Annual Report Project SB-2

Conservation and Use of Tropical Genetic Resources

CIAT
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CONTENT

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

PROJECT OVERVIEW

Project Description

Objective: To conserve the FAO Designated Collections and employ modern biotechnology tools to identify and use genetic diversity for broadening the genetic base and increasing the productivity of bean, cassava, rice and *Brachiaria*.

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. Increased efficiency of NARS breeding programs using biotechnological tools
4. *Phaseolus, Manihot,* and forage species conserved, multiplied and distributed as per international standards.
5. Germplasm available, restored, and safely duplicated.
6. Designated Collections made socially relevant.
7. NARS strengthened for conservation and use of Neotropical plant genetic resources.
8. Conservation of Designated Collections linked with on-farm conservation efforts and protected areas.

Milestones:

2005 Efficient transformation system developed for cassava.
Characterization of the bean core collections with 50 genomics and gene based microsatellite markers.
Characterization of a basic collection of tepary bean germplasm with AFLP.
1500 accession from the cassava collection genotypes with 36 SSRs markers.
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 rice.
Targeted sequencing of cassava genome.
Molecular markers developed for dry matter content and resistance to cassava green mites.
Isogenic of QTL in rice developed and tested.
Gene expression studies for insect resistance in Brachiaria.
Differentially expressed genes for adaptation of Brachiaria to acid soils isolated by microarray.
Bean cDNA libraries for drought generated.
Comparison of gene flow in bean and rice under controlled and field conditions. Technology for rapid propagation system transferred to NARS.
Testing of rice T-DNA populations for gene identification.

2006 Scaling up of marker-assisted selection and genetic transformation established for rice, bean and cassava.
Marker assisted selected for multiple traits implemented in beans, rice and cassava. Target genes for drought 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 countries in Africa.
High protein cassava lines developed and tested in Colombia and selected African countries.

Field-testing for transformed cassava with Bt gene and transformed rice with sheath blight resistance.
High through put propagation for selected tropical fruits initiated.

2007 Allele mining of *ex situ/in situ* collections of wild relatives of beans, cassava for genes of economic importance.
Gene flow studies diffused to NARS.
Candidate genes for drought tolerance identified for bean and rice.
Germplasm Upgrading Plan completed.
Safety duplicates at CIMMYT and CIP for bean and cassava germplasm.
Biofortified bean and cassava varieties in field-testing.
Methods for rapid multiplication of tropical fruit germplasm diffused to NARS.
Field-testing for cassava transgenic lines expressing inducible flowering genes for control of flowering in cassava breeding.

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

Principal Collaborators:

Africa NARS:

DRC: Mvuazi Research Center (INERA), **Ghana:** Crop Research Institute (CRI), Kumasi; **Kenya:** University of Nairobi, **Malawi:** Chitedze Research Station, Malawi; **Nigeria:** National Root Crops Research Institute (NRCRI), Institute for Agricultural Research and Training (IAR&T), Ibadan; **Rwanda:** ISAR; **Tanzania:** Agricultural Research Institute (ARI); **Uganda:** Namunlonge Agricultural and Animal Research Institute, Kampala; Medical Biotech Laboratories, Kampala.

Latin American NARS and Universities:

Bolivia: CFP - Centro Fitogenetico Pairumani; **Brazil:** Embrapa-Cenargen, Embrapa-CTA, Embrapa-CNPAF, Embrapa-CNPMF, University of Campinas; **Colombia:** Cenicaña, Cenicafe, Universidad Javeriana, CIB, COLCIENCIAS, Colombian Ministry Agriculture and Rural Development, Corpoica, Corporacion Biotech, Colombian National Biosafety Council, FEDARROZ, ICA, Instituto Humboldt, UniAndes, UniValle, Universidad Nacional at Palmira and Bogotá, Universidad del Tolima; **Chile:** INIA, REDBIO; **Costa Rica:** University of Costa Rica; **Cuba**: INIVIT, **Dominican Republic**: IDIAF, National Bean Programs of the (INIAF); **Ecuador**: INIAP, Universidad Catolica; **Honduras**: Zamorano; **Mexico:** Universidad Autonoma de Mexico, INIFAP; **Nicaragua:** Ministerio de Agricultura; **Peru:** INIA; **Venezuela:** Centro Tecnológico Polar, Simón Bolívar University.

Colombia NGOs:

CEGA, FIDAR, PBA, REDBIO-Colombia, Latin America, Small Farmers from Pescador and Tierradentro-Cauca, Cauca farmers association, Parque del Software, Cali.

Colombia private sector:

Corn product, Barranquilla; Agrobios, Bogota: LIMSYS, Cali; DATABIO, Cali; Syngenta, Cali.

