

The MOLCAS web accessible database has increased the completed country studies available for viewing to 5 and 3 more will be added before the end of the year. Two other country studies, Cuba and Mozambique are ongoing and will also be added once completed. Future perspectives for the database is to add a number of useful links, for example, sites where genetic diversity analysis software can be downloaded freely. Other perspectives is to link the MOLCAS data base to and genotyping activities on cassava in the genetic resources challenge program which is expected to begin next year.

3.2 Cassava Biotechnology Network's activities for 2003

Alfredo A. C. Alves
SB-2 Project

Introduction

The Cassava Biotechnology Network for Latin America and the Caribbean (CBN-LAC) is a network of cassava researchers and end-users united by the goal of mobilizing the development and application of biotechnological tools for the enhancement of the value of cassava for food security and economic development in the poorest rural areas of the LAC. The network is jointly funded by Directorate General for International Cooperation (DGIS) of the Netherlands' government and the Canadian International Development Research

Center (IDRC) under the project *"The Cassava Biotechnology Network in Latin America: Strategies for Integrating Small-Scale End-Users in Research Agenda-Setting, Testing and Evaluation"*, which initiated activities in 2001. The IDRC had committed funds for supporting the network for 2 years, 2001 and 2002 while the DGIS is providing funds for four years, 2001 to 2004. The CBN-LAC's objectives are to: a) integrate the needs of small-scale cassava farmers, processors and consumers into biotechnology research planning; b) stimulate research on high-priority topics; and c) stimulate the exchange of information, techniques and research materials. Its outputs will enable small-scale cassava farmers, processors and consumers to benefit from advances in cassava biotechnology

The third year of CBN-LAC (2003) was devoted mainly to the following activities:

1) Monitoring and guidance for on-going projects at pilot sites in Colombia, Brazil, and Ecuador.

2) Implementing new projects under the CBN-LAC Small Grants Scheme in Colombia, Brazil, Cuba and Ecuador.

3) Scholarships for postgraduate studies in biodiversity under the Ginés-Mera Memorial Fellowship Fund.

4) Organizing the Sixth International CBN meeting (CBN-VI)

Report on Activities at Pilot Sites

COLOMBIA

3.2.1 Application of low-cost *in vitro* propagation techniques to conserve native varieties and produce quality cassava seed in southwestern Colombia

José Restrepo (Coordinator), FIDAR ; Gloria Ospina, FIDAR; Roosevelt Escobar, CIAT
Joe Tohme, CIAT ; Carlos Hernández, Farmer

Introduction

This project has been conducted since 2001 aiming to use biotechnology tools to conserve native cassava varieties while enhancing the food security of small farmers in Colombia's Andean region. The 2003 activities focused on fine-tuning the phases of hardening in vitro-produced material and transplanting it to the field. Several native cassava varieties

were also collected and identified using morphological descriptors, and will be subsequently analyzed by AFLP technique. Since this project has been concluded this year, this analysis and other follow-up activities is being continued by a new CBN project under Small Grant Scheme, which was approved and initiated this year (see 'Projects under CBN-LAC Small Grants Scheme' section)

Achievements and constraints

- A methodology adjusted for the adaptation of *in vitro* material to the greenhouse phase and its subsequent planting in the field. Plants were placed for 8 days in water and then transferred as such to the definitive planting site, where they were planted in plastic bags containing sterilized soil. This practice eliminates the additional expenses occurred when transporting the material between the laboratory and the farms, while also reducing the possibility of lodging during transportation.

- Continued incorporation of new equipment and less expensive reagents into the process. A laminar flow chamber was built with local materials, costing 10 times less than an imported chamber.

- Advances were also made in the adjustment of the MS basal media. Cassava seedlings showed best growth when the commercial product Ferrovital–NF was used.

- Use of the rapid propagation system based on two-budded cuttings by the group of farmers to complement the *in vitro* system. With this methodology, farmers multiplied nearly 6000 plants of the variety MCOL 1468 from *in vitro* material certified by the Colombian Agriculture and Livestock Production Institute (ICA, its Spanish acronym).

- Establishment of a bank in farmers fields with *in vitro* material from 6 clones of interest to farmers (MCOL 1522, HMC 1, CMC 523-7, CM 6740-7, MBRA 383, and MPER 183) to provide FSD-free material (FSD = frog skin disease). When these clones are harvested in 2003, they will be multiplied by the rapid propagation system, using two-budded cuttings, and distributed to local farmers.

- Collection of 14 native cassava varieties in the municipalities of Caldono, Piendamó, Morales, Santander de Quilichao, and Caloto (Department of Cauca) and their identification using the morphological descriptors applied by CIAT's germplasm bank. A sample of each variety was planted on two farms in the municipalities of Caldono and Piendamó, as well as in the greenhouse at CIAT-Palmira. These varieties will be evaluated in CIAT's Biotechnology Unit Lab, using AFLP to determine the degree of diversity among materials.

- Systematization of the traditional knowledge of local men and women regarding native varieties, especially their tolerance to different stress factors and their resistance to the

most common pests and diseases affecting cassava in the region, uses, starch quality, and use of plant parts (other than roots).

- Definition of a participatory work scheme involving men and women to strengthen the formal cassava seed production system using *in vitro* technology and rapid propagation in the rural communities of Bajo Santa Ana, Alto Santa Ana, and Quinamayó.

- Easy adoption by the group of farmers of the rapid propagation technique using two-budded cuttings. This method was implemented by the project as complementary to the *in vitro* method and farmers described it as easy and simple, and said it produced immediate results.

Outstanding results

- An *in vitro* cassava seed production scheme to be managed by small farmers was defined. The scheme consists of 6 phases: (1) receipt of certified material; (2) *in vitro* multiplication in the rural laboratory; (3) adaptation in the greenhouse of the material multiplied through tissue culture; (4) planting of material in farmer fields with adequate management of irrigation, pest control, and fertilization; (5) multiplication of cuttings obtained in the field by rapid propagation (two-budded cuttings); and (6) production of good quality cuttings through the conventional system.

- During both phases of this project, rural laboratories were built with their corresponding work areas, and the use of locally available, low-cost materials in their construction was evaluated. In addition, most of the components of the culture medium, such as salts, hormones, and sugars, were replaced with products obtained in the local market. The multiplication rate (1:3-4) obtained when local inputs were used to prepare the culture media was similar to that obtained when imported raw materials were used.

- Several of the native materials collected in central northern Cauca, for example Algodona Amarilla and Algodona Grande, present good starch quality and high percentage of starch. Therefore, an agreement was reached with several farmers and starch producers to increase the area planted to these two materials using *in vitro* propagation. FIDAR assumed production costs and CIAT provided the certified materials. These two cassava clones will hopefully be able to compete better with the varieties imported by starch factories in Ecuador and other regions of Colombia.

- The women belonging to the group were able to reach a consensus about their needs and ways to solve them. Priority will be given to the search for income-generating alternatives. The *in vitro* production of cassava seed is one of these alternatives, but because of the time involved, they decided to participate in complementary production activities such as the planting of cassava, pineapple and bean, individually and in

association, and the multiplication of commercial cassava varieties using rapid propagation.

- The results of group participation reported in this phase are characterized by the empowerment and commitment of farmers, who have showed interest in undertaking other initiatives that benefit the community, for example a program to improve the quality and coverage of basic secondary education and allowing the access of young people and adults, who because of economic problems cannot attend nearby schools. An improved educational level has allowed the group to better understand and analyze the processes of *in vitro* technology.

Difficulties with the work plan

- The cost of an *in vitro* cassava plant produced in the rural laboratory was estimated at US$0.29 (see final project report 2001). This value is still quite high and not sufficiently competitive for small farmers to purchase these plants. The project continued working on this aspect during the current reporting phase and was able to integrate *in vitro* production technology with the rapid propagation system using two-budded cuttings. The data needed to determine whether the integration of these two systems reduced costs should be available at the end of 2003.

- New systems are being studied to reduce the cost of handling and transporting cassava seed in the greenhouse, its definite adaptation in farmers' fields, and the diversification of use of rural laboratories for tissue culture of banana and fruit trees.

- The sharing of a common language and the level of confidence demonstrated by the group of farmers in the facilitating farmer ensured that project participants completed the six phases of the *in vitro* cassava production process in the rural laboratory and the multiplication in the field. However, the lack of academic training prevented several farmers from converting measurements of volume and weight and from making decisions to solve problems of contamination.

- Macro and microeconomic factors affected community participation in the project, for example:

  - High fluctuations in the exchange rate of the Colombia peso versus the US dollar
  - Low price of cassava roots in Colombia over the last 10 months
  - Legal and illegal importation of sour starch by large companies in the region
  - Increase in the number of agronomic and plant health problems (whitefly and diseases such as frog skin) over the last two years

However, the adoption of several complementary measures (production projects, training in cassava cultivation) has allowed the group to continue. The importance of acting in an

organized fashion to achieve project/group objectives in benefit of the community has been recognized.

Communication and dissemination of information

Project results have been disseminated at the local and national levels through the participation in different forums and workshops on topics related to farmer application of cassava tissue technology.

- Seminars and Workshops

Researchers and technicians working with the project participated, in 2002, in the following seminars and workshop:

- First Regional Workshop on Rapid Propagation (*In Vitro*) and Genetic Transformation of Cassava. CIAT, 25 February - 2 March 2002.

- Biotechnology in the Development of Colombia. First Colombian Congress on Biotechnology. Universidad Nacional de Colombia. Bogotá, June 2002.

- Intensive training course in modern cassava production and processing systems. CIAT, June 2002.

- Rapid propagation as a technology to support the multiplication of *in vitro* cassava materials by farmers. Santa Ana (Cauca), September 2002.

- Cassava seed production workshops held for the farmer associations of El Agrado, the Toez Indigenous Council, and La Arrobleda. August, September, and October 2002.

Publications

During 2003, two articles summarizing the project's experience will be submitted to the journals *Illeia* and *Scientific American Latinoamérica*

- Tissue culture for farmers: participatory adaptation of low-input cassava propagation by a resource-poor rural community in southern Colombia. R.H. Escobar, C.M. Hernández, G. Ospina, J. Restrepo, L. Muñoz, J. Tohme, and W.M. Roca. 2003.

- Applicación de la tecnología de propagación *in vitro* para producir semilla de yuca (*Manihot esculenta* Crantz) por pequeños agricultores: descripción y análisis de la experiencia. J.M. Restrepo, G.I. Ospina, C.M. Hernández, R.H. Escobar, J. Tohme and W.M. Roca. 2003

Lessons learned and future work

By implementing and assessing this *in vitro* cassava seed multiplication technology, farmers were able to maintain informal cassava seed production systems that yielded propagation rates of 1:3-4 every 45-60 days. The system was capable of producing 3250 plants per initial explant (plant), reaching an efficiency of 400% compared with the conventional vegetative seed propagation system currently used by regional farmers. Outstanding results were also obtained in the identification of technical parameters to build a low-cost rural laboratory, that could be easily operated by farmers, as well as of equipment and inputs to prepare the culture media, achieving an efficiency similar to that of specialized laboratories.

In addition, the farmer-farmer training methodology implemented by project researchers and technicians proved to be correct because it ensured that participants understood the concepts and acquired the skills needed to operate the rural laboratory. However, more time was needed than that initially planned for the group to understand and self-manage the different processes (prepare the culture media, plant the tissues, hardening the plants, establish plantlets in the field).

Although the simplification of the tested technology significantly reduced the costs of the infrastructure, inputs, and culture media used, the labor costs implied by the different processes continue to be quite high and make it impossible for farmers to assume *in vitro* seed multiplication. It is therefore important to continue evaluating new systems and diversify the use of the laboratory with other crops to ensure its long-term sustainability. Mechanisms must also be sought to attract the participation and support of different local institutions.

The use of *in vitro* technology by farmers is an alternative that solves the problem of availability of good quality seed, especially in the case of new varieties or when seed is scarce because of climatic and plant health problems.

The rural laboratory can also be used to multiply native cassava varieties of northern Cauca. These are currently being identified and cleaned at CIAT's Biotechnology Unit Laboratory for subsequent redistribution to the communities for their *in situ* conservation and multiplication of seed of those varieties enjoying greatest acceptance by farmers and starch producers.

BRAZIL

3.2.2 Farmer participatory in vitro cleaning and multiplication of local and improved cassava varieties

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Collaborating institution
Empresa Baiana de Desarrollo Agricola (EBDA)
Farming communities of Caetité (Southeast of Bahia, Brazil)

Staff directly involved
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Wania Fukuda, Embrapa
Osvaldo Pereira da Paz, Embrapa
Osório Vasconcelos, EBDA
Josué Cerqueira, EBDA

Introduction

The project took off effectively in March 2002 with the general objective of introducing and implementing participatory biotechnology methodologies with small-scale cassava farmers in the cleaning and multiplication of cassava varieties. This is to be done using the low-cost rapid *in vitro* multiplication techniques developed at CIAT. The project site is the Maniaçu region, Caetité municipality, Bahia, Brazil.

Achievements and constraints

- Training for Osvaldo Pereira da Paz (from Embrapa) on low cost cassava in vitro rapid multiplication techniques in the Biotechnology Research Unit of CIAT. The training was performed in two farmer communities: a) Santa Ana, in Santander de Quilichao city, where is located a low-cost in vitro laboratory, and b) San Rafael, in Jamundi city, where was observed a local low-cost facilities for hardening in vitro-produced material to produce clean seeds for rapid propagation and distribution of clean plants to the farmers.

- Visit of the technician responsible for low-cost biotechnology (Osvaldo Pereira da Paz) and social scientist (Maria das Graças Sena) to seven Caetité's communities (Tanquinho, Contendas, Junquinho, Lagoa do Barro, Passagem de Areia, Ingazeira and Lagoa de Fora) in order to select the pilot site for the work of low-cost multiplication. In this oportunity, a great interest of all the communities was observed in participating actively of the work. There is a large necessity of clean and improved seeds in the

region. The farmer's time availabily for training and water quality and availability for the work were analyzed. Considering these aspects was chosen the community 'Lagoa de Fora'. This community has available area for clone multiplication, water of good quality and farmers able to receive training and transfer techniques of low-cost cassava propagation. This community housing 60 families, with approx. 300 people, which are highly dependent of the cassava crop. Two women farmers and two EBDA's technician were selected to attend a training on low-cost cassava propagation at the pilot laboratory built in the CNPMF/Embrapa.

- Construction of the pilot laboratory for low-cost cassava multiplication at CNPMF/Embrapa. This lab will be the base for training of farmers and technicians, starting with the four persons already selected.

- Cleaning and in vitro multiplication of the following clones: 003, 005, 1318, 1389, 1393, Aipim Cachorro (Local), and Lazã (Local). In CNPMF, there are 1000 plants of these clones to be transfer to the low-cost lab at 'Lagoa de Fora'.

- Seeds (cuttings) of the improved and local selected varieties were supplied to CNPMF's Biotechnology Unit for meristem multiplication and cleaning.

Difficulties with the work plan

The initial work plan was delayed on account of personnel changes at CNPMF/Embrapa leading to the late take off of the project, effectively March 2002. Since this date, however, the pace of work has moved really fast. There is ever indication that the project will expand to other regions from Caetite. This prognosis is based on the numerous requests for the sighting of the pilot site in several other places and in the general enthusiasm observed amongst the farmers, extension agents and community leaders from other regions.

Communication and dissemination of information

The project has received a lot of publicity through the radio e.g. the local radio station, Radio Educadora de Caetité, meeting with farming communities, leaders of rural associations, Caetité Municipality, women associations, and the exchange of information between the farmers

3.2.3 Diagnosis of the use and production of cassava in Manabí province

Introduction

In order to update existing information on the status of cassava in Ecuador, a diagnostic study on the production and utilization of cassava in the Manabi Province of Ecuador was carried out as a pilot study. Manabi is the major cassava producing area of Ecuador. The main objective for this study is to determine the components of the major cassava systems in Manabi and by so doing relate cassava production and use with social and environmental dynamics in the communities. In all, 650 surveys were conducted in Manabi and the data from this already inputted. The analysis is on going.

At the end of 2001 and beginning of 2002 were carried out the surveys, concluding the part corresponding to data acquisition. Due to lack of personal for data analysis and transition of CBN coordination staff, the evaluation was delayed and started at the beginning of 2003. Visits to Ecuador were performed in order to get the data and to update some informations related to cassava cultivation with communities and farmer's associations. These informacions will allow CBN to undertake actions to complete the research and to provide some support to the sector of small production of cassava.

This study will serve as means for evaluating the status of cassava projects that have already been executed in Ecuador, constitute a guide for the execution of development in the cassava cultivating community of Manabi and also serve as the base line data for planning other development projects.

CBN has in close collaboration with PRGA identified some parameters that could be used to elicit certain urgent information from the data collected so that further work in Ecuador could be commissioned and these are summarized thus: a number of questions that would give a good indication of preferences that could be analyzed by gender (detectable by the respondent's name), age, wealth (as measured by landholdings and tenure status), principal occupation and agroecological zone.

- Questions that relate to the importance of cassava overall compared to other productive activities or community priorities include:
  - preference for cassava vs. other crops,
  - importance of cassava for family subsistence,
  - importance of cassava to household well-being, and
  - indication of what are considered the most important problems faced by the community.

- Questions that indicate preferences, problems and priorities for cassava include:
  - input requirements and constraints,
  - disease problems,
  - varieties known, used, preferred and reasons,
  - yield/production,
  - preferred characteristics for cassava plants and reasons - responses may overlap with previous,
  - demand for mix of varieties,
  - might give a rough idea of the importance of cassava for marketing vs. subsistence,
  - importance of being able to delay harvest,
  - asks about seed knowledge and supply within the community. May give an indication of the importance of cassava, as well as the degree of innovation and cohesion present in the community,
  - importance of cassava by-products,
  - varietal preferences of buyers of cassava or cassava products,
  - difficulties encountered in marketing fresh cassava, cassava starch and cassava flour,
  - difficulties encountered in processing (spec grating), and
  - productivity and profitability of cassava over time.

- Questions that give an indication of for whom cassava is important and the degree of voice/asset control that person has are:
  - who decides variety selection,
  - division of labor for cassava and other household production activities,
  - control over income from sales of cassava and cassava products, and
  - control over household income and expenses)

3.2.4 Projects under CBN-LAC Small Grant Scheme

Introduction

The CBN-LAC Small Grants Scheme aims at achieving the following:

- Foster cassava biotechnology projects with developing country – developed country or advanced laboratory linkages; and

- Permit and encourage developing country laboratories to work on cassava biotechnology research topics relevant to end-users', national and overall CBN objectives.

This small grants scheme primarily supports the planning of specific proposals for international collaborative research on priority cassava biotechnology research needs as defined by cassava end-users and farmers. Some grants are also awarded in the form of grants-in-aid for developing country biotechnology research operational expenses; for

emergency biotechnology research bridging funds; or for short-term training in specific biotechnological methods.

Due to unforeseen tragic occurrences, none of these grants could be awarded earlier than early 2003 implying a reserve of funds for this line item. CBN Coordination decided to make 2 rounds of awards for 2003. The first round of the awards advertised late 2002 has been approved. In all, a total of 11 awards amounting to US$116,900 were made to cassava research groups in the 4 countries where the network maintains pilot sites. The distribution of these wards by country and by research theme is presented in Tables 1 and 2

In Table 3 is showed the projects and their status. The Small Grants Scheme was meant to be competitive but on account of the peculiarity of the region, especially with regards to relative inexperience with competing for research funds, CBN Coordination had to extend deadlines and ask for the revision and resubmission of a majority of the grants.

Table 1 – Distribution of CBN-LAC Small Grants Awards for 2003 by country

Country	No. of awardees
Brazil	4
Colombia	1
Cuba	3
Ecuador	3
Total	11

Table 2 – Distribution of CBN-LAC Small Grants Awards for 2003 by research theme

Research Area	No. of awardees
Genetic Transformation	2
Genomics	3
Germplasm Conservation and Characterization	4
Tissue Culture, Rapid Multiplication and Seed Cleaning	2
Total	11

Table 1 – Projects of the CBN Small Grants Awards 2003

Project Title	Country	Institution	Award (US$)	Period
Genetic manipulation of proline biosynthesis in cassava aiming to increased tolerance to water stress	Brazil	Embrapa Cassava and Fruits	10,000	May/03 to Apr/04
Development of protocols for genetic transformation of cassava genotypes from Northeast Brazil	Brazil	Federal University of Ceará (UFC)	10,000	May/03 to Apr/04
Isolation of genes involved in the sugary phenotype in the storage root of cassava (*Manihot esculenta* Crantz)	Brazil	Embrapa Genetic Resources and Biotechnology	10,000	May/03 to Apr/04
Maintainance and characterization of cassava living collection at the University of Brasília	Brazil	University of Brasilia (UnB)	7,000	Dec/03 to Nov/04
Use of *in vitro* technology by small farmers to clean and preserve native cassava varieties in Southern Colombia's Andean Region	Colombia	Fundación para la Investigación y Desarrollo Agrícola (FIDAR)	13,000	Mar/03 to May/04
Rescue and production of high quality seed of local cassava and cocoyam varieties by biotechnology tools adapted to Cuban rural conditions	Cuba	Instituto Nacional de Ciencias Agrícolas (INCA)	9,900	Jun/03 to May/04
Application of new techniques to control some viral diseases in cassava (*Manihot esculenta* Crantz) in combination with massive propagation technique.	Cuba	Instituto de Investigaciones de Viandas Tropicales (INIVIT)	10,000	Dec/03 to Nov/04
Application of molecular techniques for genotypes differentiation of Manihot spp.	Cuba	Instituto de Investigaciones de Viandas Tropicales (INIVIT)	10,000	Jun/03 to Mar/04
Establishing the contribution of *Manihot leptophylla* to the genetic constitution of cassava and the differentiation of sweet and bitter types	Ecuador	Pontificia Universidad Católica del Ecuador (PUCE)	13,000	Jun/03 to May/04
In situ conservation of cassava varieties cultivated by Kichwas from 'Alto Napo'	Ecuador	Federación de Organizadores de la Nacionalidad Kichwa de Napo (FONAKIN)	12,000	Dec/03 to Nov/04
Cleaning of released varieties and recovery of cassava genetic resources in production area of Ecuador	Ecuador	Instituto Nacional Autonomo de Investigaciones Agropecuarias (INIAP)	12,000	Dec/03 to Nov/04

3.2.5 Genetic manipulation of proline biosynthesis in cassava aiming to increased tolerance to water stress

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Introduction

The generation of transgenic cassava plants has a high potential as a complement to traditional plant breeding program in improving agriculturally valuable traits. However, plant genetic transformation efficacy of cassava, like many other crops, requires the establishment of efficient protocols for plant regeneration. Cassava plant regeneration efficiency is known to be genotype dependent, varying between 5 and 70% (Zhang et al., 2001) which may constrain the use of genetic transformation technology in cultivars with low regeneration frequency. Therefore, the first step of our research project was aimed to the establishment of efficient plant regeneration protocols of the selected genotypes. Simultaneously, elaboration of the gene constructs and introduction in the *Agrobacterium* was also performed.

Methodology

Plant material: Cuttings of cassava genotypes MCol 22, Aramaris, Aipim-Brasil and three genotypes selected for their high β-carotene content (BGM1153, BGM1692 e BGM1728) were grown in pots under greenhouse conditions.

Establishment of in vitro culture of explant donor plants: One month-old newly formed shoots of all six genotypes were surface decontaminated with 10% (v/v) of a commercial solution of sodium hypochlorite for 15 minutes and rinsed five times with sterile distilled water. Shoot tips were excised and transferred to culture flasks containing E4 medium (Table 1) for establishment of vitro conditions. After three weeks of culture on E4 medium, the explants were transferred to 17N medium (Table 1) for multiplication. Multiplied shoots were then transferred to MS (Murashige & Skoog, 1962) medium without growth regulators for root formation. All cultures were maintained at 27±1°C under 16 hours photoperiod.

Table 1. Macronutrient, micronutrient, vitamins and growth regulators composition of the culture media used in the establishment of in vitro explant donor plants.

Macronutrient (mg/l)	4 E	17 N
NH_4NO_3	1650	577.5
KNO_3	1900	665
$CaCl_2.2H2O$	450	154
$MgSO_4.7H_2O$	370	129.5
KH_2PO_4	170	59.5
$FeSO_4.7H_2O$	27.8	9.73
$Na_2EDTA.2H_2O$	37.3	13.055
Micronutrient (mg/l)		
KI	0.83	0.291
H_3BO_3	6.2	2.17
$MnSO_4.4H_2O$	22.3	7.805
$ZnSO_4.7H_2O$	8.6	3.01
$Na_2MoO_4.2H_2O$	0.25	0.088
$CuSO_4.5H_2O$	0.025	0.009
$CoCl_2.6H_2O$	0.025	0.009
Thiamine - HCl	1.0	1.0
Inositol	100	100
ANA	0.02	0.01
BAP	0.04	-
AG_3	0.05	0.01
Sucrose	20 g/l	20 g/l
Agar	7 g/l	7 g/l
Phytagel	1.8 g/l	1.8 g/l

Gene construct and transformation vector preparation: The binary plasmid pBI121 containing the P5CS gene from *Vigna aconitifolia* under the control of the constitutive promoter CaMV35S, with the genes *npt*II (neomycin phosphotransferase, for resistance to the antibiotic kanamycin) and *uid*A (β-glucuronidase or GUS, reporter gene) was amplified using the strain DH5α of *Escherichia coli*. Bacteria were grown overnight on 25 ml liquid LB medium. Plasmids were harvested using the mid-prep alkali lysis protocol (Sambrook et al.,1989).

The binary plasmid was transferred to the disarmed hypervirulent *Agrobacterium tumefaciens* strain EHA105 were transformed by electroporation. Bacterial cultures were grown overnight at 28°C, in liquid YMB medium (0.5 g l^{-1} K_2HPO_4, 0.1 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} NaCl, 5 g l^{-1} glucose, 10 g l^{-1} de mannitol and 0.4 g l^{-1} yeast extract) supplemented by 20 mg l^{-1} rifampicin and 30 mg l^{-1} kanamycin. Glycerol at 20% final concentration was added to the culture. The bacterial culture was separated in aliquots in 1.5 ml Ependorf tubes and stored at -80°C.

Results

In vitro culture of explant donor plants was established for the six genotypes. Plants are currently on rooting medium (Figure 1). This step is essential in order to obtain plant material for the following experiments on plant regeneration and genetic transformation using protocolos based on Roca et al., (1984) and Mathews et al. (1993) as we had proposed in the research project. Transformation vector in the *Agrobacterium* strain EHA105 was concluded and, in addition, a gene construct containing the gene *bar* (resistance to the herbicide ammonium glufosinate) instead of *npt*II is currently under way. This gene has also an agronomic interest and has been successfully used in other cassava genotypes (Sarria et al., 2000).

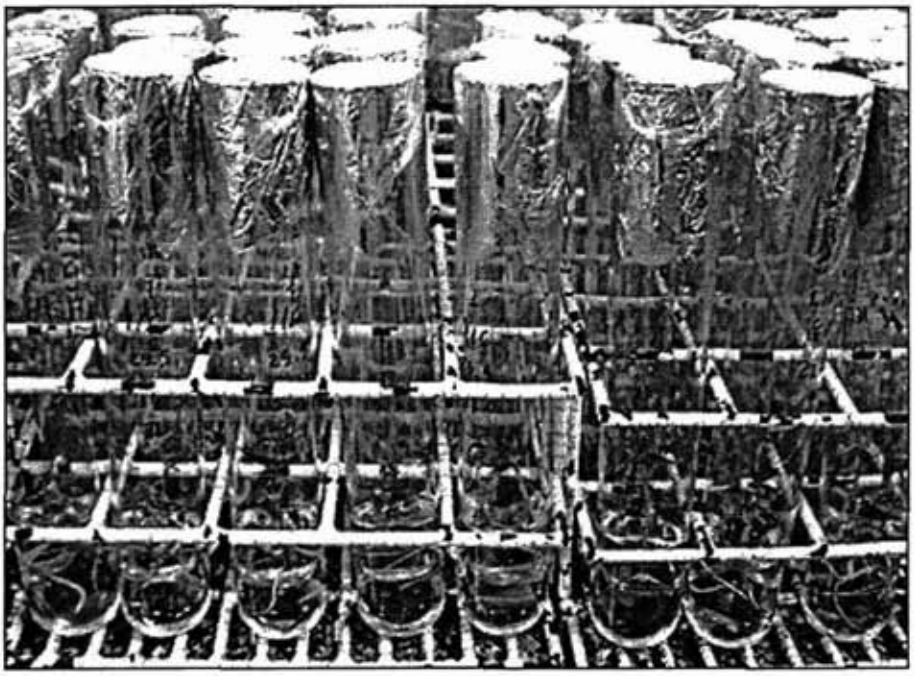

Figure 1. Explant donor plants of the six genotypes on rooting medium

Observations

The present research project is according to the proposed time table presented in the grant application.

Three genotypes of cassava with high β-carotene content were added in the experiments. Dr. Fernanda V. D. Souza (CNPMF) was included in the research group and Dr. Luiz F. P. Pereira (former researcher at IAPAR), unfortunately, had to leave the research group due to job exchange.

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Zhang, P.; Phansiri, S.; Puonti-Kaerlas, J. (2001). Improvement of cassava shoot organogenesis by the use of silver nitrate in vitro. Plant Cell Tissue and Organ Culture 67: 47-54.

3.2.6 Development of protocols for genetic transformation of cassava genotypes from Northeast Brazil

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Paul Chavarriaga, CIAT

Introduction

This is a training program in cassava transformation technology at the Biotechnology Unit of CIAT aiming to develop protocols for the genetic transformation of major cassava varieties from Northeast Brazil. The graduate student who is being trained is Terezinha Feitosa Machado who is a research scientist from Embrapa/CNPAT and PhD student at UFC.

The main steps for this project are:
a) developing friable embryogenic calli (FEC) for cassava varieties adapted to NE Brazil;
b) developing transformation protocols using *Agrobacterium* carrying marker genes; and
c) monitoring progress on modified cassava plants

Methodology

The cassava varieties that are being evaluated are listed in Table 2. The plant material prepared in Brazil and brought to CIAT were somatic embryos obtained from stem apex induced on medium MS2-50Pi (salts and vitamins MS, sucrose 2%, CuSO4 2μM, Pidoran 50μM, Agar 0.6%)

Table 2 – Cassava varieties from NE Brazil that are being evaluated for genetic transformation

CNPMF Code	BRA Code	Vulgar name	Collected in (City-State)	Institution
BGM 0365	000086	Água Morna	Pacatuba-CE	EPACE
BGM 0394	008087	Rosinha	Mage-RJ	IPEACS
BGM 0260	007277	Rosa	N.S. das Dores-SE	IPEAL
BGM 0549	012611	Amansa Burro	Itapirema-PE	IPA
BGM 0123	004502	Aparecida	Cruz das Almas-BA	EAUBA
BGM 0004	005533	Milagrosa	Cruz das Almas-BA	IPEAL
BGM 1063	073245	Tapicina	Berberibe-CE	CENARGEM
BGM 1467	102482	Bujá Preta	na	na

na = not available

The embryos are being subcultivated in the mediums MS2-50Pi, MS2-BAP 2μM (salts, vitamins MS, sucrose 2%, 0.4 mg/L of BAP and agar gelrite 3:1); and medium GD2-50Pi (salts and vitamins GD, sucrose 2%, 50μM (12mg/L) of picloram, agar-gelrite 3:1). The genetic transformation is being performed in green cotiledons using the technology of Agrobacterium tumefaciens with lineage Agl-1 1305-2 pCambia. The transformation process is going on.

3.2.7 Isolation of genes involved in the sugary phenotype in the storage root of cassava (*Manihot esculenta* Crantz)

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Claudia Regina B. de Souza, Postdoctorate Student
Julio Cezar Cascardo, UESC

Introduction

This project provide a financial suport to a post-doc student to perform a research that addresses the issue of food security in Brazil by improving food quality through exploitation of novel Amazon cassava genotypes with increased diversity of storage root quality traits. The research focuses on the biochemistry and molecular genetic analysis of recently discovered genotypes with unusual properties of accumulating sugar, and starch in the storage root. Modern genome biotechnology will be used to study the biodiversity and biology of the storage roots of cassava.

The variability of free sugar and novel starch accumulated in the storage root of identified wild clones of cassava will be explored by a) biochemical characterization of starch synthesis and degradation; b) isolation of mutated candidate genes with homologous and heterologous genes sequence coding for enzymes of the starch synthesis and degradation pathway, and gene expression (RNA) analysis as well as gene sequence variability; and c) isolation of genes differentially expressed in these contrasting phenotypes by sequencing several subtractive cDNA libraries.

The main outputs from this project are:

- Better understanding of the regulatory biochemical mechanism on the diversity of starch naturally occurring phenotypes. Identification of the molecular structure of the isolated novel starch, and amylopectin structure phenotype isolated. Identification of the molecular structure of the candidate gene coding for the enzyme responsible for the diversified amylopectin structure isolated. Obtain biochemical tools to be used in the identification and isolation of natural diversity of starch structure in cassava germplasm. Obtain molecular biology tools to be used in the identification and isolation of new clones of cassava. Offer new alternative of use of the storage root of cassava to the farmer and the industry.

- Clone and sequence the cDNA of known genes coding for starch synthesis and degradation enzyme in cassava. Gain knowledge about the presence or absence of specific gene coding for starch synthesis and degradation enzyme by gene expression analysis at the level of mRNA. Obtain molecular biology tools to be used in further advanced technology to alter starch structure in local cassava varieties and/or other crops. Gain knowledge on the structure and regulation regions of the genes of starch synthesis enzyme, needed to establish new technological strategies.

- Gain knowledge of the sequence of the genes differentially expressed, that would help to explain the storage root tissue phenotype in sugary clones. Generate a data base of EST from the diversity of the storage root of cassava. Obtain genomic tools to be used in data mine to dissect the formation of the storage root of cassava. Training scientist on the new technology as well as promote the distribution of the biotechnology to the poor.

Methodology

Plant material

Field grown plants in Embrapa Genebank collection, composed of 26 high sugar clones.

Experimental strategies

Multiple experimental strategies are being used, depending on the starch phenotype. The high sugar class of storage root allowed to identify three types starch phenotypes, being amylose free starch, phytoglycogen starch type and differentiated branched amylopectin when compared with starch from the commercial cassava. Therefore each type of phenotype is worked under appropriated framework of mutation already identified in other plant system. In the amylose free starch clone, the strategy uses the waxy mutation framework of rice and corn, and uses the candidate gene of the granule bound starch synthase as target gene. In the phytoglycogen accumulating clone the strategy use the sugary mutation framework of pea and corn, and the candidate gene coding for the debranching enzyme is the target gene. In the highly branched amylopectin it uses the soluble starch isoforms mutation framework of corn, and the candidate gene coding for the soluble starch synthase I is the target gene. The differential expressed gene of the high sugar storage root in relation to the commercial cassavas is used to build an EST database with the sequence of a subtractive cDNA library to generate the needed molecular tools.

Biochemical characterization

For the biochemical characterization will be performed the following main steps:

- Storage root tissue sampling and preparation for carbohydrate analyses;
- Reducing sugars quantification;

- Quantification of glucose, sucrose, total starch, and apparent amylose;
- Storage root tissue sampling and preparation for enzyme activities and protein quantification;
- Soluble protein quantification;
- Estimation of Proteins bound to the starch granule;
- Activity of the enzymes involved in the starch synthesis: ADP-Glucose Pyrophosphorylase (AGP), Soluble Starch Synthase (SSS), Granule-Bound Starch Synthase (GBSS), Starch Branching Enzyme (SBE), and Starch Debranching Enzyme (SDE);
- Storage root tissue sampling and preparation for starch granules characterization;
- Microscopy of the starch granules observation; and
- Estimation of size and specific area of starch granules.

Results

Functional genomics applied to the diversity of storage root of cassava

Six subtractive cDNA libraries were assembled and are being sequenced. The results so far, can be summarized as follow:
1) A collection of more than 22000 cDNA clones has been assembled.
2) A pilot sequence initiative generated more than 2500 high quality sequence (PHRED>20).
3) Conventional RNA blots probed with cDNA probes belonging to several classes of genes confirmed the efficiency of the subtraction techniques used to building the differential expressed cDNA library.
4) Several classes of new genes have been revealed in our preliminary analysis of the sequence of our EST bank.

These new tools resources will be used in this project to isolate the genes related to *sugary* phenotype.

3.2.8 Maintainance and characterization of cassava living collection at the University of Brasília

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Staff directly involved
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Introduction

The target of this project is a cassava living collection, housed in University of Brasilia, which consists of rare wild *Manihot* species, that have been extincted in their natural habitats, and some interspecific hybrids. In order to preserve this collection, which has 16 wild species and 13 hybrids of cassava with wild species, this project aims for the following objectives:

- To propagate old and degenerated accessions of both wild and interspecific cassava hybrids
- To characterize these introductions morphologically and cytogenetically
- To improved the field facilities for maintaining the living collection
- To create an adequate database system for data characterization
- To create a seed genebank

Outputs

The main outcome of the project will be the maintainance of wild species of cassava that already disappeared in their natural habitats from complete extinction, saving the rare examples of interspecific hybrids from degeneration and extinction to enable use them in the future for cassava improvement, using their invaluable gene pools.

3.2.9 Use of *in vitro* technology by small farmers to clean and preserve native cassava varieties in Southern Colombia's Andean Region

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Results

Collection of varieties

Thirteen new native materials of yuca were collected in the municipalities of Buenos Aires, Caldono, Morales and Santander of Quilichao. These materials with the 14 gathered at the end of the second semester of the 2002 were sowed in three nurseries in the farmers' parcels in the municipalities of Caldono (1.500 m), Piendamó (1.700 m) and Santander de Quilichao (1.000 m). For each one of these materials fields in the nurseries were evaluated the following morphological describers: color of apical leaf, pubescens, shape of central lobe, plant height, height of first ramification, degree of branching, length of the filament, branching habit, habit of stem growth, color of the stem epidermis, color of the root cortex, color of the terminal ramification, flowering, plant type, peduncle in root, cortex color, root pulp color, texture and constrictions of the root. For each one of these collected materials a collection record was elaborated, with the purpose of comparing them with the database of the Genetic Resources Unit of CIAT.

Cleaning and identification of the varieties

In different nurseries, the technicians of CIAT have taken samples of each varieties with the purpose of carrying out meristems cultivation starting from mature stakes of each genotype. With the in vitro materials will begin a termoterapy process and of the materials coming from the selected stakes young leaves were harvested and total DNA was extracted by protocol of Dellaporta (Figure 1). The DNA was quantified by fluorometer.

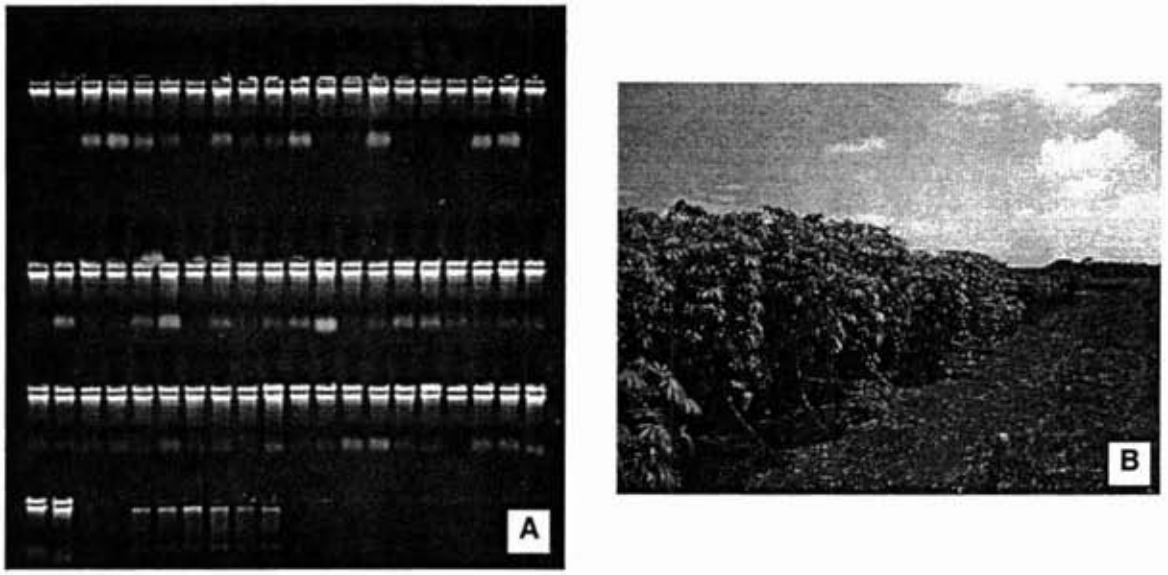

Figure 1 – A) Gel showing the total DNA extracted from young leaves of mature stakes of the local varieties collected in Cauca. B) Plants of six varieties of commercial interest ready to be propagated and distributed to the farmers.

Farmers' verbal registration about the collected cassava varieties

With the purpose of systematizing the local farmers knowledge about the native and introduced varieties of cassava collected in the North of the Cauca, meetings were carried out with some groups using open type questions and obtaining the following answers:

Question 1: What is the opinion that you have about the native cassava varieties in relation to those introduced or improved?

Opinions are divided, 60% of the farmers has preference for the native materials, arguing that they have seed permanently and most of the varieties have double purpose (starch and fresh consumption). However, they consider that some native varieties have diminished their growth and development and many of them are disappearing.

In relation to the improved or introduced varieties they consider that they are more precocious, they have higher production and they get less disease and pests, but some of them after being cultivated during 3 or 4 cycles show low yield. Another difficulty is the lack of planting material.

Question 2: What are the main characteristics of the collected varieties?

In Table 1 a summary is presented on the farmers' opinions about the collected varieties.

Question 3: What are the main factors that the farmers keep in mind to cultivate a native variety?

- Farmers from medium and high zones (1400 - 1700 m)

- The seed should be obtained in the region.
- The variety should be for double purpose (fresh consumption and starch).
- Good content and quality of starch.
- Good yield and resistance to pests and diseases.
- Do not need so much fertilizers.
- The varieties of more acceptance in order of importance for the farmers from high zones are the following: "algodona grande" (verdadera algodona), "bajuna", "chirosa" y "parroquiana".

- Farmers of low or warm area (900-1300 m)

- The variety should be for double purpose.
- Good yield and resistant to pests and disease, especially to white fly and frog skin.
- Varieties with more acceptance in this area are: "Rojita", "Raya 7", "Falsa chirosa".

Question 4: What other parts of the cassava, different to the roots, are used by the communities?

- The root peel is used as organic fertilizer.
- The leaf is used for feeding of cattle and pigs, the same as the by-products of starch processing.
- The hearts are used as greenness for the sausages or stuffs.
- The water from starch processing is used to control stomach aches and diarrheas.