Asia NARS:

China: Academy of Agricultural Sciences (CAAS), SCIB; **India:** Central Tuber Crops Research Institute (CTCRI) Thiruvananthapuram, Kerala; **Thailand:** Rayong Field Research Center.

Biodiversity Institutes:

Colombia: Instituto Humboldt; **Costa Rica:** Inbio; **Mexico:** Conabio; US: Smithsonian Museum of Natural History

Advanced Research Institutes:

Australia: Center for Applied Molecular Biology in International Agriculture (CAMBIA)
Europe: **Belgium:** University of Ghent; **Denmark:** University of Aarhus; **France:** CIRAD, Genoplants, IRD, INRA, Universite de Perpignan; **Germany:** University of Freiburg, University of Hanover, University of Hohenheim, Federal Biological Research Centre for Agriculture and Forestry (BBA); **Netherlands:** PRI-Wageningen; **Sweden**: USLU, Uppsala; **Switzerland:** Universite de Geneve, ETH; **UK**: University of Bath; **Japan:** JIRCA-Tsukuba; **United States:** Clemson University, Cornell University, Danforth Center, Kansas Sate University, Louisiana State University, Michigan State University, National Center for Genome Research, (NCGR), Ohio State University, Penn State University, Rutgers University, Smithsonian Molecular Systematic Lab, University of Nebraska, University of Puerto Rico; University of Chicago, USDA-Plant Soils and Nutrition Lab at Cornell University, USDA at Children Hospital Baylor University, USDA-Soybean Genomics, at Beltsville, Yale University.

Regional networks:

ASARECA, SACCAR, AfNet, ECABREN and SABRN (Africa); SIGTTA (Central America); REDBIO (Latin America); CATIE and EAP-Zamorano (Central America), Cassava Biotechnology Network (CBN-LAC); FLAR, CLAYUCA.

CGIAR, and International organizations:

CIP, CIMMYT, FAO, IAEA ICARDA, ICRISAT, IFPRI, IITA, IPGRI, IRRI, TSBF, WARDA.

CGIAR Challenge Programs:

HarvestPlus; Generation

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 (2005-2007)

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 to the conservation of agrobiodiversity in tropical countries.	# of CIAT scientists and partners using biotechnology information and tools in crop research. Germplasm and Genetic stocks available to key CIAT partners.	**CIAT and NARS publications.** Statistics on germplasm exchange.	
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.	A database 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 bean, cassava, rice and Brachiaria and of associated organisms.	*Molecular Genetic Techniques and molecular information on diversity*		
Development of genome-wide anchored PCR based markers for marker assisted selection, germplasm characterization, and fine mapping, and gene flow analysis.	*2005:* SNP markers for bean and rice developed.	Method available, Publication of SNP primers and protocols	Availability of up-to-date genomics equipment Collaboration with NARS maintained and expanded
	Characterization of core collection and national collections of bean with fifty genomic and gene-based microsatellite markers.	Reports on marker analysis and articles describing the genetic structure of the bean and cassava core collection of the world germplasm collection vis-à-vis other accessions of the collection.	
Identification and mapping of useful genes and gene combinations for agronomical and nutritional traits.	Characterization of a basic collection of tepary bean germplasm with AFLP and microsatellite markers.		
Markers assisted selection for multiple traits for bean, cassava and rice	1500 cassava accessions genotyped with 36 SSR markers.	Availability of a laboratory information management system (LIMS).	
Bioinformatics tools for data mining	Cassava core collection screened for carotenes and true retention of carotenes after processing	Reports and primers made available in databases	

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
	determined LIMS developed and implemented 2006-07 Cross-legume and single-nucleotide polymorphism markers in CIAT mapping population integration of legumes cross-collection diversity data Selection of a set of genes for the development of cassava COS markers *Identification and mapping useful genes and QTLs for agronomical traits in bean, cassava, rice and Brachiaria* 2005 QTL analysis completed in two bean populations for nutritional traits including iron, zinc and tannin content Molecular markers tightly linked to CMD resistance identified and BAC library of TME3 constructed Two advanced backcross with wild AA Oryza genomes genotyped and QTLs for yield component in rice identified Generation of rice mapping populations for nutritional traits including iron and zinc 2006-07 QTL analysis completed on two populations of common bean for drought traits and nutritional traits.	Genes suitable for the development of COS markers in cassava databases. Articles describing molecular markers associated with agronomical traits. Populations, markers and BAC library available for distribution and further analysis by partners Publications, reports, and data on population posted on web. Databases shared Map position sof targer genes indicated	