Table 1 - Main characteristics of the collected varieties according to the farmers

VARIETY (local name)	*COLLECTION'S PLACE*	*PRODUCTION'S POTENTIAL*	*ADVANTAGES OF THE VARIETY*	*LIMITATIONS*
1- PARROQUIANA	Vereda La Unión (Piendamó). 1700 m. It has been known for 30 years in the region.	Thick roots and of good size when it is harvested after 16 months. Yield is good when fertilizer is applied.	It is known in the region since 30 years. It is tolerant to the excess of humidity and has little disease incidence. It is used for fresh consumption.	It is very late, needs more than 1 months to obtain good yields.
2- VARITA	Vereda La Unión (Piendamó). 1700 m. It has been known for 10 years in the region.	Regular production and it produces little planting material.	Big and small roots stuck to the stem, favorable to the harvest.	Its yield in the last years is not ver good.
3- FALSA CHIROSA	Vereda Pescador (Caldono). 1500 m. It has been known for 12 years in the region.	Good yield and formation of roots.	It is well accepted in the market due to cortex pink color. It has good market for fresh consumption.	It is susceptible to Phytopthora.
4- ALGODONA GRANDE	Vereda El Mango (Piendamó). 1700 m. It has been known for 40 years.	The yield is good when the weeds are controlled and is fertlized. It resists the drought. Good ramification and seed production.	It is a variety of high acceptance for those that produce starch, for its quantity and quality. The true 'algodona'.	It is late, it needs from 16 to 18 month to produce good starch.
5- ROJITA	Vereda San Miguel (Buenos Aires) 1100 m. It has been known for 14 years.	Using stake of good size its yield is better.	It is used for fresh consumption and starch. In the last years the processors of starch prefer it for starch quality and precocity.	It produces little planting material.
6- ALGODONA GIGANTE	Vereda El Mango (Piendamó). 1700 m. It has been known for 12 years.	It presents good yield when fertilized and weeds controlled. It adapts better above 1500 m.	It has high acceptance by the processors of starch for its quality and quantity.	It is susceptible to bacteriosis. It need between 16 and 17 months to produc well.
7- VENENOSA O MATASUEGRA	Vereda Mary López (Buenos Aires – Cauca). It has been known for 20 years.	It is a rustic variety that supports drought.	It is a variety for industrial use (production of starch).	Its production is low when the see doesn't have good quality. It is no good for fresh consumption.
8- ALGODONA RAPIDA	Vereda La Independencia (Piendamó). 1500 m.	Variety of good yield when fertilized and when doesn't receive a lot of water.	Good quality of starch.	It is susceptible to Phytopthora.
9- SATA	Vereda La María (Piendamó). 1500 m. It has been known for 15 years.	It presents good yield, especially when using thick seed of good size.	It presents early branching avoiding the proliferation of weeds. It is tolerant to bacteriosis.	Thin stems and it is a late variety (1 months to be harvested).
10- AMARILLA	Loma de los reyes (Piendamó) 1700 m. It has been known for 25 years.	Plant of good size, stems of good diameter and short internodes. Good yields especially when fertilized with chicken manure.	Good acceptance for fresh consumption due to its thick roots with conical shape.	It takes between 16 and 18 months t obtain good yield.
11- VALLUNA	Vereda El Mango (Piendamó). 1600 m. It has been known for 15 years. It was brought from coffee area of the 'Valle'.	It has good yield when fertilized and the weeds controlled.	Variety of double purpose (starch and fresh consumption).	It presents not well formed roots, but i is very accepted for fresh consumptio by the families.
12- TOTOQUEÑA	Vereda el Mango (Piendamó). 1600 m. It has been known for 12 years.	Variety of good yield, big and medium roots.	It is good for fresh consumption and to process starch. It is a precocious variety, can be harvested at 12 months.	It presents some difficulties fo harvesting (pull out the roots)
13- CHIROSA	El Porvenir (Caldono) 1500 m. It has been known for 12 years. It was introduced by several farmers from	Variety of good yield. It produces big, medium and small roots.	It is very good for the fresh consumption market. It gets better prices than other varieties.	The plant material found in the regio of 'Caldono' and 'Piendamó' is ver deteriorated and its production is low.

VARIETY (local name)	*COLLECTION'S PLACE*	*PRODUCTION'S POTENTIAL*	*ADVANTAGES OF THE VARIETY*	*LIMITATIONS*
	Armenia (Quindío).			
14- VERDE O VERDECITA	Vereda Independencia (Piendamó). 1500 m. It was introduced from Buenos Aires to Caldono.	Its yield in this area is not the best, it behaves well between 1000 and 1300 m.	It is a very good variety for fresh consumption.	It doesn't present a lot of starch. It is very susceptible to caterpillars.
15- PATA PAVA	La María (Piendamó). 1600 m. It has been known for 10 years.	It presents good yield with big and medium roots.	It is very accepted for the fresh consumption in the farm.	Late variety, needs between 16 and 18 months to obtain good production.
16- BAJUNA	Piendamó. 1400 m. It has been known for 40 years. It is one of the oldest varieties.	Good yield when fertilized and the weeds is controlled in the first months.	It is good for fresh consumption and starch production.	Late variety, needs from 15 to 18 months. The plant material is deteriorated..
17- CORREITA	Pescador (Caldono) 1500 m. It has been known for 10 years.	Its yield is not very good. The planting material is deteriorated.	Variety recommended for fresh consumption in the farm. No very good acceptance by pests.	Late variety, needs between 16 and 18 months. It doesn't produce so much planting material.
18- MEJORADA INDEPENDENCIA	Vereda Independencia (Piendamó), 1500 m. Variety introduced by Fidar and CIAT since 4 years.	It presents good yield and thick roots of good size.	Variety of double purpose (fresh consumption and starch production).	It needs good fertilizers. The cortex color is not very attractive for the market.
19- CHIROSA ROJA or BATATA	La María (Piendamó) 1700 m. It has been known for 20 years.	It has an acceptable yield when using planting material of good quality.	It presents very good acceptance for the fresh market.	The plant material is not of good quality. Before it produced more.
20- BLANQUITA	El Turco (Santander de Quilichao) 1300 m. It has been known for more than 10 years.	Good yield when using planting material of good quality.	I has very good acceptance for processing due to quality and quantity of starch.	This variety is disappearing for the loss of the quality of the plant material.
21- YUCA PAPA	Mondomo (Cauca) 1300 m. It has been known for 15 years.	Good yield when the weeds is controlled in the first months.	It is a variety of double purpose (fresh consumption and starch). It is used for family consumption.	It is not very attractive for market due to shape and color of the roots.
22- REGIONAL MORADA	Piendamó (S. José) 1700 m. It has been known for 10 years.	Its yield has been good (25 ton/ha) but in the last years have diminished.	It is used for fresh consumption and has very good acceptance for market.	Susceptible to bacteriosis.
23- PANAMEÑA	Cajibío (Cauca) 1800 m. Not very well-known variety, it was introduced by CIAT.	It presents good production potential.	It is used for fresh consumption and starch.	Susceptible to bacteriosis.
24- SM 850-1	Cajibío (Cauca) 1800 m. Variety introduced by CIAT.	It has good yield especially when fertilized.	It is a variety of double purpose (fresh consumption and starch).	It doesn't produce so much plant material.
25- CHIROSA (2)	La María (Piendamó). 1650 m. It is known as 'Chirosa' but is not the true one.	It presents good yield.	Good quality for fresh consumption in the farm.	The color of the cortex is not pink and therefore it is not very good for market
26- YUCA BLANCA	Vereda El Carmen (Piendamó). 1700 m. It has been known for more than 10 years.	It presents a good yield (18 ton/ha). The plant material is being deteriorated.	It is a variety of double purpose. Good content of starch.	It doesn't produce so much plant material. Many branching.
27- SAUCE	Villa Clemencia (Piendamó). 1700 m. It has been known for more than 15 years.	Low yield.	Can be used for fresh consumption and starch.	Susceptible to bacteriosis.

Evaluation and maintenance of cassava varieties multiplied *in vitro*

During the last 8 months the evaluation and monitoring of the materials multiplied *in vitro* by farmers, in the first phase of the project, were continued. The varieties or materials that are available to be harvested in September and then multiplied by fast progatation technique through rooting induction of 2 nodes stakes, are the following: HMC-1, MBRA 383, MPER 183, MCOL 1522, CM 6740-7 and CM 523-7.

These materials multiplied by fast propagation will be planted in the farmers' plots in San Rafael, Santa Ana and Caldono with the purpose of evaluating their yield and sanitary quality of the planting material by technicians and farmers.

Communication and socialization of the information

The diffusion of the project's results has been carried out at local level in forums with the schools of Santander of Quilichao, where the project's advances were presented to a group of 12 teachers interested to know how to use the different tools used for cassava *in vitro* propagation.

During the second semester a group of 6 teachers from 'Fernández Guerra' Scholl, in Santander of Quilichao will be trained in *in vitro* seed propagation of cassava.

With the participation of the farmers'group of Santa Ana and Caldono the training on cassava seed quality and the need for conserving local varieties, have continued. Emphasis is also been focused in aspects related to better nutritional content of cassava and beans varieties, to include them in the diet and to strengthen the food security and the nutritio of the families. All these activities are guided to strengthen the organization and the labor participation of men and women in equal form in the different home and community activities.

Also, some ideas have been discussed on low cost cassava in vitro propagationt, with University's professors (National University and Nariño University, from Colombia) and with Dr. James Gorges from CTCRI, India.

Difficulties in the work plan

The main difficulties during this phase of the project were related to the following aspects:

- Some collected materials were confounded in their identification due to different vulgar names in the same region. This situation was corrected identifying them in the field using morphological descriptors by CIAT's expert.

- The materials sent to CIAT and multiplied in vitro have presented a slow growth and it has been necessary to change the cultivation medium to stimulate their growth. These two difficulties have retarded the proposed chronogram of activities.

- In relation to the participation of the farmers' groups (Santa Ana and Pescador) some farmers' demotivation has been reported due to low prices of the cassava roots and starch, situation that has diminished the planted area.

Future activities

- To begin AFLP's evaluation.

- To continue the thermoterapy treatments and *in vitro* cultivation until finding negative plants for frog skin.

- To certify for frog skin by graft with 'Secundina' variety.

- Once certificated, the multiplication activities should start to give the materials to the groups of Santa Ana, Caldono and Piendamó.

- To begin the activities of fast propagation and to plan a field day to delivery materials to the farmers involved in the project.

- To facilitate the delivery to CIAT of the native materials for low areas.

- To elaborate a new proposal to present to a donor in order to keep in mind the advances achieved by the project.

- To strengthen the participation and the farmers' organization linked to the project keeping In mind the activities that men and women carry out in the communities.

- To consolidate and to strengthen the training of the farmers for handling *in vitro* technology.

3.2.10 Rescue and production of high quality seed of local cassava and cocoyam varieties by biotechnology tools adapted to Cuban rural conditions

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Background

The main objective of this project is the rescue and multiplication of high quality planting material of cassava and cocoyam varieties selected according to farmers' requirements and preferences, through biotech tools adapted to the conditions of the selected rural communities.

The application of *in vitro* techniques for micropropagation of cassava and cocoyam varieties will facilitate the increment of the yields in these crops due to high quality of the seed produced and better tolerance to main pests and diseases, since the planting material will be free of contaminations. This effort is a high priority for food security in Cuba and also will open new possibilities for incorporation of the experience from rural women and technicians in the rural communities of Cuba.

Methodology and results

Diagnostic

This phase had two activities: 1) Survey'application to know how the flow of cassava and cocoyam seeds is performed within communities, its entrance and exchange points, and leadership in these crops. With the results of this survey will be evaluated the relationship among farmers' groups and their roles within the processes of acquisition, distribution and use of planting material; and 2) Realization of the first workshop with participation of the farmers from several regions involved in the project, as well as researchers, farmers' association representatives, local decision-makers politicians, and other stakeholders; the

objective of this workshop is to divulgate the project, discuss the general problems related to cassava and cocoyam cultivation, and establish the priorities of future actions. These two activities have been accomplished and the data analysis is in process.

Farmers' training and farmer participatory research on in vitro *plant adaptation*

During the first workshop many farmers have visited facilities utilized for micropropagation, perceived more details about this technique, and observed the genetic diversity of cassava and cocoyam when visiting INIVIT's collections.

In this phase was accomplished the second project's workshop aiming to establish the characteristics of *in vitro* plants and to arrange local conditions for *in vitro* plants of cocoyam, which currently are being multiplied and will be distributed to the actors in November 2003. Also, an adjusted timetable of activities and agreements were established.

Clones of cassava and cocoyam, selected by farmers, have been micropropagated. *In vitro* cocoyam plants and cassava stakes from micropropagated plants from INIVIT's elite clones will be cultivated in farmers' experimentation fields.

Two actors from INCA have attended a training at INIVIT about cassava and cocoyam micropropagation and farmers from La Palma, after training, will assemble the facilities for *in vitro* plants climatization.

These activities have been performed in the municipality of Santo Domingo, province of Villa Clara (homogeneous environment), and La Palma, community of San Andrés, province of Pinar de Río (heterogeneous environment).

3.2.11 Application of new techniques to control some viral diseases in cassava (*Manihot esculenta* Crantz) in combination with massive propagation technique

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Introduction

The systemic pathogenic organisms, as virus, spread with the planting material generation after generation; these agents not only affect the yield of the local cassava cultivars but also limit the maintenance of the germplasm bank, the regional and international exchange of clones and the supply of certified seed for extensive cassava production. Such pathogens are the cassava vein mosaic virus (CVMV), cassava common mosaic virus (CsCMV), cassava virus X (CsVX) and the agent of the cassava frog skin disease (CFSD).

This project will become available an alternative technology for virus elimination, with less time and less infrastructure requirements, which will decrease the cost of basic seed production of cassava. This outcome will facilitate the adjsutment of biotech tools for massive multiplication of certified seeds by cassava farmers, mainly those involved with cassava production for industrial starch.

Outputs

- Alternative technology for cassava virus elimination. The procedure (electrotherapy) would replace the current thermotherapy method applied in cassava (40° C at day-light and 35° C at night, during 3 to 4 weeks) to obtain, in combination with apical tissue culture, plants free of virus and other systemic pathogens. This technique will speed the growth of *in vitro* plants and get more regenerated plants.
- Personnel trained on application of electrotherapy at CIAT and other institutions interested in production of cassava planting material free of virus.
- Reduced cost of production of free disease cassava plants.
- Communications in specialized forums.

3.2.12 Application of molecular techniques for genotypes differentiation of *Manihot* spp.

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Introduction

With the development of molecular biology, several molecular markers have been used for identification of genetic variability in cassava cultivars from different regions and countries (Fregene et al., 1997; Fregene et al., 2003). The microsatellite technique or SSR (Simple Sequence Repeats) is attractive to study due to its abundance in the plant genome, its high polimorphism and adaptability to the automation. It has been appropriate for germplasm cassava characterization (Dixon et al., 2002; Azudia et al., 2002), and can contribute to evaluate the genetic diversity within cassava Cuban collection and to establish the relationships between the accessions and their wild or cultivated relatives.

The objective of this study is to evaluate the cassava genetic diversity and its phylogenetic relationships with cultivated relatives of Africa, South and Central America, in order to assist a sustainable management of the Cuban genetic resources.

Methodology

A total of 94 cultivars was collected in May 2003 in the Cuban cassava collection, according to its economic and/or genetic importance. Also 54 clones from Africa and America were incorporated (12 from Nigeria, 10 from Tanzania, 12 from Guatemala and 20 of South America), and other 13 genotypes of genetic interest.

Total DNA was extracted following Dellaporta et al. (1983); DNA concentration and quality were evaluated by fluorometry and electroforesis in gel, respectively. The samples of DNA were diluted to a concentration of 10 ng/ml for the subsequent analyses of PCR and to evaluation of 36 SSR markers that represent a wide covering of cassava genome. The PCR amplification for diversity studies with SSRs, the electroforesis in poliacrilamida gel and the dye with silver were developed according to adjusted approaches from Fregene et al. (2002).

Preliminar results

A total of 18 SSR has been evaluated, which have showed a high polimorphism, high amplification level and a great number of bands per primer (Figures 1 and 2), except the SSRY-132 primer that was monomorphic not allowing differentiation among the individuals.

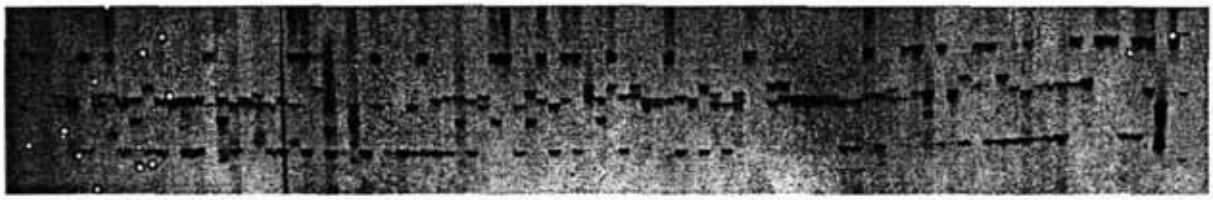

Figure 1 – PCR amplification of cassava cultivares from Cuba, Nigeria, Tanzania, Guatemala and South America with primer SSRY51.

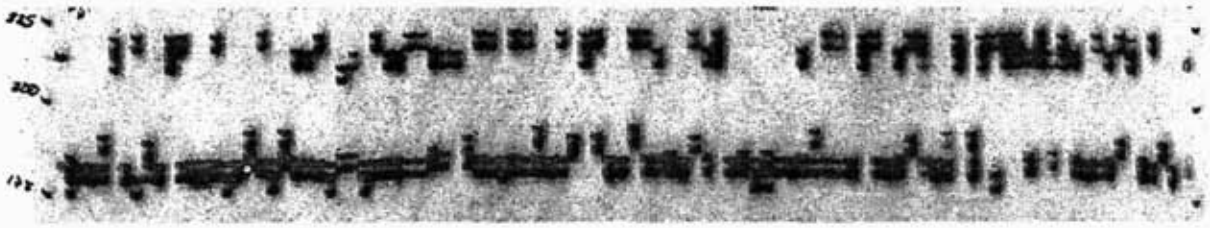

Figure 2 - PCR amplification of cassava cultivares from Cuba, Nigeria, Tanzania, Guatemala and South America with primer SSRY151.

Other SSRY primers will be evaluated up to complete 36. The reading and determination of band's weight will be carried out by the program 'Quantity one' and the statistic analysis will be performed using the following softwares: "Microsat","Gensurvey" and "FSTAT". A comparison matrix among countries will be designed with Fst values and the relationship will be analyzed by a cluster analysis, using the method UPGMA of NTSYS-PC.

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3.2.13 Establishing the contribution of *Manihot leptophylla* to the genetic constitution of cassava and the differentiation of sweet and bitter types

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Introduction

Allem (2002) proposes that *M. leptophylla*, a species whose distribution range includes southern Colombia, coastal Ecuador, Perú and reaches to Belém in Brazil, could be part of the synonymous complex of sub-species which includes *M. esculenta* ssp. *esculenta*, *M. esculenta* ssp. *flabellifolia*, and *M. esculenta* ssp. *peruviana*. The *M. leptophylla* botanical type is reported from the coastal province of Manabí in Ecuador.

Research dedicated to elucidate the position of *M. leptophylla*, and whether it may have contributed to the genetic constitution of cassava on the western side of The Andes was based on the following initial observations: i) extensive review of herbarium specimens indicated that only specimens from the western coast of Ecuador and the southwestern part

of Colombia are morphologically similar to the *M. leptophylla* type reported from the Manabí Province of Ecuador and ii) specimens of the species available from the Amazon Basin are morphologically distinct from those from the West leading us to believe that there may be a misidentification of specimens, given the plasticity of *Manihot* species. In this case, specimens form the eastern side of the Andes more probably correspond to *M. flabellifolia* than to *M. leptophylla*, which would be restricted to the lowlands on the western side of the Andes in Ecuador and Colombia. Molecular marker studies using AFLPs (Narváez & Second, 2002) clearly indicated that *M. leptophylla* is genetically very distant from cassava and from *M. flabellifolia*. Eventhough AFLPs give a total evaluation of molecular diversity given that they are distributed in the total genome and are considered reliable markers for diversity studies, their potential in an outbreeding plant such as cassava may be limited. Furthermore, as one of the objectives of this study is to evaluate possible hybridization and/or introgression microsatellite markers were chosen for their reproducibility, enabling compilation of data from different data sets and for their high allelic diversity as co-dominant markers which could generate markers that indicate specific introgressed sites on the genome of the species under evaluation.

The objectives of this project include: a) To determine the relation of *Manihot leptophylla* to the other species of the genus and in particular to cassava (*Manihot esculenta ssp. esculenta*) and b) To provide proof that *M. leptophylla* has contributed to the genetic constitution of cassava through hybridization and introgression, events that may have also led to the genetic differentiation between sweet and bitter cassava types.

Methodology

Plant samples

Samples from wild putative *M. leptophylla* populations at different geographical sites in Ecuador were collected in silica gel throughout 2002-2003. Feral plants encountered at these sites were also collected. Collection number and site are listed in Table 1. *M. esculenta* samples were collected in traditional fields in the western provinces of Manabí and Esmeraldas, collection number and site are listed in Table 2. A selection of samples for SSR analysis was made considering geographical location and ecological characteristics. The final data sets (Data 1 and Data 2) are listed in Tables 3 and 4.

Primers

An initial set of 15 mapped primers (Mba, et al 2001) were selected based on previous studies on cassava (Morillo, 2002) for preliminary evaluation of the data sets. These primers are: SSRY3 (CA) 17, SSRY9 (GT) 15, SSRY30 (CT) 22, SSRY31 (GA) 21, SSRY38 (CA) 17, SSRY40 (GA) 16, SSRY55 (GA) 16, SSRY68 (CT) 12 CC(CT) 17, SSRY80 (GA) 25, SSRY103 (GA) 22, SSRY108 (CT) CCT, SSRY100 (CT) 17 TT(CT) 7, SSRY135 (CT) 16, SSRY169 GA19A3GAA2, SSRY179 (GA) 28.

PCR conditions were based on Mba et al (2001); silver staining was performed according to Promega®.

Data analysis included principal coordinate analysis using NTSYS v. 2.1.

Results

Results were obtained for each of the data sets. Data set 1 included samples from different geographical origin within Ecuador for *M. leptophylla* in order to establish genetic relations to *M. esculenta*, which included samples of bitter and sweet types, the first represented by samples from French Guiana and the latter from western and eastern localities in Ecuador. Samples of *M. flabellifolia* included ones from French Guiana and from the CIAT world collection. Samples for Central american species were also obtained from CIAT. As can be seen in Figure 1, the first axis clearly shows a differentiation of *M. leptophylla*, as an extreme in the PCoA analysis in regard to *M. esculenta* (bitter and sweet types). *M. flabellifolia* samples, from French Guiana, is found at a center position. *M. leptophylla* could actually be genetically more similar to *M. aesculifolia*, although these two species differentiate on the second axis of variation.

Regarding *M. leptophylla*, there seems to be a geographical differentiation. The samples Ant1, Ant2 and Ant7, the most extreme in the PCoA analysis (Figure 1), are from the geographical site from were the botanical type is reported, Camarones, Manabí Province, Ecuador; therefore these can be considered "true" *M. leptophylla*. The samples from other localities, especially those from Guayas and Los Rios Provinces warrant further analysis.

Analysis of allele diversity shows that *M. flabellifolia* has the largest number of alleles (Table 5), followed by *M. esculenta* and *M. leptophylla*. Of the species analyzed, *M. flabellifolia* shares a large number of alleles with *M. esculenta*, while *M. leptophylla* and *M. esculenta* have few common alleles (data not shown).

The second sample set (Data set 2), includes samples of *M. leptophylla* and *M. esculenta*, especially traditional varieties grown in the coastal provinces of Manabí and Esmeraldas, as well as putative hybrids and feral plants. In this case, there is also a clear differentiation of *M. leptophylla* from *M. esculenta*. Within *M. leptophylla* there is not a high genetic diversity (Figure 2) while within *M. esculenta* there is a large diversity (revealed on the second axis of variation); especially noteworthy are the feral plants that may represent hybrid plants and, within *M. leptophylla*, sample Ant17 which has unique alleles and also an interesting position in the PCoA analysis (Figure 2).

These results give way to considering a new sample set, combined from the two described here. For this purpose, further sampling of Ríos and Manglares-Churute (Guayas) sites must be carried out.

Table 1 - List of wild (*Manihot leptophylla*) or feral cassava

Sample No.	Reference code	Species	Locality
Ant01-04-01		*M. leptophylla*	Tabuga, Manabí
Ant02-04-01		*M. leptophylla*	Tabuga, Manabí
Ant03-04-01		*M. leptophylla*	Camarones, Manabí
Ant04-04-01		*M. leptophylla*	Camarones, Manabí
Ant05-04-01		*M. leptophylla*	Tabuga, Manabí
Ant06-04-01		*M. leptophylla*	Camarones, Manabí
Ant07-04-01		*M. leptophylla*	Tabuga, Manabí
Ant08-04-01	Ecu 1- Gerard	*M. leptophylla*	San Mateo, Esmerladas
Ant09-04-01	Ecu 2- Gerard	*M. esculenta*	Esmeraldas
Ant10-04-01	Ecu 3- Gerard	*M. esculenta*	Caimito, Esmeraldas
Ant11-04-01	Ecu 4- Gerard	*M. leptophylla*	Quingue, Esmeraldas
Ant12-04-01	Ecu 5- Gerard	*M. leptophylla*	San Mateo, Esmeraldas
Ant13-04-01	Ecu 6-Gerard	*M. esculenta*	Unión del Toachi, Pichincha
Ant14-04-01		*M. brachyloba*	Jatun Sacha, Napo
Ant15-04-01	CB-Guayas	*M. leptophylla*	BP Cerro Blanco, Guayas
Ant17-02-02	Mgl1- Manglares 1	*M. leptophylla*	Cerro Pancho Diablo, Guayas
Ant18-02-02	PP1- Patricia Pilar 1	*M. leptophylla*	Patricia Pilar, Los Ríos
Ant19-02-02	PP2- Patricia Pilar 2	*M. leptophylla*	Patricia Pilar, Los Ríos
Ant20-02-02	Mgl2- Manglares 1	*M. leptophylla*	Cerro Pancho Diablo, Guayas
Ant21-02-02	Mgl3- Manglares 2	*M. leptophylla*	Cerro Pancho Diablo, Guayas
Ant22-20-12-01	ET1-Cerro Blanco	*M. leptophylla*	BP Cerro Blanco, Guayas
Ant23-20-12-01	ET2-Cerro Blanco	*M. leptophylla*	BP Cerro Blanco, Guayas
Ant24-20-12-01	ET3-Cerro Blanco	*M. leptophylla*	BP Cerro Blanco, Guayas
Ant25-20-12-01	ET4-Cerro Blanco	*M. leptophylla*	BP Cerro Blanco, Guayas
Ant26-12-09-02	#9 colección de la Tola	*M.esculenta*	La Tola del Indio, Guayas
Ant27-12-09-02	#8 colección de la Tola	*M.esculenta*	La Tola del Indio, Esmeraldas
Ant28a	Ecu 7.1-Gerard	*M.esculenta*	germinations
Ant28b	Ecu 7.2-Gerard	*M.esculenta*	
Ant28c	Ecu 7.3-Gerard	*M.esculenta*	
Ant28d	Ecu 7.4-Gerard	*M.esculenta*	
Ant28e	Ecu 7.5-Gerard	*M.esculenta*	

Table 2 - List of local cassava varieties from Manabí and Esmeraldas Provinces

Sample No.	Local name	Species	Localidad	Province	Coordenates	
M001-03-2002	Mcol 2215	*M. esculenta*	Bijahual	Manabí	N 00 02 29.1	W 77 19 36.9
M002-03-2002	Cascaruda "2"	*M. esculenta*	San Vicente	Manabí	S 01 04 32.3	W 80 19 32.2
M003-03-2002	Cascaruda	*M. esculenta*	San Vicente	Manabí	S 01 04 32.4	W 80 19 32.3
M004-03-2002	Blanca	*M. esculenta*	San Vicente	Manabí	S 01 04 32.5	W 80 19 32.4
M005-03-2002	3 Meses	*M. esculenta*	Sapaniyal	Manabí	S 01 05 13.2	W 80 20 17.5
M006-03-2002	Peruana	*M. esculenta*	Quebrada Guillén	Manabí	S 01 04 38.7	W 80 18 28.5
M007-03-2002	Negra	*M. esculenta*	Quebrada Guillén	Manabí	S 01 04 38.8	W 80 18 28.6
M008-03-2002	Tres meses	*M. esculenta*	Charapotó	Manabí	S 00 49 51.6	W 80 29 34.9
M009-03-2002	Yuca de año	*M. esculenta*	Charapotó	Manabí	S 00 49 51.7	W 80 29 34.1
M010-03-2002	Tres meses	*M. esculenta*	San Clemente	Manabí	S 00 45 59.7	W 80 30 03.5
M011-03-2002	Colorada Colombiana	*M. esculenta*	San Clemente	Manabí	S 00 45 59.8	W 80 30 03.6
M012-03-2002	Yema de huevo	*M. esculenta*	Tablones, Junín	Manabí	S 00 57 13.5	W 80 13 56.0
M013-03-2002	Tres meses	*M. esculenta*	Tablones, Junín	Manabí	S 00 57 13.6	W 80 13 56.1
M014-03-2002	Blanca	*M. esculenta*	Junín	Manabí	S 00 56 24.6	W 80 14 10.6
M015-03-2002	Colorada	*M. esculenta*	Junín	Manabí	S 00 56 24.7	W 80 14 10.7
M016-03-2002	Tres meses	*M. esculenta*	Junín	Manabí	S 00 56 24.8	W 80 14 10.8
M018-03-2002	Huevo cambiado	*M. esculenta*	Potrerillo, Calceta	Manabí	S 00 52 24.2	W 80 10 58.7
M019-03-2002	Huevo cambiado	*M. esculenta*	Potrerillo, Calceta	Manabí	S 00 52 24.3	W 80 10 58.8
M020-03-2002	La babita	*M. esculenta*	Potrerillo, Calceta	Manabí	S 00 52 24.4	W 80 10 58.9
M021-03-2002	Seis meses	*M. esculenta*	Salida de Calceta	Manabí	S 00 50 40.1	W 80 10 22.1
M022-03-2002	Yema de huevo	*M. esculenta*	Pay Pay	Manabí	S 00 48 40.9	W 80 13 33.1
M023-03-2002	Espada	*M. esculenta*	Pitahaya	Manabí	S 00 48 17.9	W 80 15 00.6
M024-03-2002	Mulata	*M. esculenta*	Pitahaya	Manabí	S 00 48 17.10	W 80 15 00.7
M025-03-2002	Yuca de año	*M. esculenta*	Jama	Manabí	S 00 12 16.5	W 80 15 35.4
M026-03-2002	Tres meses	*M. esculenta*	Jama	Manabí	S 00 12 16.6	W 80 15 35.5
M027-03-2002	Blanca	*M. esculenta*	Jama	Manabí	S 00 12 16.7	W 80 15 35.6
M028-03-2002	Pata de Paloma	*M. esculenta*	Pedernales	Manabí	N 00 04 06.3	W 80 03 20.5
M029-03-2002	Espada	*M. esculenta*	Pedernales	Manabí	N 00 04 06.4	W 80 03 20.6
M030-03-2002	Cimarrona 1	*M. esculenta*	Bilsa	Esmeraldas	N 00 17 54.7	W 79 57 04.3
M031-03-2002	Cimarrona 2	*M. esculenta*	Bilsa	Esmeraldas	N 00 17 54.8	W 79 57 04.4
M032-03-2002	Cimarrona 3	*M. esculenta*	Bilsa	Esmeraldas	N 00 17 54.9	W 79 57 04.5
M033-03-2002	Cimarrona 4	*M. esculenta*	Bilsa	Esmeraldas	N 00 17 54.10	W 79 57 04.6
M034-03-2002	Amarilla	*M. esculenta*	Estero Ancho	Esmeraldas	N 00 42 07.3	W 79 54 53.8
M035-03-2002	Blanca	*M. esculenta*	Estero Ancho	Esmeraldas	N 00 42 07.4	W 79 54 53.9
M036-03-2002	Negra	*M. esculenta*	Agua Clara	Esmeraldas	N 00 46 16.4	W 79 55 49.9
M037-03-2002	Añera	*M. esculenta*	Albergues, Tonsupa	Esmeraldas	N 00 52 43.5	W 79 48 57.3
M038-03-2002	Blanca	*M. esculenta*	Albergues, Tonsupa	Esmeraldas	N 00 52 43.6	W 79 48 57.4
M039-03-2002	Negra	*M. esculenta*	Albergues, Tonsupa	Esmeraldas	N 00 52 43.7	W 79 48 57.5
M040-03-2002	Yema de huevo	*M. esculenta*	Albergues, Tonsupa	Esmeraldas	N 00 52 43.8	W 79 48 57.6
M17-03-2002	Serrana	*M. esculenta*	La Mijarra	Manabí	S 00 53 46.0	W 80 10 55.7

Table 3 - Data set 1 - wild Manihot species and M. esculenta

Sample No.	Species	Observation	Origin
VRH3	M.esculenta	sweet	Napo, Ecuador
VRH12	M.esculenta	sweet	Napo, Ecuador
M20	M.esculenta	sweet	Manabí, Ecuador
M37	M.esculenta	sweet	Manabí, Ecuador
G1	M.esculenta	bitter	French Guiana
G20	M.esculenta	bitter	French Guiana
G21	M.esculenta	bitter	French Guiana
G28	M.esculenta	bitter	French Guiana
G31	M.esculenta	bitter	French Guiana
G34	M.esculenta	bitter	French Guiana
G92	M.esculenta	bitter	French Guiana
G112	M.esculenta	bitter	French Guiana
G125	M.esculenta	bitter	French Guiana
GUY15	M. baccata		French Guiana
GUY17	M. baccata		French Guiana
GUY22	M. baccata		French Guiana
GUY23	M. baccata		French Guiana
GUY25	M. baccata		French Guiana
GUY 32-1	M. flabellifolia		French Guiana
GUY1	M. flabellifolia		French Guiana
GUY36	M. flabellifolia		French Guiana
GUY41-2	M. flabellifolia		French Guiana
Fla 318	M. flabellifolia		CIAT world collection
Per 4-12	M. peruviana		CIAT world collection
Ant 1	M.leptophylla		Manabí, Ecuador
Ant 2	M.leptophylla		Manabí, Ecuador
Ant 6	M.leptophylla		Manabí, Ecuador
Ant 7	M.leptophylla		Manabí, Ecuador
Ant12	M.leptophylla		Manabí, Ecuador
Ant19	M.leptophylla		Manabí, Ecuador
Ant 21	M.leptophylla		Manabí, Ecuador
N°1	M.leptophylla		Manabí, Ecuador
N°3	M.leptophylla		CIAT world collection
Chl2	M. chlorostica		CIAT world collection
Chl8	M. chlorostica		CIAT world collection
Chl9	M. chlorostica		CIAT world collection
Maes 1	M. aesculifolia		CIAT world collection
Maes 3	M. aesculifolia		CIAT world collection
Maes 7	M. aesculifolia		CIAT world collection
Rub 4	M. rubricaulis		CIAT world collection
Rub 29	M. rubricaulis		CIAT world collection
Fla 19	M. flabellifolia		CIAT world collection
Fla 61	M. flabellifolia		CIAT world collection
Fla68	M. flabellifolia		CIAT world collection
Ecu75	M. esculenta		CIAT world collection

Table 4 - Data set 2 - *M. esculenta* local varieties and wild *M. leptophylla*

Sample No.	Species	Origin
Blo 001	M.brachyloba	CIAT world collection
Ecu 82	M.esculenta	CIAT world collection
Ecu116	M.esculenta	CIAT world collection
M31	M.esculenta	Esmeraldas, Ecuador
M32	M.esculenta	Esmeraldas, Ecuador
M33	M.esculenta	Esmeraldas, Ecuador
M10	M.esculenta	Manabí, Ecuador
M24	M.esculenta	Manabí, Ecuador
M25	M.esculenta	Manabí, Ecuador
M26	M.esculenta	Manabí, Ecuador
Ant14	M. brachyloba	Napo, Ecuador
M02	M.esculenta	Manabí, Ecuador
M04	M.esculenta	Manabí, Ecuador
M05	M.esculenta	Manabí, Ecuador
M09	M.esculenta	Manabí, Ecuador
M15	M.esculenta	Manabí, Ecuador
M17	M.esculenta	Manabí, Ecuador
M18	M.esculenta	Manabí, Ecuador
M19	M.esculenta	Manabí, Ecuador
Ant 1	M. leptophylla	Manabí, Ecuador
Ant 2	M. leptophylla	Manabí, Ecuador
Ant 3	M. leptophylla	Manabí, Ecuador
Ant 5	M. leptophylla	Manabí, Ecuador
Ant 8	M. leptophylla	Esmeraldas, Ecuador
Ant 9	M. leptophylla	Esmeraldas, Ecuador
Ant 10	M. leptophylla	Esmeraldas, Ecuador
Ant11	M. leptophylla	Esmeraldas, Ecuador
Ant 13	M. leptophylla	Pichincha, Ecuador
Ant 15	M. leptophylla	Guayas, Ecuador
Ant 17	M. leptophylla	Guayas, Ecuador
Ant 18	M. leptophylla	Los Ríos, Ecuador
Ant 20	M. leptophylla	Guayas, Ecuador
Ant 22	M. leptophylla	Guayas, Ecuador
Ant 25	M. leptophylla	Guayas, Ecuador
Ant28a	M. esculenta	germinations
Ant28b	M. esculenta	germinations
Ant28c	M. esculenta	germinations
Ant28d	M. esculenta	germinations
M28	M. esculenta	Manabí, Ecuador
M36	M. esculenta	Manabí, Ecuador

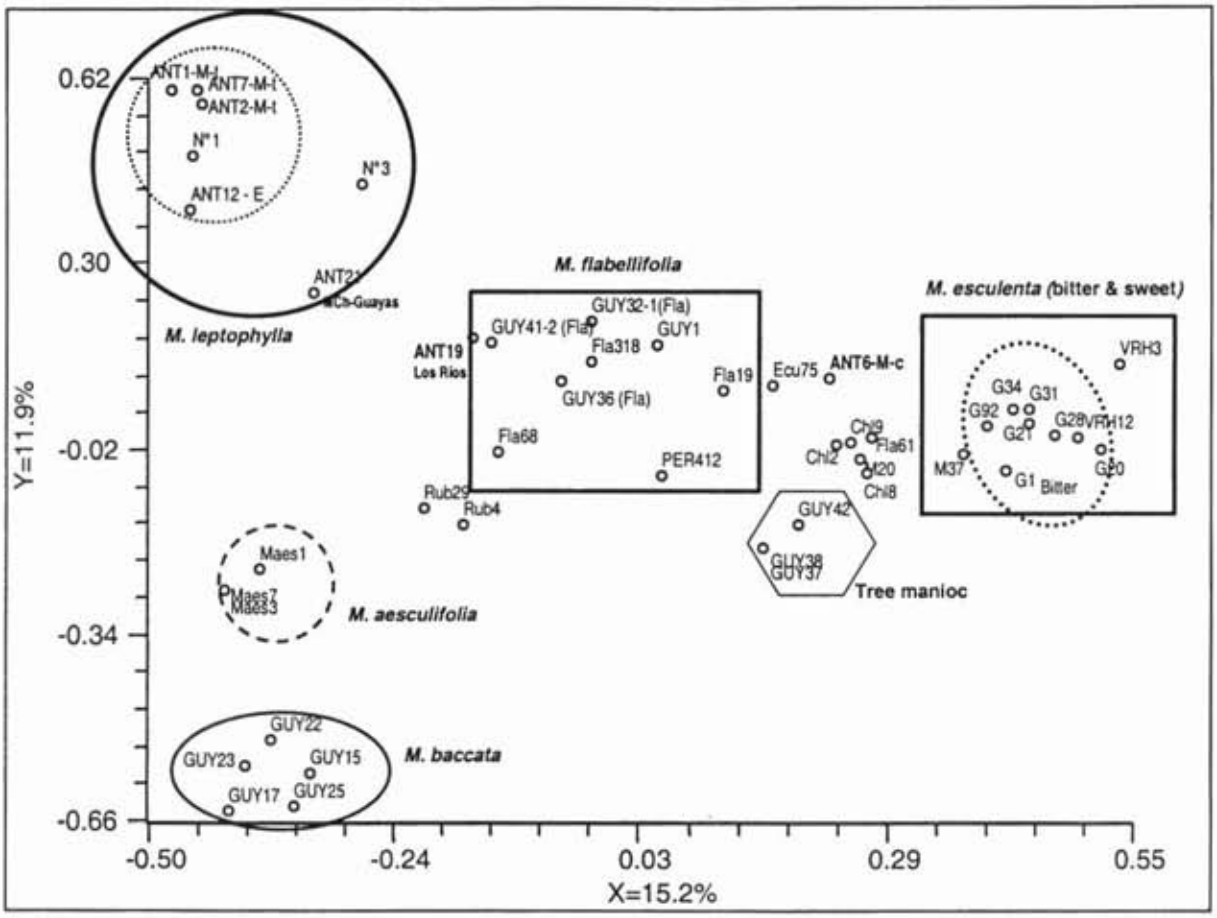

Figure 1 - PCoA analysis showing relations among seven Manihot species (*M. leptophylla, M. esculenta, M. flabellifolia, M. baccata, M. aesculifolia,* M. chlorostica *and* M. rubricaulis), based on analysis with 15 SSR primers (see text for details).

Table 5 - Total number of alleles per species corresponding to Data set 1

	SSR Primer (Mba et al, 2001)															Total Number
Species	9	100	31	3	169	68	55	108	103	38	88	135	40	179	30	of Alleles
M esculenta	6	5	5	3	3	6	8	4	6	3	5	5	3	6	4	72
M. baccata	1	2	4	2	1	1	3	2	2	2	1	1	3	3	2	30
M. flabellifolia	4	7	7	5	6	6	7	5	6	5	3	4	5	6	4	80
M. peruviana	1	1	1	1	1	1	2	1	2	1		1	1	1	1	16
M. leptophylla	3	6	6	5	6	4	5	3	4	3	2	5	2	4	3	61
M. chlorostica	2	2	1	1	2	2	2	2	2	2	1	2	3	1	1	26
M. aesculifolia	1	2	2	2	2	2	1	2	2	1	1	2	2	2	2	26
M. rubricaulis	1	2	1	1	1	1	3	2	2	1	1	1	1	2	1	21

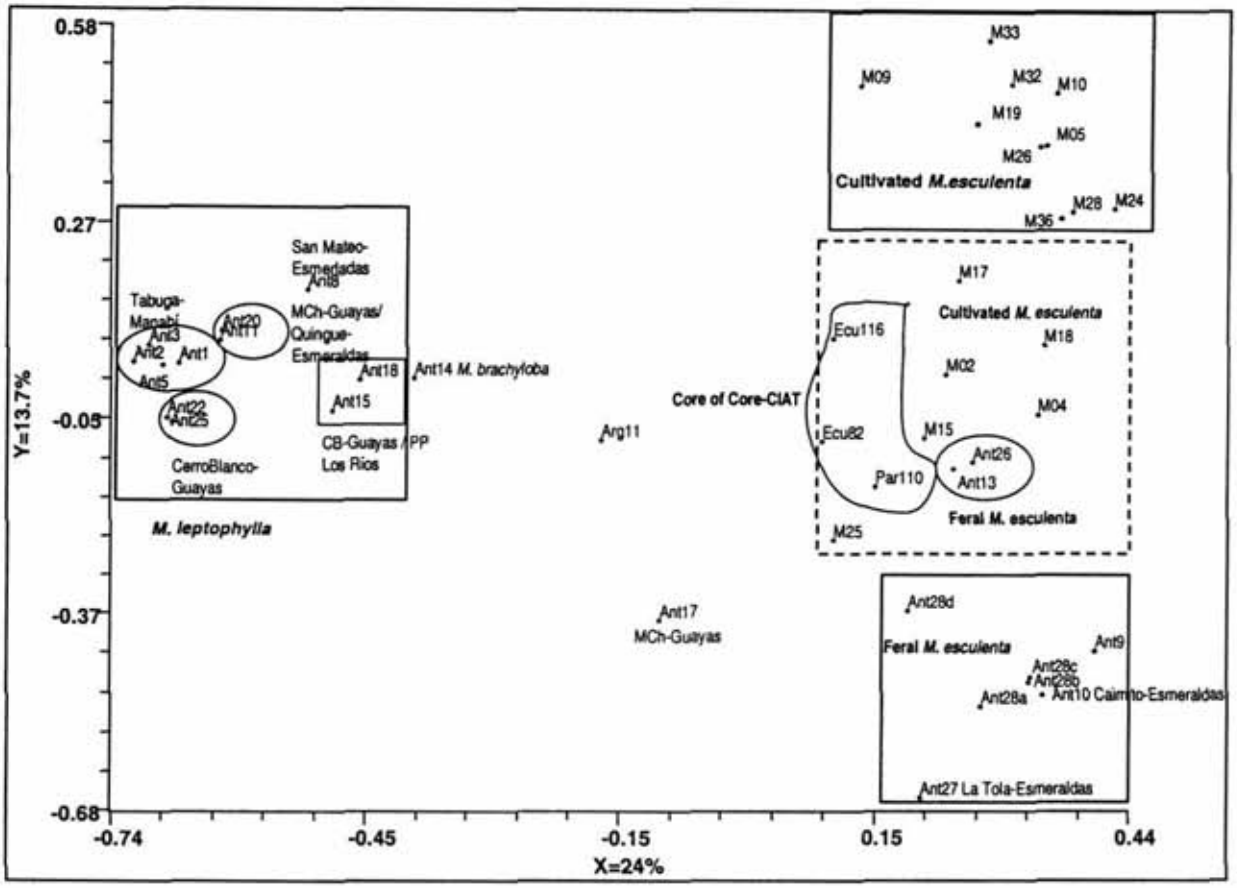

Figure 2 - PCoA analysis showing structuring of *M. leptophylla* and *M. esculenta*, based on analysis with 15 SSR primers (see text for details).

Conclusions

SSR data confirm AFLP data that indicated that *M. leptophylla* is genetically distinct from *M. flabellifolia*. Additionally, there seems to be a closer relationship to *M. aesculifolia* than to *M. esculenta* or *M. flabellifolia*.

Within species diversity of *M. leptophylla* could be considered small, although there seems to be a geographical differentiation, being the samples found in Camarones, Manabí Province the most extreme on the first axis of variation, which is in agreement with the botanical data that point to this locality as the botanical type for the species.

Samples from other geographical sites, specially from Guayas Province on the western coast and from Los Ríos on the Andes foothills may be candidates for introgression.

Up to the moment there is no clear evidence for intogression of *M. leptophylla* and *M. esculenta* or for differentiation of sweet/bitter types based on genetic contribution of *M. leptophylla.*

Future Plans

Based on current results, further sampling from interesting sites is needed in order establish a more comprehensive data base that will enable a better evaluation of introgression.

Additional primers to be included in the analysis are those used by Dr. Martin Fregene in cassava diversity studies (CIAT Anual Report 2002). Contact was established with Dr. Fregene in June of this year in which was requested an aliquot of these rutine primers in order to lower costs, Dr. Fregene also recommended using internal controls, that would be supplied by himself, in order to make our data compatible with other data sets.
These last points are the present constraints in order to continue work; the number of SSR loci analyzed up to date is low given that we had a failure with our power pack and therefore had to send it for repair. We have overcome this and have full capacity to advance quickly.

Additional data analysis is necessary, especially related to genetic diversity. This will be carried out on the final data.

Contact was established with the Instituto Nacional de Investigaciones Agropecuarias (INIAP), the Ecuadorian NAR, in order to transfer a copy of the material we have collected. It seems that there is an interest to make a new collection of cassava for which our collection data was made available to them.

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3.2.14 *In situ* conservation of cassava varieties cultivated by Kichwas from 'Alto Napo'

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Introduction

Although cassava is the main crop for the nationality Kichwa of the Amazonía, it has not been considered in research and development projects in Ecuador. In this region the cassava is perceived as part of the life: it has always been and it will be present in the life of the Kichwas especially of the women. This lack of cassava studies is due to the wrong idea that cassava is an abundant and unperishable crop.

However a reduction of the cultivated varieties is observed. This reduction is due to the devaluation of the culture (the boys and girls are educated more and more to live a "modern" style in detriment of traditional life style). Considering these circumstances, FONAKIN has proposed this project aiming the importance of cassava crop for the family subsistence and for the culture, and the narrow relationship among the chakra (cassava agroforesty system) and the woman.

This project correspond to the first phase of the project "Documentación del conocimiento Kichwa sobre la Chakra y Fortalecimiento al Grupo de Mujeres de Base de la FONAKIN", designed with participation of the FONAKIN's Women Group and has the following main objectives:

- To document the knowledge of Kichwa's Nationality from 'Alto Napo about production systems in chakra.
- To promote women participation in planing, execution and evaluation of FONAKIN's projects.

Outputs

- Diagnostic socioeconomic of the chakra, including the systemization of cassava management.
- Characterization of the plant diversity within representative chakras.
- *In situ* conservation of cassava varieties of high basin of the river Napo.
- Genetic and morphological characterization of cassava varieties.
- Invigoration of the administration and negotiation capacity of FONAKIN regarding to property rights on genetic resources.
- Women kichwas with capacity of formulating projects aiming gender analysis, including a training for 25 women on several topics, such as molecular biology and support to women's events.

3.2.15 Cleaning of released varieties and recovery of cassava genetic resources in production area of Ecuador

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Introduction

The INIAP, in Ecuador, has released two cassava varieties since 1992. According to a survey, performed in the main production areas of Manabí, those varieties have decreased their good characteristics. The importance of cassava for human food, animal feeding and agroindustry makes necessary to clean the released and local varieties to recovery their best performance, which will allow to have these valuable genotypes as source of diversity to obtain cassava products and by-products required by national and international markets.

Objectives

- To clean the released cassava varieties 'INIAP-Portoviejo 650' and 'INIAP-Portoviejo 651'.
- To adjust a methodology for conservation and management of the cleaned materials under farm facilities belonging to five 'Asociaciones de Productores y Procesadores de Yuca' (APPY) by training processes, participatory research and gender analysis.
- To recover genetic materials of local cassava varieties through collection and *ex situ* conservation..

The direct beneficiaries will be the partners of the APPYs from: Tablones (Junín), Bijahual y San Vicente (Portoviejo), Jaboncillo y San Miguel (24 de Mayo), of Manabí province.
The indirect beneficiaries will be the non partners farmers and their families.

3.2.16 Other CBN Activities

Recruitment of CBN coordination staff

A Regional Coordinator, Dr. Alfredo A. C. Alves has been appointed and have resumed duties at the Cali Headquarters of CIAT in February 2003. On his resumption of duty, the recruitment of the Social Scientist have been concluded with the recruitment of Elizabeth Caicedo in May 2003.

The Ginés-Mera memorial fellowship fund for postgraduate studies in biodiversity

The Canadian International Development Research Center (IDRC) has generously provided funding in the total sum of US$750,000 spread over 5 years for "*The Ginés-Mera memorial fellowship fund for postgraduate studies in biodiversity*". This fund is aimed memorializing Dr. Maria Jesús (Chusa) Ginés and Ms. Verónica Mera, CBN Coordinator and Social Scientist, respectively, who lost their lives in a tragic airplane accident (Jan 2002) while on an official trip from their base in Quito, Ecuador, to the headquarters of the International Center for Tropical Agriculture (CIAT), Cali, Colombia. Specifically, the fund aims at achieving the following over the 5-year span:

To provide opportunities and support to female and male master's students from the developing countries of the world to undertake thesis research addressing key elements of the sustainable use and conservation of agricultural biodiversity, in particular:
a) intellectual property rights and access to agricultural genetic resources
b) molecular characterization of agrobiodiversity
c) community-based conservation of genetic agrobiodiversity.

To promote the bridging of the research/ development divide, by encouraging researchers and their home universities to develop linkages with research for development projects, and to undertake applied research which informs development processes.

To explore opportunities for further expansion of this initiative in order to involve other stakeholders.

To encourage and support the exchange of information, knowledge and technology between the stakeholders in agricultural biodiversity conservation in these countries.

A total of 22 proposals were evaluated and 7 fellowships were approved and started in October/2003 (Table 1)

References

Thro, AM and C. Spillane (2003). Biotechnology-assisted Participatory Plant Breeding: Complement or Contradiction? Working Document No. 4. CIAT, Cali, Colombia.

Fauquet, CM and Taylor, NJ, eds (2003). Cassava: An Ancient Crop for Modern Times. [4 Compact Discs] Proceedings of the 5th International Meeting of the Cassava Biotechnology Network. 2001 November 4-9, St. Louis MO, USA.

Web based information dissemination

A website, http://www.ciat.cgiar.org/biotechnology/cbn/index.htm, dedicated to highlighting the activities of CBN-LAC has been launched. The site is also aimed at dissemination information to network members and in due course would also serve as an interactive forum for the exchange of ideas.

Capacity building of NARS partners

In order to ensure more efficient performance by NARS partners involved in projects at pilot sites the following have received CBN support to perform training at CIAT:

Yoel Beovides García, Researcher (INIVIT), Cuba

Terezinha Feitosa Machado, Researcher (Embrapa/CNPAT) and Doctorate Student (Federal University of Ceará), Brazil

Maryluz Folgueras, Researcher (INIVIT), Cuba

Table 1 - Projects supported by the "Ginés-Mera Memorial Fellowship Fund for Postgraduate Studies in Biodiversity". 2003

Student	Project Title	University	Award (US$)
Adriana Mercedes Alzate	Identificación por medio de marcadores moleculares de la diversidad genética de yuca de pequeños agricultures y evaluación del nivel de adopción e impacto de varieddaes mejoradas por CIAT en la Costa Atlántica Colombiana	Universidad Nacional de Colombia	12000
Astrid Johanna Arango Ulloa	Uso de la diversidad del totumo (*Crescentia cujete*): un árbol multipropósito para pequeños agricultures en regiones secas	Georg-August-Universitat-Goettingen, Germany	8000
Constanza Maria Quintero Valencia	Aplicación de una metodología para caracterizar germoplasma de frijol común (*Phaseolus vulgaris* L.) mediante polimorfismo de un solo nucleótido (SPN)	Universidad Nacional de Colombia	12000
Javier Llacsa Tacuri	Factores de distribución y fluctuación de la variabilidad de papas natives en las familias de las comunidades de Amaru, Chahuyatire y Viacha del Distrito de Pisac-Calca, Peru	Universidad Nacional San Antonio Abad del Cusco–Peru	12000
Juliana Chacón Pinilla	Patrones filogenéticos inter-e-intra específicos de la yuca (*Manihot esculenta* spp. Esculenta, *Euphgorbiaceae*): biogeografía y ecología compararada de las especies de la amazonía y la region andina	Universidad de los Andes, Colombia	14000
Nelson Arturo Royero Moya	Caracterización molecular de la variabilidad genética de guanábanos (*annona muricata* L.) y especies de anonáceas ralacionadas de importancia hortícola	Universidad Nacional de Colombia	8000
Roosevelt Escobar Pérez	Establecimiento de técnicas biotecnológicas a bajo costo para la limpieza y conservación de variedades locales de yuca y uso sostenido en programas de seguridad alimentaria	Universidad Nacional de Colombia	8000

Familiarization with Projects at Pilot Sites and Monitoring of on-going Activities

The CBN coordination staff has traveled to visit on-going projects at Cauca, Colombia; Bahia, Brazil; and Ecuador. Plans are underway for initiating visits to the on-going projects under small grants scheme.

Participation in Scientific Meetings

The network's activities have been highlighted at the following meetings:

Needs Assessment Study on Cassava, Ghana, 12-14 May 2003.

Worshop on Needs Assessment Study on Cassava, Cali, CIAT, 19-23 May 2003.

Planning Workshop on the Development of a Protocol for the Generation of Cassava Doubled-Haploids and their Use in Breeding, Cali, CIAT, 11-12 June 2003.

Organization of the Sixth International CBN meeting (CBN-VI)

The CBN is organizing its Sixth International Scientific Meeting (CBN-VI), which will take place at CIAT, Cali, Colombia on 8-14 March 2004. The theme of the meeting is: Adding Value to a Small-Farmer Crop. The aim is to discuss the better uses of biotechnology tools to add value to this small-farmer crop. Presentations at the meeting will focus how biotechnology can assist cassava farmers by developing, for example, more suitable varieties, disease-free planting materials, and better ways to conserve and process cassava after harvesting.

The announcements and other informations about this meeting can be found at CBN website:
http://www.ciat.cgiar.org/biotechnology/cbn/index.htm

Activity 3.3 Database and Libraries

3.3.1 Databases about distribution of wild relatives of crops

D.G. Debouck
SB-1 Project

We have continued with the establishment of databases about the distribution of wild relatives of beans (*Phaseolus*), cassava (*Manihot*) and rice (*Oryza*) in the Neotropics. This year we have done an inventory at the following herbaria: COL, EBUM, INB, NY, QCA, and US. The data basically include: taxonomic identification, location, date, phenology and notes. For easy consultation, types are in red, collectors in blue, and state/ province in green (examples below).

To date, the following herbaria have been visited: ARIZ, BAA, BM, BR (part), BRIT, COL, CR, CUZ, DES, ENCB, F (part), G, HAO, HUT, K, LIL, LPB, MA, MEXU, MICH, MO, MSC, NY, QCA, SGO, SI, US, USCG, USJ, and USM. This work has been developed bearing in mind the following perspectives:

Correct identification of materials collected and kept in *ex situ* conservation facilities (namely CIAT genebank, and from there collaborating institutions).
Geographic distribution of wild relatives of direct interest in breeding activities (namely acquisition of germplasm useful to the breeders).
Distribution of wild relatives genetically compatible with the crop, in view of introduction and management of transgenical crops.
Monitoring of modification/ destruction of natural habitats and disappearance of populations.

Along perspective # 1, a major outcome of that work has been a revision of *Phaseolus* (Freytag & Debouck 2002).

Example 1

Flora de Costa Rica, Fabaceae/ Pap. *Vigna umbellata* (Thumb.) Ohwi & Ohashi, det. N Zamora, enero 1996. San José, cantón de Aserrí. Z.P. Cerros de Escasú. Cerros de Escasú – La Carpintera. Cedral. Bosque primario y secundario en la falda norte del Cerro Pico Alto. Cuenca del Río Poás. 09°50'57"N 84°08'25"W. 1600-2300 m. Planta rastrera, flores fucsia. JF Morales 194. 13 December 1991. Instituto Nacional de Biodiversidad, en colaboración con el Missouri Botanical Garden (MO). /// Instituto Nacional de Biodiversidad. Fabaceae/ Pap. JF Morales 194. *Phaseolus coccineus* L. ssp. *darwinianus* Hern-Xol & Miranda, identifica N Zamora, octubre 1996. /// DGD: *costaricensis*, 1 racème, début floraison, exemplaire pauvre non typique, peu vigoureux. [INB; 21-VIII-2003].

This example shows a population of a new species described for Costa Rica (Freytag & Debouck 1996), that has been shown to belong to the phylum of the common bean (Schmit et al. 1993), thus widening possibilities for wide crossing.

Example 2

EBUM7394. Fam, Euphorbiaceae. *Manihot intermedia* Weatherby. n.v. "teyapu". Loc. Rancho Galeana. Edo. Michoacán. Mpio. Apatzingán. Hab. Terreno plano, rocoso basáltico, suelo arcilloso. 310 msnm. Selva baja caducifolia. Col. X Madrigal Sánchez no. 3167. Obs. Latex muy irritante. Det. X Madrigal Sánchez. Fecha Dic 6/ 1978. /// DGD: *tomatophylla*, végétatif, fe palmatilobées, à 5 lobes arrondis, pétioles 12-16 cm long. [EBUM; 11-II-2003].

This example shows an additional record to the few known for this wild species of cassava, the distribution of which seems restricted to Michoacan, Mexico. It does not exist in genebanks.

Example 3

Plants of Louisiana. Herbarium of Southern Methodist University. *Oryza sativa* L.. Allen Parish, 1.7 miles northeast of Oberlin. Shallow water, roadside ditch, and silty clay; bordering rice field. Several plants. Lloyd H Shinners 22092. 8 October 1955. /// Herbarium of Northern Kentucky University (KNK), det. John W Thieret 1984. /// DGD: *sativa*, la panicule a perdu pratiquement tous ses épillets, certains épillets ont les glumes brunâtres, type 'red rice' ! [BRIT; 10-VI-2002].

This example opens the possibility of the presence of 'red rice' on borders of rice fields in the southern USA, as early as 1955.

Example 4

Herb. Le Jolis [s.n.], *Phaseolus*, Mexique Occidental, Acapulco, [Guerrero], presqu'île Griffon, Legumin. Oct. 1866. /// Herbier Barbey-Boissier. /// Durand 1913 (acquisition ou cession?!). /// DGD: *mcvaughii*, go vertes, 3 racèmes, dimorphisme dans les gousses! [G; 15-II-2002].

This case shows how modification of habitats affects wild population, because this site has now been converted into a tourist resort. It has been shown elsewhere (Bayuelo et al. 2002) that this species is promising for salinity tolerance.

References

Bayuelo, JS, DG Debouck & JP Lynch. 2002. Salinity tolerance in *Phaseolus* species during early vegetative growth. Crop Sci. 42: 2184-2192.

Freytag GF & DG Debouck. 1996. *Phaseolus costaricensis*, a new wild bean species (*Phaseolinae, Leguminosae*) from Costa Rica and Panama, Central America. Novon 6: 157-163.

Freytag GF & DG Debouck. 2002. Taxonomy, distribution, and ecology of the genus *Phaseolus* (Leguminosae-Papilionoideae) in North America, Mexico and Central America. SIDA Bot. Misc. 23: 1-300.

Schmit, V, P du Jardin, JP Baudoin & DG Debouck. 1993. Use of chloroplast DNA polymorphisms for the phylogenetic study of seven *Phaseolus* taxa including *P. vulgaris* and *P. coccineus.* Theor. Appl. Genet. 87: 506-516.

3.3.2 Updating the database and maintaining the ceparium of the Biotechnology Research Unit

J. L. Bocanegra[1], A. F. Salcedo[2], F. Rojas[2], P. Chavarriaga[2] and A. Mejia[2].
Universidad del Tolima, Ibagué, Tolima, Colombia
SB2 Project, CIAT, Cali, Colombia

Introduction

The ceparium was established in 1996 to maintain a collection of *Agrobacterium* and *E. coli* strains. It consisted of 97 accessions of *A. tumefaciens*, 17 of *A. rizhogenes* and 156 of *E. coli.* The strains are kept frozen at –80 °C, in four boxes, whit 3 copies per box, two of which are the stocks and one is the working collection.

The last renovation of the entire ceparium was done in 1998. Since then new accessions have been added, in some cases without a back up copy, or lacking crucial information. Due to the overwhelming increase in information on strains, vectors and genes, it was necessary to create a database in year 2000, using Oracle developer 5, Edition Release 8.1.7.4.0 (Figure 1). The data base was not completely filled due mainly to the lack of a full time person devoted to this activity.

We hired a visiting scientist and a "pasante" from the *Universidad del Tolima* to update the ceparium, renew part of the collection andintroduce new strains.

Objectives

Update the ceparium and database
Renew and purify existing strains in critical shape
Enter new accessions into the ceparium and database.
Review and collect references relevant to the ceparium
Digitalization of information (pictures, maps, MTA, etc) available for strains, vectors and genes.

Materials and Methods

All boxes, stocks and working collections, were checked and all damaged accessions of *A. tumefaciens, A. rizhogenes,* and *E. coli,* were renewed by growing them on LB media with the proper antibiotics. Bacterial cultures were mixed with conservation media (Glycerol 30%, $MgSO_4$ 7-H_2O 100 mM, TrisHCl pH 7.5 10 mM) and stored in cryogenic vials. All available drawings, maps, pictures, restriction patterns of vectors and MTAs (Material Transfer Agreements) were scanned and included in the database. Using an Oracle tool called TOAD (Figure 1), the available information on strain, vector and genes was also included in the ceparium database.

To eliminate contaminants in *Agrobacterium* strains, a genus-specific Ketolactose test (Bernaerts & DeLey , 1963) was used to purify and renew strains.

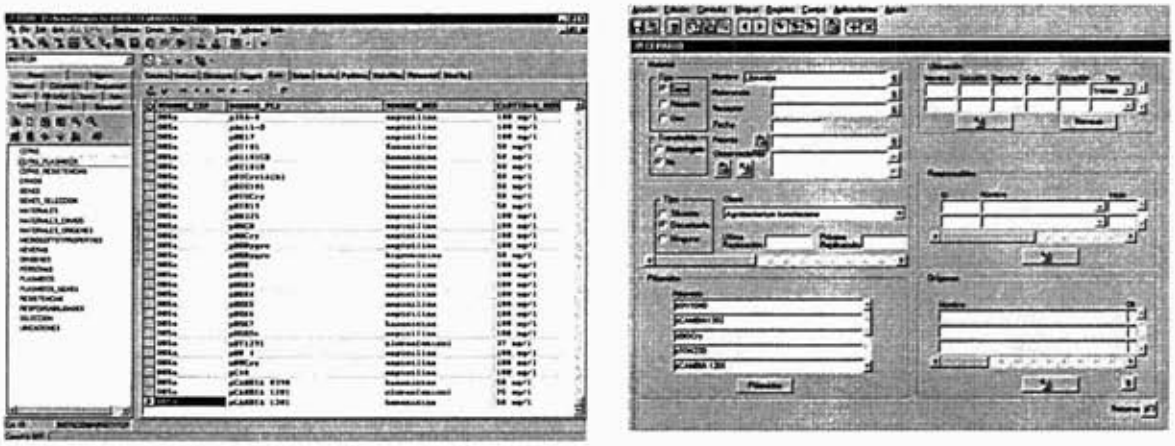

Figure 1. TOAD, a tool from Oracle that facilitates entering the data in the ceparium database. On the right is the actual application of the ceparium, as it shows on the screen, used also to enter and search for data.

Results

The ceparium was partially renovated by replacing damaged accessions, or reincorporating lacking ones in all boxes. Currently the collection is maintained by triplicate (Stock 1, Stock 2 and Working Collection) at –80°C. Each strain is in triplicate within each box. There 67 strains of *A. tumefaciens*, 7 of *A. rhizogenes* and 36 strains of *E. coli*. A ketolactose test for all renovated *Agrobacterium* accessions confirmed that , in fact, they belong to this genu.

All the information available about strains, vectors and genes was digitized and entered into the ceparium database application. Ten New articles related to strains, vectors, were filed.

The plasmids pBSK 7, pUC9 ps1, pTo 76, pUC9#44, pUC9 GA, pT3T7, pBI22i were introduced in *E. coli* DH5□ by electroporation, creating seven new accessions.

Filling a data sheet established for such purpose will monitor each new strain that enters the ceparium (Figure 2).

Future Activities

Entering to the ceparium collection and database new accessions; these included the plasmid vectors of JIRCAS (Dreb) and pRIp.

Incorporated into ceparium collection and database the accessions belong to rice transformation collection.

Training transformation personal in ceparium database application manage.

References

Bernaerts, M. J. and DeLey, J. (1963) A biochemical test for crown gall bacteria Nature (*London*) 197, 406-407

Figure 2. Data sheet to fill in when entering new strains in the ceparium.

Activity 3.4 Training and Workshops

3.4.1 National and international Collaboration

Joe Tohme and Howdy Bouis visited the Gates Foundation January 2003 to discuss the biofortification proposal.

Joe Tohme visit to the Danforth Center, Kansas State University and Yale University, January 2003 to discuss collaborative project on Cassava and rice

Joe Tohme. January, 2003 Corpoica, Ministerio de Agricultura. Bogotá –Colombia

Daniel Debouck, Costa Rica, January 2003. Observations about gene flow in wild-weed-crop complexes in the Central Valley of Costa Rica. Field work for the BMZ supported project on gene flow analysis. University of Costa Rica.

Steve Beebe. January, 2003. Guatemala. SDC follow up workshop on the future of the Central American networks

Daniel Debouck, Mexico, February 2003. Annual meeting of the SGRP; upgrading plan for the genebanks of the CGIAR. University of Morelia: Conference: "Evolución temprana del frijol en el Occidente de México". Invited seminar at the University of Guadalajara: "Entre Maguey y Cempoalxochitl. Observaciones sobre los recursos fitogenéticos del Occidente de México".

Mathew Blair. Alexandria, Egypt, February 2003, to attend Genetic Resource Challenge Program stakeholders meeting

Steve Beebe. February, 2003. Uganda. Training workshop on Marker Assisted Selection on project funded by Rockefeller Foundation.

Steve Beebe. February, 2003. Rwanda. Field visits.

Steve Beebe. February, 2003. Kenya. Planning workshop for a bioefficacy trial with the University of Nairobi.

Mathew Blair. Kisumu and Nairobi, Kenya, February, 2003 to attend Bean Biofortification project meeting.

Mathias Lorieux. March 2003. III Genoplante Congress. Presentation of one PostersPoitiers, France.

Mathew Blair. Medellin, Colombia, March, 2003.To visit field experiments and plan for collaborative activities with CORPOICA.

Mathew Blair. Santa Cruz, Bolivia, March – April, 2003, to visit field experiments and plan for collaborative activities with PRONOLAG

Martín Fregene. March, 2003 Trip to CRI, Kumasi, Ghana, on the collaborative project, " Analysis of genetic diversity of local land races from Africa and Latin America and the search for heterosis", being carried out as a Ph.D. study by Ms Elizabeth Okai.

Steve Beebe. March, 2003. Haiti. Field visit and training of local CIAT staff.

Joe Tohme. March, 2003. Meeting CIO on Biofortification. Biosafety Meeting. Washington. Organizer and participation.

Steve Beebe. April, 2003. Honduras. To attend annual meeting of Honduran CIALs (Local Agricultural Research Committees)

Steve Beebe. April, 2003. Honduras. To attend annual meeting of PCCMCA.

Martín Fregene. April 2003 Trip to NARO Namulonge, Uganda, on the collaborative project " Genetic mapping of cyanogenic potential (CNP) in cassava, being carried out as a Ph.D. study by Ms Elizabeth Kizito, under the BIOEARN program (SLU with Sida funding)

Manabu Ishitani. April, 2003. Objective: to build research networks with research institutions in Japan and to discuss specific research projects to seek for future collaborations Institution: April. RIKEN Plant Science Center ; Mitsui Global Strategic Studies Institutes ; JIRCAS ; RIKEN; Okayama University ; TOYOTA R & D Center ; Nagoya University ; National Institute for Basic Biology

Mathew Blair. Cusco, Peru, April, 2003 to visit field experiments and plan for collaborative activities with INIA

Daniel Debouck, Bogotá, April, 2003 (with Dr J Tohme of CIAT). Meeting with Genetic Resources and Biotechnology Program of CORPOICA: planning and future activities.

Mathias Lorieux. May 2003. Planning Meeting. CIO – CIAT. Montpellier, France.

Martín Fregene. May 2003 Trip to ARI, Mikocheni, Tanzania, on discussions of the project "A Molecular marker-assisted, farmer-partipatory breeding project to improve local cassava varieties in Tanzania with resistance to pest and diseases"

Martín Fregene. May 2003 Trip to ILRI, Nairobi, to visit the bioscience facility particularly the molecular marker capacity and ongoing cassava work (by Dr Morag Ferguson, IITA).

Martín Fregene. May 2003 Trip to RF-Nairobi, in company of Dr Ferguson to present to Dr Joe DeVries, RF Assistant Director of Food Security, outcome of the meeting in Tanzania.

Manabu Ishitani. May, 2003. To meet staff and exchange information. Japanese Embassy. JICA Colombia Office

Mathew Blair. Chicago, USA, May, 2003 attend technical meeting of the Bean/Cowpea CRSP.

Daniel Debouck, New York, USA, June 2003, invited seminar at the New York Botanical Garden: "Bees and beans: diversity of sweets, tongues, mutualisms, and consequences"; review of NY herbarium.

Martín Fregene. June 2003. Visit to Nigeria, in company of Bernardo Ospina and Luis Fernando Cadavid to assess the farming and seed multiplication operations of the Nigerian Starch Mills (NSM).

Joe Tohme. June, 2003. Visiting Instituto von Humboldt. Ministerio de Agricultura

Joe Tohme. June, 2003. Coffee Genomics. Meeting Coordinator. Cenicafé. Manizales – Colombia.

Joe Tohme. July, 2003. GEF Project Meeting. Bogotá – Colombia

Daniel Debouck, Lima Peru. July 2003, invited presentation (with Dr W Roca of CIP) at the Regional Workshop on Access to Genetic resources in the Andean Region: "Mecanismos de intercambio de germoplasma de los Centros del CGIAR"; review of QCA herbarium.

Daniel Debouck, Costa Rica, August 2003, meeting with INBio Staff in view of future collaborative activities; review of INB herbarium.

Daniel Debouck, Washington, USA, August 2003, invited seminar at the Smithsonian Institution, National Museum of Natural History: "*Phaseolus* beans in the Pliocenic transit

lounge of Panamá: last findings of markers and cpDNA analyses"; review of US herbarium; meetings with Smithsonian Staff in view of future collaborative activities.

Manabu Ishitani. August, 2003. To attend technical planning workshop for Genetic Resources Challenge Program . Wageningen Science Center

Joe Tohme. August, 2003. PBA Meeting. Bogotá – Colombia

Martín Fregene. August 2003. Visit to the John Innes Center (JIC), Norwich, to attend a one day conference in honour of Prof Mike Gale on his retirement from JIC; and to discuss a collaborative project with Prof Allison Smith.

Mathias Lorieux. August, 2003. Planning Meeting of the Challenge Program. "Unlocking Genetic Diversity of Crops for the Resource Poor". Wageningen, The Netherlands,

Daniel Debouck, Bogota, Colombia, September 2003, invited presentation (with Dr E Torres of IvH) at the Regional Workshop on Access to Genetic resources and protection of indigenous knowledge: "Propuesta de mecanismo de seguimiento y control, luego de un contrato de acceso a recursos genéticos".

Mathew Blair. Davis, USA, September, 2003 to organize Phaseomics meeting.

Mathew Blair. Nairobi, Kenya, September – October, 2003 to attend Harvest Plus Challenge Program – bean project meeting.

Martín Fregene. September 2003. Visit to Swedish Agricultural University (SLU), Uppsala, to attend the 2nd MOLCAS meeting and discuss with Dr Eva Ohlsson of Sida a proposed visit in October by Hernan and Joachim.

Martín Fregene. September 2003. Visit to Mukono, Uganda on the work planning meeting of the cassava biofortification challenge program (BCP).

Martín Fregene. September 2003. Return visit to CRI, Kumasi, to review progress made with establishing the crossing block in the project " Analysis of genetic diversity of local land races from Africa and Latin America and the search for heterosis" .

Cesar Martínez. September, 2003. Attend Plant and Animal Genome XII Conference. Miami - USA.

César Martínez. October, 2003. IRRI – Philippines. Attend the Rice Crop Meeting of the Biofortification Challenge Program.

Mathias Lorieux. October, 2003. III International Conference on Rice Breeding. Universidad Central de Venezuela. Maracay, Venezuela.

Zaida Lentini. October 2003. International Biosafety Workshop. Agrobio. Canada.

Steve Beebe. October, 2003. Biofortification Challenge Program planning meeting for common bean, in Nairobi, Kenya.

Manabu Ishitani. September, 2003. To discuss possibility of research collaboration and to have scientific discussion with Drs. Leon Kochian and Mark E. Sorrells
Cornell University.

Joe Tohme. November, 2002. Workshop on Biosafety. Ministerio del Medio Ambiente. Bogotá –Colombia.

Steve Beebe. November, 2002. Guatemala. SDC workshop on the future of the Central American networks.

Joe Tohme. September , 2003. – Biofortification Sweet Potato and Cassava crop meeting, Uganda. Visiting ILRI Nairobi

Joe Tohme. October, 2003. Meeting Biofortification. Gate Announcement. Visiting World Bank. Washington – USA.

Joe Tohme, October 2003. Meeting PBA. Santa Marta – Colombia

Joe Tohme. November 2003. Agronomic Meeting. Attend PAC. Washington – USA.

Joe Tohme. November 2002. Meeting on Biofortification. Rio de Janeiro, Brazil.

Myriam C. Duque, Apoyo a Instituciones de Agronatura. IPGRI. Análisis de colecciones de *Capsicum* y de *Phaseolus vulgaris* en Cuba y Guatemala: Dra. Carmen de Vicente, Sr. Félix A. Guzmán.

Myriam C. Duque. Apoyo a Instituciones de Agronatura. Biotec: Análisis de ensayos en guanábana: Sres. Nelson Royero y Juan Jairo Ruíz.

Myriam C. Duque. Análisis de redundancia /CANOCO. Ofrecido por el Dr. Chris Blackwood en curso coordinado por el Dr. Edmundo Barrios.

3.4.2 Training, Workshops, International and National Conferences for CIAT Personnel

SB-2 Staff. September 2002 . XXXVIII ACCB Congress. Pasto – Colombia

SB-2 Staff. October,2002. Second International Meeting on Agricultural Biotechnology. Cartagena – Colombia.

Palmira, Colombia, presentation during the Open House at the Genetic Resources Unit, 7 December 2002: "A race, a revolution, a treaty".

Fort Collins, USA, 31 January 2003, invited seminar at the Genetic Resources Conservation Center of USDA: "Searching for new bean germplasm in the Americas".

Matthew Blair - Kampala, Uganda, February 5-8, 2003. to lecture at Rockefeller Foundation sponsored marker workshop at CIAT-Africa

SB-2 Staff.. March 7, 2003. Meeting CEGA – PBM. Bogotá – Colombia

SB-2 Staff. March 31– Abril 4, 2003. Taller Gestion y conservación de recursos genéticos . Cartagena – Colombia

SB-2 Staff. March 24, 2003. Cita con representante agregado agropecuario de USDA en la embajada de los USA en Bogotá. Bogotá Colombia

Matthew Blair - Bogotá, Colombia, April 30-May 1, to attend the meeting of the National Congress of Food Scientists – ACTA "Asociacion Colombiana de Ciencia y Tecnologia de Alimentos", and present a keynote address on biofortification.

ACTA. Asociación Colombiana de Ciencia y Tecnología de Alimentos. April 30-May 1, 2003. Bogotá – Colombia

Biosafety Workshop for Journalists: J. Tohme, P. Chavarriaga, Z.Lentini,. Coordination of Workshop on Agriculture Biosafety for Journalists. April, 2003. CIAT, Cali, Colombia. A total of 15 participants.

Matthew Blair - Dakar, Senegal, May 15-21, to attend the Forum on Agricultural Research in Africa and present a talk on biofortification for a USAID sponsored workshop.

SB-2 Staff. May 18-22, 2003. Asistencia y presentación de los trabajos en el Molecular Breeding of Forage and Turf, Tirad Symposium. Dalas. USA.

SB-2 Staff. June 18, 2003. Semi Quantitative RT-PCR. Training. Perpignan – France

SB-2 Staff . June 9, 2003. Course CSHL and training on Microarrays. Illinois University. USA.

SB-2 Staff. June 26, 2003. Course on Bioinformatics and Genomics. Madrid – España.

SB-2 Staff. June 10, 2003. Visiting Bath University.

Myriam C. Duque. Conferencia Magistral en el XXIV Congreso Nacional de Fitopatología y Ciencias Afines. Armenia, Colombia. June 27, 2003

Mauricio Soto Suárez was a speaker in the "XXIV Congreso Nacional de Fitopatología y Ciencias Afines". June. 25-27. 2003.

Carmenza Muñoz – RNA extraction for common bean nodular cortex and physiological studies on nitrogen fixation potential of tepary beans under phosphorous deficiency. INRA-ENSA, Montpellier, France – June - July, 2003.

Congreso Sociedad Colombiana de Fitomejoramiento y Producción de Cultivos. July 2-5, 2003. Bogotá – Colombia

Paul Chavarriaga, XXX Congreso de SOCOLEN en calidad de conferencista. July 17-19, 2003.

SB-2 Staff. July 1, 2003. Monitoring and guidance for the Project "Limpieza y Multiplicación *in vitro* de variedades de yuca tradicionales y mejoradas con la participación de agricultores. Salvador, Brazil.

Matthew Blair - Bogotá, Colombia, July 3-5, to attend Plant Breeding Association "Congreso Nacional de Fitomejoramiento" meetings and give a keynote address on plant biotechnology in the one-day genomics symposium.

Soto-Suárez, M, Restrepo, S, Mosquera, G., Lopez, C., Tohme, J., and Verdier, V. 2003.Gene expression profiling of cassava responses to *Xanthomonas axonopodis* pv. *manihotis* infection. 11-th International Congress on Molecular Plant-Microbe Interactions - July 18-27 2003, St.-Petersburg, Russia.

SB-2. Staff. August 1, 2003. Agroexpo. Bogota – Colombia

Matthew Blair - Wageningen, Netherlands, August 24-30, to attend the Genetic Resource Challenge Program technical meeting.

Matthew Blair - Clemson, USA, Sept 3-7, to organize genomic resources and conduct hybridization experiments

SB-2 Staff. Oracle Linux Day. ASVOC. Bogotá – Colombia

SB-2 Staff. Oct 27, 2003. Universidad del Tolima.

SB-2 Staff. Oct, 23, 2003 Ministerio de Agricultural. Conference. Bogotá – Colombia

J. Salcedo. Universidad del Valle, Colombia. BSc thesis. October 2002-September 2003.

R. González. Universidad Nacional de Colombia. MSc. thesis. October 2002-September 2003.

Silvia Restrepo . October, 2002. Workshop on the application of Marker Assited Selection to bean breeding, at CIAT headquarters. Attended by 10 national program breeders. Myriam C. Duque support "Fundamento y Explicación de los procesos estadísticos de análisis".

Master's Degree Programme in Plant Genetic Resources, Universidad Nacional de Colombia, October 2002.

Biosafety Workshop for the Ministry of Environment: Z.Lentini, P. Chavarriaga, J. Tohme. Coordination of Workshop on Agriculture Biosafety for the Colombian Ministry of Environment. November, 19-20, 2002. CIAT, Cali, Colombia. A total of 25 participants.

Carlos Cesar Caula - training in microsatellite mapping, marker assisted selection and gene tagging, at CIAT.

Gloria Santana – CORPOICA – Rionegro, Antioquia, Colombia (Aug – Sept 2003) – training in molecular marker techniques and indirect selection for BCMV resistance, at CIAT.

Emeterio Payro - Departamento de Biologia, Centro de Investigación Fitogenética de Yucatán, Mexico (August 2003). training in molecular marker techniques for diversity assessment of wild beans, at CIAT.

Carlos Andrés Hidalgo, Adrian Alejandro Carrera, César Augusto Vera - Escuela Politécnica del Ejercito del Ecuador, Quito, Ecuador (September 1 - 26, 2003). training in molecular marker techniques and indirect selection for BCMV resistance at CIAT.

Ms Elizabeth Okai (Ghana) Ph.D. student, University of the Free State, Bloemfontein, South Africa (6 months, left January 2003)

Ms Elizabth Kizito (Uganda) Ph.D. student, University of the Free State, Bloemfontein, South Africa (June-December 2003)

Henry Ojulong (Uganda) Ph.D. student University of the Free State, Bloemfontein, South Africa (6 months, left January 2003)

Martha Isabel Moreno (Colombia) M.Sc. student, Universidad de Valle, Cali (September 2002 – August 2003)

Wilson Castel Blando (Colombia) B.Sc. student, Universidad Nacional de Colombia, Sede Bogota (Jan-Dec 2003)

Liliana Cano (Colombia) B.Sc. student, Universidad de Santander, Bucaramanga (Jan-Dec 2003)

Ana-Maria Correa Colombia) B.Sc. student, Universidad de Valle (June 2003 – May 2004).

Catalina Oviedo. Universidad Eafit Medellín. Process Engineering. Six Months: August 2003 – January 2004. Developing of a process for sterilizing *Phaseolus* sedes with CL_2 vapors

Joel Beovides, INIVIT, Cuba. CBN small grant recipient (June – December 2003)

Dr Chiedozie Egesi, NRCRI, Nigeria. One week on breeding methods at CIAT after the doubled haploid workshop (June 2003).

Catalina Oviedo. Universidad Eafit Medellín. Process Engineering. Six months: Augst 2003- January 2004 . Developing of a process for sterilizing *Phaseolus* seeds with Cl_2 vapors

Juan Jairo Ruiz M.Sc. Thesis; 3 years. National University of Palmira, Colombia; – Corporación Biotec . Field evaluation of *in vitro* propagated trees of soursop

Silvio Cadena; M.Sc. Thesis; 3 years. National University of Palmira, Colombia – Corporación Biotec. Greenhouse management of *in vitro* propagated plants of soursop

Nelson Royero M.Sc. student. National University of Palmira, Colombia – Corporación Biotec Molecular characterization of accessions of soursop and related annonaceus species.

Jhon Alex Cambindo. Instituto Técnico Agropecuario (ITA). Buga – Valle del Cauca. Specialization as technician in agronomy. Six months.

Sandra Lorena Acosta Lopez. System Plus. Palmira. Valle del Cauca. Database management and large data set organization. Two months.

International Biosafety Workshop: Organized by Agrobio, Canada. October 2003. Cartagena, Colombia. Z.Lentini Lecturer. A total of 50 participants

Adriano Alejandro Carrera. B.Sc. Military Polytechnic School. Quito, Ecuador. Training on tissue culture, anther culture, and genetic transformation.

Carlos Hidalgo B.Sc. Military Polytechnic School. Quito, Ecuador. Training on issue culture, anther culture, and genetic transformation.

Cesar A. Vera. B.Sc. Military Polytechnic School. Quito, Ecuador. Training on tissue culture, anther culture, and genetic transformation.

Ángela Mina. B.Sc. El Valle University. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Paola Olaya. B.Sc. El Valle University. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Margarita Pineda. B.Sc. National University of Colombia, Palmira. Training on tissue culture.

Ines de Alba Aragon Nieto, BIOMOL. Training course of Real Time PCR technology from September 8 to September 12.

February, 2003. Uganda. Training workshop on Marker Assisted Selection. Attended by about 15 national program breeders.

Myriam C. Duque, Curso Métodos estadísticos aplicados en Biología Molecular. Fundación DANAC – Venezuela. January 13-17, 2003

Myriam C.Duque, Curso "Métodos estadísticos aplicados en ensayos de campo. Fedearroz, Valledupar – Colombia. February 5-7, 2003

Myriam C. Duque, Curso "Análisis de variables categóricas por tablas de frecuencia"Fondo para el desarrollo del Recurso Humano. CIAT . May 15 – 16, 2003

Myriam C. Duque. Capacitación en Biometría para el Ing. Edwin Iquize, funcionario del Instituto Boliviano de Tecnología Agropecuaria-Proyecto. IBTA-Chapare.

Actvity 3.5 Publications

3.5.1 Refereed Journals, Books

Aluko,G.K., CP Martinez, Carolina Castaño, J. Tohme, J.H.Oard.2003. QTL, non-parametric, and population structure analysis of agronomic traits in doubled haploid lines derived from the intespecific cross O.sativa (L)/O.glaberrima(Steud). Poster. PAG meeting. San Diego ,California. January,2003.

Anderson J., Delseny M., Fregene M., Jorge V., Mba C., Lopez C., Restrepo C., Piegu B., Verdier V., Cooke R., Tohme J., Horvath D. 2003. An EST Resource for Cassava and Other Species of Euphorbiaceae. Plant Molecular Biology (submitted)2.

Bayuelo, J. JS, DG Debouck & JP Lynch. 2002. Growth, gas exchange, water relations, and ion composition of *Phaseolus* species grown under saline conditions. Field Crops. Research 80: 207-222.

Bayuelo, J. JS.; D.G. Debouck, Lynch, J.P. 2002. Salinity tolerance in *Phaseolus* species during early vegetative growth. Crop Science 42(6):2184-2192.

Beebe, Steve. Improvement of common bean for mineral nutritive content at CIAT. Chapter for an encyclopedia.

Blair MW, Pedraza F, Buendia HF, Gaitán-Solís E, Beebe SE, Gepts P, Tohme J (2003) Development of a genome-wide anchored microsatellite map for common bean (*Phaseolus vulgaris* L.) Theor Appl Genet (accepted)

Blair MW, Garris AJ, Iyer AS, Chapman B, Kresovich S, McCouch SR. (2003) High resolution genetic mapping and candidate gene identification at the xa5 locus for bacterial blight resistance in rice (*Oryza sativa* L.). Theor Appl Genet. 107: 62-73

Blair MW, Beebe S, Tohme J (2003) CIAT Bean Genomics. In Legume Genomics. Special issue, ed. G. Stacey, K Vendenbosch. Plant Physiology

Broughton WJ, Hernandez G, Blair MW, Beebe SE, Gepts P, Vanderleyden J (2003) Beans (*Phaseolus* spp.) – Model Food Legumes. Plant and Soil 252: 55-128. K

Borrero,J. Martínez,CP.,Almeida,A., Duque,MC.,Correa,F., Carabalí,SJ., Delgado D., Tohme,J., Silva,J. 2003.Incorporando genes valiosos de parientes silvestres al arroz cultivado.Paper presented at VIII Congreso Soc. Col. Fitomejoramiento y Producción Cultivos. Bogotá, Julio 2-5,2003.

Buendia, HF, Beebe SE, Blair MW, Terán H, Pedraza F (2003) Identificación de marcadores moleculares asociados a genes de rendimiento en una población RC2F3.7 de frijol común *Phaseolus vulgaris* L. (DOR390 x G19892). Fitotecnia Colombiana 3: 57-64.

Coulibaly, I; J. Louarn, M. Lorieux , A. Charrier, S. Hamon, M. Noirot. 2003. Pollen viability restoration in a Coffea canephora P. and C. heterocalyx Stoffelen backcross. QTL identification for marker-assisted selection. Theoretical and Applied Genetics 106:311-316

Cortés, D.F.; Reilly, K.; Okogbening, E.; Beeching, J.R.; Iglesias, C.; Tohme, J. 2002. Mapping wound-response genes involved in post-harvest physiological deterioration (PPD) of cassava (*Manihot esculenta* Crantz). Euphytica. 128(1);47-53

Chavarriaga P, Pachico D and Tohme J (2003) Transgenic cassava as a tool to control the stemborer Chilomima clarkei in Colombia. *In*: "Forging Links: Southern Perspectives on Biotechnology and Trade", Chapter X. International Centre for Trade and Sustainable Development ICTSD, Switzerrland (*In press*).

Coulibaly, I. B. Revol, M. Noirot, V. Poncet, M. Lorieux, C. Carasco-Lacombe, J. Minier, M. Dufourand P. Hamon. 2003. AFLP and SSR polymorphism in a *Coffea* interspecific backcross progeny [(*C. heterocalyx* x *C. canephora*) x *C. canephora*]. Theoretical and Applied Genetics 107:1148-1155

Dorn, B.; Mattiacci, L. Bellotti, A.C.; Dorn, S. 2003. Effects of a mixed species infestation on the cassava mealybug and its encyrtid parasitoids. Biological Control 27(1):1-10. May Elsevier Science.

Echeverry M, Mancilla LI, Cortes DF, Chavarriaga P y Tohme J (2003) Evaluación preliminar de la expresión del gen bar en plantas transgénicas de yuca (*Manihot esculenta*) mantenidas en reproducción vegetativa por cerca de diez años. Revista de la Asociación Colombiana de Ciencias Biológicas 15:43-52.

Freytag GF & DG Debouck. 2002. Taxonomy, distribution, and ecology of the genus *Phaseolus* (Leguminosae-Papilionoideae) in North America, Mexico and Central America. SIDA Bot. Misc. 23: 1-300.

Fregene M., Mba C., Buitrago C., Zarate A., Garcia T., Tohme J. 2003 A Predominantly Simple Sequence Repeat (SSR) Marker Map of Cassava (*Manihot esculenta* Crantz). Plant Molecular Biology (submitted)

Fregene M., Suarez M., Mkumbira J., Kulembeka H., Ndedya E., Kulaya A., Mitchel S. Gullberg U., Rosling H., Dixon A., Kresovich S. (2003) Simple Sequence Repeat (SSR) Diversity of Cassava (*Manihot esculenta* Crantz) Landraces: Genetic Structure in a Predominatly Asexually Propagated Crop Theor Appl Genetics 107:1083-1093

Fregene M., Matsumura H., Akano A., Dixon A., Terauchi R. 2003. Serial Analysis of Gene Expression (SAGE) of Host Plant Resistance to the Cassava Mosaic Disease Resistance (CMD) Plant Molecular Biology (submitted)

Gaitan, S.E.; Duque, MC.; Edwards, K.J.; Tohme, M.J. 2002. Microsatellite repeats in common bean (*Phaseolus vulgaris* L.): Isolation, characterization, and cross- species amplification in Phaseolus ssp. Crop Science 42(6):2128-2136

Gómez OJ, Blair MW; Frankow-Lindberg BE, Gullberg U (2003) Molecular and phenotypic diversity of common bean (*Phaseolus vulgaris* L.) landraces from Nicaragua. Genetic Resources and Crop Evolution (accepted)

González-Torres, R.I., E. Gaitán, M.C. Duque, O. Toro, J. Tohme & DG Debouck. 2003. Monitoring gene flow between wild relatives and landraces of common bean in Costa Rica. Annu. Rept. Bean Improvement Coop. (USA) 46:1-2.

House, W.A.; Welch, R.M.; Beebe, S. Cheng, Z. 2002. Potential for increasing the amounts of bioavailable zinc in dry beans (*Phaseolus vulgaris* L) through plant breeding. Journal of the Science of Food and Agriculture. 82(13):1452-1457.

Langar, K. ; M. Lorieux, E. Desmarais, Y. Griveau, L. Gentzbittel, A. Bervillé. 2003. Combined mapping of DALP and AFLP markers in cultivated sunflower using F9 recombinant inbred lines. Theoretical and Applied Genetics 106:1068 - 1074

Lentini Z., Lozano I, Tabares E., Fory L., Domínguez J., Cuervo M., Calvert L. 2002. Expression and inheritance of hypersensitive resistance to rice hoja blanca virus mediated by the viral nucleocapsid protein gene in transgenic rice. Theoretical and Applied Genetics 106: 1018-1026. Published online: 14 December 2002.

Lopez, C.E.; Acosta, I.F.; Jara, C.; Pedraza, F.; Gaitan-Solis, E.; Gallego, G.; Beebe, S.; Tohme, J. 2003. Identifying resistance gene analogs associated with resistances to different pathogens in common bean. Phytopathology. 93(1): 88-95.

Lorieux M.; G. Reversat, S. X. Garcia Diaz, C. Denance, N. Jouvenet, Y. Orieux, N. Bourger, A. Pando-Bahuon, A. Ghesquière. 2003. Linkage mapping of *Hsa-1*^{Og}, a resistance gene of African rice to the cyst nematode, *Heterodera sacchari.* Theoretical and Applied Genetics 107:691-696

Mahuku, G.S. , C. Jara, C.E.; Cajiao, V.; Beebe, S. 2002. Sources of resistance to angular leaf spot (*Phaeoisariopsis griseola*) in common bean core collection, wild *Phaseolus vulgaris* and secondary gene pool. Euphytica. 130(3):303-313.

Mahuku, G.S. , C. Jara, C.E.; Cajiao, V.; Beebe, S. 2002. Sources of resistance to *Colletotrichum lindemuthianum* in the secondary gene pool of *Phaseolus vulgaris* and in crosses of primary and secondary gene pools. Plant Disease. 86(12):1383-1387. The American Phytopathological Society.

Martinez, C.P., Borrero, J., Almeida, A., Duque, M. C., Correa, F., Carabalí, S. J., Delgado. D., Tohme, J., Silva, J.2003.Análisis de la adaptación regional de líneas interespecíficas. Paper presented VIII Congreso Sociedad Col. Fitomejoramiento Y Producción Cultivos. Bogota, Julio2-5,2003.

Martinez, A.K.; Gaitán-Solis, E.; Duque, MC.; Bernal, R.; Tohme, J. 2002. Microsatellite *loci* in Bactris gasipaes (*Arecaceae*): their isolation and characterization. Molecular Ecology Notes. 2(4):408-410.

Okogbenin E. and Fregene M (2002). Genetic Análisis and QTL mapping of early root bulking in an F1 population of non-inbred parents in cassava (*Manihot esculenta* Crantz). Theoretical and Applied Genetics. 106(1):58-66

Sallaud, C. ; M. Lorieux, E. Roumen, D. Tharreau, R. Berruyer, P. Svestasrani, O. Garsmeur, A. Ghesquière, J.-L. Nottéghem. 2003. Identification of five new blast resistance genes in the highly blast-resistant rice variety IR64 using a QTL mapping strategy. Theoretical and Applied Genetics 106:794-803

Sperling, L. 2002. Emergency seed aid in Kenya: Some case study insights on lessons learned during the 1990s. Disasters. 26(4):329-342

Sperling, L.; Longley, C. 2002. Beyond seeds and tools: Effective support to farmers in emergencies. Disasters. 26(4):283-287

Tomkins J., Fregene M., Main D., Kim H., Wing R., and Tohme J. 2003. Bacterial Artificial Chromosome (BAC) Library Resources for Positional Cloning of Pest and Disease Resistance Genes in Cassava (*Manihot esculenta* Crantz). Plant Molecular Biology (submitted)

Thomson M.J., Tai T.H., McClung A.C., Hinga M.H., Lobos K.B., Xu Y., Martínez C., McCouch S.R. 2003. Mapping quantitative trait loci for yield components, and morphological traits in an advanced backcross population between *Oryza rufipogon* and the *Oryza sativa* Jefferson. Theoretical and Applied Genetics 107:479-493.

Wenzl, P.; Chavez, A.L.; Patiño, G.M.; Mayer, J.E.; Rao, I.M. 2002. Aluminium stress stimulates the accumulation of orgnic acids in root apices of Brachiaria species. Journal of Plant Nutrition and Soil Science. 165(5):582-588.

3.5.2 Proceedings, Abstract and Others

Blair MW, Giraldo MC, Duran L, Beaver J, Nín JC (2003) Phaseolin characterization of Caribbean common bean germplasm. Annual Report of the Bean Improvement Cooperative.

Blair MW, Iriarte G, Beebe SE (2003) QTL analysis of an Andean advanced backcross population for yield traits derived from wild P. vulgaris. Annual Report of the Bean Improvement Cooperative

Blair MW, Pantoja W, Muñoz LC, Hincapie A (2003) Genetic analysis of crosses between cultivated tepary bean and wild *Phaseolus acutifolius* and *P. parvifolius*. Annual Report of the Bean Improvement Cooperative

Blair, MW (2003) "Mejoramiento nutricional mediante biotecnologia" ACTA; Asociacion Colombiana de Ciencia y Tecnologia de Alimentos, April 30-May 1, Bogotá, Colombia (Abstract).

Blair, MW (2003) "Introducción a la Genómica Vegetal" VIII Congreso Sociedad Colombiana de Fitomejoramiento y Produccion de Cultivos. July 2-5, 2003. (Abstract).

Checa O, Blair MW (2003) Trait correlations in climbing beans. Annual Report of the Bean Improvement Cooperative.

Checa O, Blair MW (2003) "Evaluacion de caracteres asociados con la capacidad trepadora y rendimiento en frijol voluble (*Phaseolus vulgaris*)" VIII Congreso Sociedad Colombiana de Fitomejoramiento y Produccion de Cultivos. July 2-5, 2003. (Abstract).

Florez, C.P.; Emmerling, M.; Fory, L.F.; Spangenberg, G. and Lentini, Z. 2003. Isolation and characterization of a caffeic acid O-methyl transferase from signal grass. Molecular Breeding of Forage and Turf, Third International Symposium, May 18-22, 2003. Dallas-Texas, USA. Poster # 136, p. 131.

Florez, C.P.; Arroyave, J.A.; Duque, M.C.; and Lentini, Z. 2003. Improving Somatic embryogenesis and plant regeneration in *Brachiaria decumbens*. Molecular Breeding of Forage and Turf, Third International Symposium, May 18-22, 2003. Dallas-Texas, USA. Poster # 129, p. 124.

Iriarte, GA, Blair MW, Beebe SE (2003) "Identificacion de qtls en una retrocruza avanzada entre una accesion silvestre y una variedad cultivada de frijol comun" VIII Congreso Sociedad Colombiana de Fitomejoramiento y Produccion de Cultivos. July 2-5, 2003. (Abstract).

Lentini, Z. 2003. Genetic transformation as a tool for germplasm development at CIAT. Models of Food Safety Assessment of Transgenic Crops. May 6-8, 2003. IFPRI, Washington DC. USA

Lentini, Z. 2003. The use of doubled-haploids in breeding of a self-pollinated crop: The case of rice. Planning Workshop on Development of a Protocol for the Generation of Cassava Doubled-Haploids and their Use in Breeding. June 11-12, 2003. Cali, Colombia.

Lentini, Z. 2003. Developing cassava haploid technology. Planning Workshop on Development of a Protocol for the Generation of Cassava Doubled-Haploids and their Use in Breeding. June 11-12, 2003. Cali, Colombia.

Lentini, Z. 2003. Unique Challenges and Opportunities for Environmental Assessment of GMOs in the Tropics. *In*: Craig R. Roseland. (Ed.). LMOS and the Environment. Proceedings of an International Conference (2002). November 27-30, 2001. Raleigh, North Carolina, USA. OECD, Paris.

Muñoz C, Blair MW, Debouck D "Diversidad Genetica en Frijol Tepari (*Phaseolus acutifolius*)" VIII Congreso Latinoamericano de Botánica, Cartagena, Colombia. October 13-18, 2002 (Abstract).

Santana G, Blair M, Morales F, Mahuku G, Jara C, Castaño M (2003) "Uso de tecnicas clasicas y avanzadas para identificar genotipos de frijol resistentes a antracnosis y mosaico común" VIII Congreso Sociedad Colombiana de Fitomejoramiento y Produccion de Cultivos. July 2-5, 2003. (Abstract) .

3.5.3 Thesis

Andrea Frei – (January – July 2003, thesis submitted Sept 2003) ETH, Switzerland – studying the quantitative trait loci involved in resistance to the leaf-feeding insect, *Thrips palmi* in common bean (collaboration C. Cardona, S. Dorn, H. Gu)

Andrés Salcedo. 2003. BSc. Clonaje y caracterización parcial de genes implicados en la biosíntesis de carotenos en raíces de yuca. Universidad del Valle.

Catalina Romero. 2003. Aproximación genómica al fenómeno de resistencia de *Brachiaria* al salivazo (*Aeneolania varia*):Correlación con homólogos de genes de resistencia (HGR) y aislamiento de genes expresador diferencialmente en la respuesta de defensa. Universidad Nacional de Colombia, Sede Bogotá. **(Laureada).**

Claudia Patricia Flores. Ph.D. Thesis. Desarrollo y uso de la transferencia genetica de *Brachiaria decumbens* stapf en fitomejoramiento.Universidad Nacional de Colombia. Sede Palmira. **(Meritoria).**

Elizabeth Okai (Ghana) Ph.D. student, University of the Free State, Bloemfontein, South Africa (6 months, left January 2003)

Elizabth Kizito (Uganda) Ph.D. student, University of the Free State, Bloemfontein, South Africa (June-December 2003)

Hernando Ramírez. Ph.D. Thesis. Universidad Nacional de Colombia. Sede Palmira (graduated June 2003). Incorporación de resistencia al cogollero "tuta absoluta" en la variedad de tomate UNAPAL-Arreboles, por transformación genética, via *Agrobacterium*. Universidad Nacional de Colombia, sede Palmira – Colombia. 193p.

Henry Ojulong (Uganda) Ph.D. student University of the Free State, Bloemfontein, South Africa (6 months, left January 2003)

Ivan Ochoa – (Jan 2003 – Feb 2003) Pennsylvania State University, USA – conducting laboratory and field studies to understand the inheritance and mechanisms of low

phosphorous tolerance in common bean and the role of adventitious rooting in adaptation to low phosphorous stress (collaboration with J. Lynch).

Juan Jairo Ruiz M.Sc. en Fitomejoramiento. 2003. Evaluación del comportamiento agronómico de clones de guanábano "*Annona muricata* L." propagados por microinjertación *in vitro*. Universidad Nacional de Colombia, Sede Palmira. 146p.

Martha Isabel Moreno (Colombia) M.Sc. student, Universidad de Valle, Cali (September 2002 – August 2003)

Manuel Quintero, Under-Graduate Thesis. Universidad Nacional de Colombia. Sede Palmira graduated September 2003). Ajuste del sistema RITA para la inducción de callos embriogénicos y regeneración de plantas a partir del cultivo de anteras de arroz. **(Meritoria).**

Maria Eugenia Buitrago. 2003. Evaluación de la tolerancia al aluminio, de una población segregante de *Brachiaria decumbens* x *Brachiaria ruziziensis*. Universidad del Valle.

Mauricio Soto. 2003. Herramientas de genomica funcional: Análisis de ESTs, Librerías sustractivas y microarreglos de ADN para avanzar en el conocimiento de las respuestas de defensa de la yuca a la infección x *Xanthomonas axonopodis* pv *manihotis*. Universidad Nacional de Colombia, Sede Palmira. **(Meritoria).**

Oscar Checa – (complete year, from 2001 onward) Universidad Nacional de Colombia – Palmira, Colombia – studying the inheritance of climbing ability in common bean and the importance of genotype x environment interaction in this trait.

Paola Ruíz. 2002. Caracaterización fenotípica y genética del arroz rojo (*Oryza sativa* f. *Spontanea* L.) de Saldaña (Departamento del Tolima) y Huila. Universidad Javeriana, Bogotá.

Rosana Pineda, M.Sc. Thesis. Universidad Nacional de Colombia. Sede Medellín. Evaluación de Flujo de genes desde arroz transgénico (*Oryza sativa* L) y no transgénico (*V. Purpura*) hacia la maleza arroz rojo (*Oryza sativa F. Spontanea*) bajo condiciones experimentales de campo. Universidad Nacional de Colombia, Sede Medellín. 2003.

Silvio Cadena; M.Sc. Thesis; 3 years. National University of Palmira, Colombia – Corporación Biotec. Greenhouse management of *in vitro* propagated plants of soursop

Silvio James Carabalí. Master Thesis: Genetic gains in rice grain quality obtained through several cycles of recurrent selection. Universidad Nacional de Colombia Seccional Palmira. Febrero 2000-Diciembre 2002.

Vanessa Segovia. M.Sc. Thesis. Universidad Real de Madrid, Spain. Optimización de la regeneración de lulo (*Solanum quitoense*) orientada a la transformación genética de plantas. Universidad Internacional de Andalucia, Sede Rabida-España. Maestría en Biotecnología de Plantas. April 2003.

Yina J. Puentes Páramo. Ensayos preliminaries para la transformación genética con orientación antisentido del gen Waxy en yucca (Manihot esculenta Crantz) para la obtención de un almidón 100% amilopectina. Universidad Nacional de Colombi, Sede Palmira. (**Meritoria**)

3.6 Projects

3.6.1 Project approved or on going

High through-put genetic diversity characterization of germplasm with a DNA chip. Donor: IPGRI.

Expanding the range of uses of cassava starch: A source of income generation. Donor: USAID

Development of strategies for better targeting of seed relief and linking relief and rehabilitation. Donor: FAO

Models of food safety assessment of transgenic crops – Workshop. Donors; USAID, Rockefeller Foundation

Nutritional Genomics. Donor: USAID

Maize – Vit A Biofortification. Donor: USAID

Model of Food Safety Assessment of Transgenic Crops. Donor: USDA

Biofortified Crops for Improved Human Nutrition. Donor: WB/IFPRI

Bean Genomics for Improved drought Tolerance in Africa and Latin America. Donor: GTZ

A molecular marker-assited, farmer-participatory breeding project to improbé local cassava varieties in Tanzania with resístanse to pest and disease (Rockefeller Foundation).

Genetic Mapping of the Linamarin biosynthetic genes CYPD1 and D2 and the development of markers for CNP in Cassava, in collaboration with Prof. Birger Moller, Royal Agriculture and Veterinary University, Copenhagen (DANIDA).

Genoplante. Project for phenotypic and characterization of a series of T-DNA mutants.

3.6.2 Project Submitted, in preparation and concept notes

Combating hidden hunger in Latin America: Biofortified crops with improved Vitamin A, essential minerals and quality protein A collaborative project submitted to CIDAon behalf of A Partnership of International Agricultural Research Centers, and National Agricultural Research Systems in Latin America

Using biotechnology tools and GIS to conserve biodiversity in Colombia

Development of micro-satellite markers to facilitate use of the cassava

Molecular genetic maps by African collaborators working on gene mapping resístanse to CMD

Towards the development of industrial cassava varieties: genetic and molecular análisis of early bulking in cassava germplasm collection

Marcadores moleculares asociados a resistencia a pudrición radical por *phytophthora drechsleri*, *Phytophthora nicotianae* y *Phytophthora cryptogea* en una población segregante de yuca.

Sustainable oil palm production as a source of employment and income for rural communities and small-scale farmers in tropical Latin America.

Molecular Characterization of Genetic Diversity and the Definition of Heterotic Groups in Cassava.

Applications of Spatial Statistics and GIS to Cassava Bacterial Blight Management.

Developing and exploiting expressed séquense Tags for cassava Starch and Bacterial Blight Resístanse.

Expanding the range of uses of cassava starch: A source of income generation.

Seed aid and germplasm restoration in disaster situations: síntesis of lessons learned and promotion of more effective practices.

Identificación de marcadores moleculares para la resistencia a la enfermedad de la hoja blanca del arroz en programas de mejoramiento.

Development of an *in vitro* protocol for the production of cassava doubled-haploids and its use in breeding.

Combating hidden hunger in Latin America: Biofortified crops with improved Vitamin A, essential minerals and quality protein.

"Comparative genomics and genetics in legumes" a collaborative research Project between CIAT and University of Aarhus, concept note prepared for DANIDA.

"Phaseomics" wRUIG-GIAN. (Submitted by Univ. Of Geneva with CIAT collaboration)

"Utilización de hierro y zinc en modelo animal y respuesta clínica al consumo habitual de fríjol de alta densidad mineral en mujeres y niños" Submitted by Universidad del Valle (with CIAT) to Colciencias.

The molecular diversity network of Cassava (MOLCAS).

Mutagenesis of Cassava (Manihot esculenta Crantz) for the generation, identification and Molecular Análisis of Novel traits. Research Contract submitted to the International Atomic Energy Agency (IAEA), Viena, Austria.

Workplan in sub-programs of the Genetic Resources Project for 2004. Fregene

Challenge Program "Unlocking Genetic Resources in Crops fort the Resource-Poor".

Genoplante. Project for phenotypic and characterization of a new series of T-DNA mutants.

Development and use of inbred lines in cassava breeding. Submitted to the Rockefeller Foundation. New York .

Development of an In vitro Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding. Submitted to ZIL, Switzerland.

3.6.3 Projects funded and their Donors (Oct 2002 – Sept 2003)

Canada

International Development Research Centre. (IDRC)

Strategies for integrating small-scale end-users in cassava biotechnology research (Latin America)

Colombia

Fundación para la Investigación y el Desarrollo Agrícola. (FIDAR)

Rice Functional Genomics Consortium

Ministry of Agriculture and Rural Development. (MADR)

Regeneration capacity and genetic transformation potential of commercial cassava varieties in Colombia

Propagation and certification of FSD-free cassava

Biotech Fruits

Corporación BIOTEC

Molecular and agromorphological characterization of native genetic variability of soursop and related Annonaceae species

Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología. (COLCIENCIAS)

Characterization of cassava resistance to vascular bacteriosis and its use in breeding

Instituto de Investigaciones de Recursos Biológicos Alexander von Humboldt.

Use of morphological and molecular techniques to study the diversity and conservation of endangered Colombian palm trees

Investigación sobre etiología, epidemiología y control de la Mancha Anular de la Palma de Aceite de la Zona Occidental de Colombia productora de Palma de Aceite

Belgium

Belgian Administration for Development Cooperation. (AGCD/BADC)

Genetic Improvement of common beans using exotic germplasm and biotechnology

France

Advanced Research Platform. (AGROPOLIS)
Genoplante (IRD)

Developing and exploiting expressed sequence tags for cassava starch and bacterial bligh resistance
Genoplante – evaluation and multiplication of 5000 lines of TDNA-mutants

Germany

German Agency for Technical Cooperation. (GTZ)

An integrated approach to genetic improvement of aluminum resistance in crops on low-fertility acid soils

Gene flow analysis for assessing the safety of bio-engineered crops in the tropics

New Zeland

Government of New Zeland (NZ)

Rome

Food and Agriculture Organization. (FAO)

Meeting to measure baselines of genetic diversity

International Plant Genetic Resources Institute (IPGRI)

Hihg through-put genetic diversity characterization of germplasm with a DNA chip.

The Netherlands

Ministry of Foreign Affairs and Trade. (MFA)
Directorate General International Cooperation. (DGIS)

Cassava Biotechnology Network III – CBN

United Kingdom

Wallace Genetic Foundation (WGF)

Department for International Development (DfiD)

Knowledge and tools for the modulation of post-harvest physiological deterioration in cassava.

USA

Rockefeller Foundation. (RF)

Legume genomics meeting between US and CGIAR

Research development of a molecular maps of cassava (*Manihot esculenta*)

Delivery of transgenic rice cultivars to seed producers and farmers in tropical America, following a multi-step approach involving biosafety assessment, nutritional testing, and negotiations on intellectual property rights

Molecular marker-aided analysis of traits of agronomic importance in cassava

Rice biotechnology Research

Agency for International Development. (USAID)

Crop Biofortification Initiative

Yale University

Rice Functional Genomics Consortium
Development of molecular markets for the breeding of sustainable pest resistance in common beans – a novel strategy

Department for International Development. (DFID)

Reviving the agricultural base of a region: Use of genetic transformation and interactive testing to restore predominant locally adapted cassava varieties.

Knowledge & tools for the modulation of post-harvest physiological deterioration in cassava.

IFPRI

Biofortified Crops for Improved Human Nutrition

Venezuela

Centro Tecnológico Polar

Ensuring stable and durable resistance of rice to pathogens and pests: rice Hoja Blanca Virus, *Rhizoctonia solani*, and Sogata

Activity 3.7 Project SB-2 Project

3.7.1 Current SB-2 Investigators: Discipline, position and time fraction

Name	Discipline	Time dedication%
Alves Alfredo	CBN Regional Coordinator	100
Beebe Steve	Bean Breeding	30
Bellotti Anthony	Cassava Entomology	20
Blair Mathew	Bean Genetics and breeding	70
Ceballos Hernan	Cassava Breeding	40
Chavarriaga, Paul	Transgenesis, Cassava	100
Debouck Daniel	Botany	20
Fregene Martin	Cassava Genetics and breeding	60
Ishitani Manabu	Molecular Biologist	100
Lentini Zaida	Biology/Genetics	80
Lorieux Mathias	Rice Genetics and Biotechnology	50
Martínez César	Breeding	49
Mejía Alvaro	Cell Biology	100
Sperling Louise	Seed Systems	20
Tohme Joe	Genomics, Project manager	100

Tissue Culture/Cryopreservation/Plant Transformation

Escobar, Roosevelt – Biochemists/Education	Research Assistant
Galindo, Leonardo F. – Agron. Engineering	Research Assistant
González, Eliana – Biologist	Research Assistant
Ladino, Janeth Julieta – Agron. Engineering	Research Assistant
Manrique, Norma - Agron. Engineering	Research Assistant
Muñoz, Liliana - Biochemists/Education	Research Assistant
Segovia, Vanesa - Agron. Engineering	Research Assistant
Tabares, Eddie - Biologist	Research Assistant
López, Danilo - Agron. Engineering	Research Assistant
Echeverry, Morgan - Biologist	Research Assistant
Juan Jairo Ruiz	Research Assistant
Fory, Luisa – Biologist	Rice Biotechnology Research Coordinator
Rios, Auradela – Biochemistry Tech.	Technician
Bolaños, Eugenio	Technician
Dorado, Carlos	Technician
Herrera, Pablo	Technician
Ríos, Alexander	Technician
Tigreros, Humberto	Technician
Muñoz, Carmenza -	Visiting Researcher

Genome Diversity

Gallego,Gerardo. - Biologist	Genome Research Coordinator
Gaitán, Eliana. - Biologist	Research Assistant
Barrera, Edgar. – Biologist	Research Assistant
Gutiérrez, Janeth P. - Biologist	Research Assistant
Bohorquez, Adriana. - Biologist	Research Assistant
Vargas, Jaime. – Biologist	Research Assistant
Quintero, Constanza. –Biologist	Research Assistant
Muñoz, Monica. -Biologist	Research Assistant
Cortés Diego F. – Biologist	Research Assistant
Giraldo,Olga X. – Biologist	Research Assistant
Galindo, Lonardo M. - Biologist	Research Assistant
Reyes, Nidia	Technician
Londoño, Claudia	Technician

Plant-Stress interactions

Chaves Alba L. – Chemistry	Research Associate
Soto Mauricio. - Microbiologist	Research Assistant
Leonardo Miguel Galindo	Research Assistant
Andrés Felipe Salcedo	Research Assistant
Maria Eugenia Recio	Technician

Administrative

Cruz, Olga L.	Bilingual Secretary
Zuñiga, Claudia S.	Bilingual Secretary
Duque, Myriam C.	Statistical Consultant

Institute A. von Humboldt

Palacio, Juan D. – Agron. Engineer	Visiting Researcher
Carolina Villafañe	Visiting Researcher
Tania García	Visiting Researcher

Corporación BIOTEC

Royero, Nelson. – Biologist	Visiting Researcher
Cadena, Silvio. – Agron. Engineer	Visiting Researcher
García Victor Hugo – Biologist	Visiting Research

CORPOICA

Sanchez, I., PhD.	Visiting Researcher

3.7.2 Current Graduate Students

Andrea Frei – PhD. ETH, Switzerland – studying the quantitative trait loci involved in resistance to the leaf-feeding insect, *Thrips palmi* in common bean (collaboration C. Cardona, S. Dorn, H. Gu)

Constanza Quintero. Recursos Fitogenéticos Neotropicales. MSc. Plant Genetic Resources. Universidad Nacional de Colombia Sede Palmira

Eliana Gaitán; Molecular markers and diversity of palm trees. PhD. Plant Breeding Program, Universidad Nacional de Colombia, Palmira, Colombia.

Eliana González. BSc. 2001. Univalle. Diversidad genética de 3 poblaciones de *colombo balanus* excelsa (fagacia) especie endémica de los Andes Colombianos

Edgar Barrera; Molecular markers for ACMD resistance- MSc Plant Breeding, Universidad Nacional de Colombia, Palmira, Colombia.

Eyvar Andrés Bolaños Vidal. BSc. Caracterización de la diversidad genética en cuanto a contenidos de caroteno de raíces y hojas de 682 genotipos de yuca.

Fabio Escobar; Molecular markers to certify seeds of rice - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

Gerardo Gallego. Gene cloning of rice disease resistance genes. PhD. Plant Breeding Program, Universidad Nacional de Colombia, Palmira, Colombia.

Iván Ochoa. PhD. Pennsylvania State University. USA. Conducting laboratory and field studies to understand the inheritance and mechanism of low phosphorous tolerance in common bean and the role of adventitious rooting in adaptation to low phosphorus stress (collaboration with J. Lynch).

Jaime Vargas. MSc. Plant Breeding. Identificación de marcadores moleculares microsatélite asociados con el gen de resistencia a mosca blanca en yuca. Universidad Nacional de Colombia, Sede Palmira.

Leonardo Fabio Galindo. Master Bussiness Administration. Universidad del Valle.

Martha Isabel Moreno, Post Graduate (M.Sc). Universidad del Valle. Gene Cloning of CMD2)

Meike Anderson. PhD. 2002. Genetic diversity and core collection approaches in the multipurpose shrub legumes Flemingia macrophylla and Cratylia argentea. University of Hohenheim

Nelson Royero; Molecular markers and diversity of *Anonna* spp - MSc Plant Breeding, Universidad Nacional de Colombia, Palmira, Colombia.

Oscar Checa – PhD. Plant Breeding. Universidad Nacional de Colombia, Sede Palmira. Studying the inheritance of climbing ability in common bean and the importance of genotype x environment interaction in this trait.

Paola Fory. 2002. MSc. Plant Genetic Resources. Improving the breeding of lulo (*Solanum quitoense* LAM) through an understanding of the species genetic diversity . Universidad Nacional de Colombia, Sede Palmira

Roosevelt Escobar. Genotypic stability of cryopreserved cassava plants- MSc. Plant Genetic Resources Program , Universidad Nacional de Colombia, Palmira, Colombia.

Rosana Paola Pineda. 2002. MSc. Medición de flujo de genes con microsatelites en arroz. Universidad Nacional de Colombia, Sede Medellín.

Vanessa Segovia. 2001 M.Sc. Thesis.. Optimización de la regeneración de lulo (*Solanum quitoense*), orientada a la transformación genética. Universidad Internacional de Andalucía, Spain

Yamileth Cortés. MSc Plant Breeding. Analyzing the genetic diversity of the Colombian plantain collection (*Musacea collection*) using microsatellites. Universidad Nacional de Colombia, sede Palmira

An Argentine PhD candidate was hosted for two weeks to prepare data analysis for his thesis work, involving a statistical analysis of multi-locational trials carried out over a 15 year period in the north-west of Argentina.

A thesis plan, including field work, was established with a Cuban MsC student. The student will carry out a physiological analysis of lines derived from the cross of DOR 364 x BAT 477, the latter of which has expressed resistance to multiple abiotic stresses. The study will reveal the physiological relationship between resistances to low P, nitrogen and drought stress.

3.7.3 Undergraduate students (current)

Andrés Bolaños, Universidad Nacional de Colombia, Sede Palmira
Angela Zarate, Universidad de Tolima, Ibague (Conversion of RFLP to SSCP markers)
Carolina Castaño, Universidad de los Andes, Bogotá
Carolina Astudillo, Universidad del Valle
Carolina Ramirez Rodríguez, Universidad del Tolima
Catalina Romero, Universidad Nacional de Colombia, Sede Bogotá
Gloria Iriarte, Universidad de Tolima
Hector Fabio Buendía, Universidad de Tolima
Jaime Marin, Universidad de Tolima, Ibague. (QTL mapping of early bulking)
Juan Felipe Calderon, Universidad Nacional de Colombia, Sede Palmira (Humboldt)
Leonardo Bocanegra, Universidad del Tolima (Micros, Rice)
Manuel Quintero, Universidad Nacional de Colombia, Sede Palmira
Maria Eugenia Buitrago, Universidad del Valle, Cali. (Plant Stress)

Paola Ruíz. Universidad Javeriana, Bogotá
Paula Andres. Universidad Javeriana, Bogota (Gene tagging of CBB resistance)
Paola Cardenas, Universidad Javeriana Bogotá (Humboldt)
Sergio Prieto – Universidad Nacional de Colombia
Wilfredo Pantoja, Universidad del Valle

3.8. List of Acronyms and Abbreviations Used in the Text

Acronyms

ADB	Asian Development Bank
AHI	African Highland Initiative
Bean/Cowpea CRSP	Bean/Cowpea Collaborative Research Support Program (*of the* University of Georgia, USA)
BoT	Board of Trustees (*of* CIAT)
CA	Département des Cultures Annuelles (*of* CIRAD)
CARDER	Corporación Autónoma Regional de Risaralda, Colombia
CARE	Cooperative for American Relief Everywhere
CATIE	Centro Agrónomico Tropical de Investigación y Enseñanza, Costa Rica
CBN	Cassava Biotechnology Network
CENIPALMA	Centro de Investigación en Palma de Aceite, Colombia
CIALs	Comités de Investigación Agrícola Local, Colombia
CIFOR	Centre for International Forestry Research, Indonesia
CIMMYT	Centro Internacional para Mejoramiento de Maiz y Trigo, Mexico
CIP	Centro Internacional de la Papa, Peru
CIPASLA	Consorcio Interinstitucional para una Agricultura Sostenible en Laderas, Colombia
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement, France
CLODEST	Comité Local para el Desarrollo Sostenible de la Cuenca del Río Tascalapa, Honduras
CNPMF	Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical (*of* EMBRAPA)
CODESU	Corporación para el Desarrollo Sostenible de Ucayali, Peru
COLCIENCIAS	Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología "Francisco José de Caldas", Colombia
CONDESAN	Consorcio para el Desarrollo Sostenible de la Ecorregión Andina, Peru
CORPOICA	Corporación Colombiana de Investigación Agropecuaria
CSIRO	Commonwealth Scientific and Industrial Research Organisation, Australia
CURLA	Centro Universitario Regional del Litoral Atlántico, Honduras
DANIDA	Danish International Development Agency, Denmark
DFID	Department for International Development, UK
DGIS	Directorate-General for International Co-operation, the Netherlands
DICTA	Dirección de Ciencia y Tecnología Agropecuaria, Honduras
DNP	Departamento Nacional de Planeación, Colombia
EAP-Zamorano	Escuela Agrícola Panamericana *at* Zamorano, Honduras
EC	Economic Commission (*of the* European Union)
ECABREN	Eastern and Central Africa Bean Research Network
ECLAC	Economic Commission for Latin America and the Caribbean
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária, Brazil
EPMR	External Program and Management Review (*of* CIAT)
ETH	Eidgenössische Technische Hochschule, Switzerland
FAO	Food and Agriculture Organization of the United Nations
FCRI	Field Crop Research Institute, Thailand
FLAR	Fondo Latinoamericano y del Caribe para Arroz de Riego, *based at* CIAT
FONAIAP	Fondo Nacional de Investigaciones Agropecuarias, Venezuela
GRU	Genetic Resources Unit (*of* CIAT)
GWG	Gender Working Group (*of the* CGIAR Systemwide Programme on Participatory Research and Gender Analysis for...)
IBSRAM	International Board for Soil Research and Management, Thailand

ICA	Instituto Colombiano Agropecuario, Colombia
ICARDA	International Center for Agricultural Research in the Dry Areas, Syria
ICER	Internally Commissioned External Review (*of* CIAT)
ICIPE	International Centre of Insect Physiology and Ecology, Kenya
ICRAF	International Centre for Research in Agroforestry, Kenya
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics, India
IDEAM	Instituto de Hidrología, Meteorología y Estudios Ambientales, Colombia
IDIAP	Instituto de Investigación Agropecuaria de Panamá
IDRC	International Development Research Centre, Canada
IFDC	International Fertilizer Development Center, USA
IFPRI	International Food Policy Research Institute, USA
IGAC	Instituto Geográfico "Agustín Codazzi", Colombia
IGDN	Inter-American Geospatial Data Network
IGER	Institute of Grasslands Environment Research, UK
IIA	Instituto de Investigaciones Agropecuarias, Venezuela
IIASA	International Institute for Applied Systems Analysis, Austria
IICA	Instituto Interamericano de Cooperación para la Agricultura, Costa Rica
IILA	Instituto Italo-Latino Americano, Italy
IITA	International Institute of Tropical Agriculture, Nigeria
ILRI	International Livestock Research Institute, Kenya
INBIO	Instituto Nacional de Biodiversidad, Costa Rica
INIA	Instituto Nacional de Investigación Agraria, Peru (*now* INIAA)
INIAA	Instituto Nacional de Investigación Agraria y Agroindustrial, Peru (*formerly* INIA)
INIAP	Instituto Nacional Autónomo de Investigaciones Agropecuarias, Ecuador (*formerly* Instituto Nacional de Investigaciones Agropecuarias)
INIFAP	Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias,
Mexico	
INIVIT	Instituto de Investigaciones de Viandas Tropicales, Cuba
INTA	Instituto Nacional de Tecnología Agropecuaria, Argentina
IPGRI	International Plant Genetic Resources Institute, Italy
IPRA	Investigación Participativa en Agricultura/*Participatory Research in Agriculture* (CIAT)
IRRI	International Rice Research Institute, the Philippines
IVITA	Instituto Veterinario de Investigaciones Tropicales y de Altura, Peru
IWMI	International Water Management Institute, Sri Lanka (*formerly* International Irrigation Management Institute)
JIRCAS	Japan International Research Center for Agricultural Science
LSU	Louisiana State University, USA
MT	Management Team (*of* CIAT)
NARO	National Agricultural Research Organization, Uganda
NRI	Natural Resources Institute, UK
NRMG	Natural Resource Management Group (*of the* CGIAR Systemwide Programme on Participatory Research and Gender Analysis for...)
OFI	Oxford Forestry Institute, UK
ORSTOM L'	Institut Français de Recherche Scientifique pour le Développement en Coopération, France (*now* L'Institut de Recherche pour le Développement)
PABRA	Pan-Africa Bean Research Alliance
PASOLAC	Programa de Agricultura Sostenible de Laderas en Centro América
PBG	Plant Breeding Group (*of the* CGIAR Systemwide Programme on Participatory Research and Gender Analysis for...)
PROCITROPICOS	Programa Cooperativo de Investigación y Transferencia de Tecnología para los Trópicos Suramericanos
PRODAR	Programa para el Desarrollo Agroindustrial Rural, Costa Rica
PROFRIJOL	Programa Cooperativo Regional de Frijol para Centro América, México y el Caribe
PROFRIZA	Proyecto Regional de Frijol para la Zona Andina
RIVM	Rijksinstitut voor Volksgezondheid en Milienhygiene (National Institute of Public Health and Environmental Protection), The Netherlands
SABRN	South Africa Bean Research Network
SDC	Swiss Agency for Development and Cooperation
SINCHI	Instituto Amazónico de Investigaciones Científicas, Colombia
SINGER	The CGIAR System-wide Information Network for Genetic Resources
SP-IPM	Systemwide Program on Integrated Pest Management (*of the* CGIAR)
SP-PRGA	The CGIAR Systemwide Programme on Participatory Research and Gender Analysis for Technology Development and Institutional Innovation

SWNM Management	The CGIAR Systemwide Program on Soil, Water & Nutrient
TAC	Technical Advisory Committee (*of the* CGIAR)
TCA	Tratado de Cooperación Amazónica
TSBF	Tropical Soil Biology and Fertility Programme, Kenya
UNEP	United Nations Environment Programme
UNIVALLE	Universidad del Valle, Colombia
USDA	United States Department of Agriculture
WARDA	West Africa Rice Development Association, Cote d'Ivoire
WRI	World Resources Institute, USA
WWW	World Wide Web

Abbreviations

ACMV	African cassava mosaic virus
AES	Agroecosystem
Al	Aluminum
ARIs	Advanced research institutes
AROs	Advanced research organizations
C	Carbon
CBB	Common bacterial blight of bean; Cassava bacterial blight
CD-ROM	Compact disk—read-only memory
CLOs	Comités locales
DCs	Developed countries
DS	Decision support
ESTs	Expressed sequence tags (biotechnology)
FM	Forest margins
FPR	Farmer participatory research
FTE	Full-time equivalent
GA	Gender analysis
GIS	Geographic information systems
GOs	Governmental organizations
HS	Hillsides
IARCs	International agricultural research centers (the CGIAR system)
INIAs	Instituciones nacionales de investigación agropecuaria
IPM	Integrated pest management
IPR	Intellectual property rights
LA	Latin America (n)
LAC	Latin America and the Caribbean
LDCs	Less-developed countries
LoRSDIs	Local rural sustainable development initiatives
M&E	Monitoring and evaluation
MTA	Material transfer agreement (used in germplasm exchange)
MTP	Medium-Term Plan (CIAT)
N	Nitrogen
NARES	National agricultural research and extension systems
NARIs	National agricultural research institutes
NARS	National agricultural research systems
NGOs	Nongovernmental organizations
NRM	Natural resource management
P	Phosphorus