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
	One advanced backcross populations (BC2F5) from wild beans is genotyped to determine if nutritionally superior genotypes can be obtained. Integration of drought and nutrition QTLs across multiple populations Populations segregating for dry matter, cyanogenic glucosides content, leaf retention, resistance to hornworm developed, characterized and evaluated with molecular markers QTL analysis completed on one population for Al tolerance in *Brachiaria*.		
Output 2 Genomes modified: genes and gene combinations used to broaden the genetic base of crops (bean, rice, and cassava) and forage species (*Brachiaria*) Identification of points of genetic intervention and mechanism of plant stress interaction Improved methods for genetic transformation for bean, rice and cassava Develop and obtain gene constructs for traits related to plant disease-insect resistance, plant stress and nutritional traits. Acquisition of rice T-DNA and Ac/Ds mutants populations for testing and gene	*Candidate genes identified for agronomical traits* 2005 Cloning of candidate genes involved in tolerance to acid soils: full length cDNA libraries developed; Differentially expressed genes isolated by microarray Molecular characterization of spittlebug insect resistance in Brachiaria using cDNA subtractive differential expressed libraries Comparative genomics and gene discovery drought in bean by gene expression profiling; cDNA libraries produced under drought conditions	Publications, reports, and project proposals. Germplasm Libraries and candidate genes available Libraries and candidate genes available Libraries and candidate genes available Microarray chips available for distribution	Phenotypic-biochemical analysis conducted prior to molecular analysis Continued access to biosafety field

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
discovery Implement biosafety regulation for greenhouse and field condition	Cassava bacterial blight interaction characterized using cassava cDNA plant defense microarray chip of 6000 cassava unigene sets T-DNA rice 10000 mutants collections characterized under field condition 2006-07 Consolidated genes sequence data for drought and acid soils stress response pathways; gene chips for candidate genes developed; over 5000 genes arrayed and 25 candidate genes putatively related to drought tolerance identified Modification of flowering to increase the efficiency of breeding in cassava by using constructs for *LFY, API* and *FLC* flowering genes under the control of an ethanol-inducible promoter. Cloning of the cassava mosaic disease (CMD) resistance gene CMD2 by positional cloning *Transformation* 2005 Degree of gene flow from rice and bean into wild/weedy relatives measured in tropical centers of diversity. Identification, evaluation and diffusion of molecular markers for assessing rate and direction of gene flow Field performance biosafety evaluation for	Characterization data made available on web. Databases Libraries and sequence made available. Databases Technical reports on sites and distribution of wild/weedy/ landraces, and cases of gene flow Number of advanced lines and crosses with RHBV resistant transgenic source Publications, reports, and protocols made available and published.	testing and collaboration with CIRAD, IRD and genoplantes Phenotypic-biochemical analysis conducted prior to molecular analysis Funding from Rockefeller Foundation, access to genes. IPR management to access genes and gene promoters. Biosafety regulations in place. IPR management to access genes and gene promoters. Biosafety regulations in place. Request extension of field permit to other field locations in Colombia approved IPR management to access genes and gene promoters. Biosafety regulations in place. Already received approval for field testing. Field Biosafety inspected and approved

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
	agronomic traits of advanced generations of crosses between RHBV-N resistant transgenic rice and selected rice commercial varieties Transgenic *Agrobacterium* strains generated with constructs from JIRCAS containing different versions of *DREB* gene encoding for drought tolerance. Transgenic lines of cassava with Bt constructs and or rice with resistance to sheath blight tested under biosafety field conditions and evaluated for agronomical traits. 2006-07 Protocol developed for generating transgenic plants based on mannose selection system in place for rice and cassava Scaling up of rice and cassava transformation efficiencies; incorporating new genes, cultivars, and regeneration methods, and testing plants in the field. Field testing for rice and cassava transformed genotypes Optimization of bean transformation protocols Optimization of low cost alternative system using temporary immersion system principle in place for rice anther culture callus induction and plant regeneration for a scaling up system Adaptation and optimization of protocols for rice isolated microspore culture as an alternative for high efficiency generation of doubled haploids		

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Output 3 Increased efficiency of NARS breeding programs using biotechnological tools	2005 Marker assisted selection for Cassava Mosaic Disease (CMD) transferred to NARS in Tanzania CIAT partners in LDCs using information and genetic stocks. LAC NARS involved in biofortification effort for iron, zinc and beta carotene Improved capacity of Colombian NARS to deal with biosafety 2006-07 New partnerships with private sector. Agreement on technology and gene constructs access	Publications. Training courses and workshops. Project proposals. Regional workshop MTA established and joint publication	Government and industry support national biotech initiatives. Freedom to operate obtained
Output 4 Bean, Cassava and forage species conserved and multiplied as per international standards.	2005 Germination rates for long-stored materials. Cost per accession/year, compared with other gene banks. 2006-07 Safety duplicates at CIMMYT and CIP for bean and cassava germplasm.	Visits to GRU substations and conservation facilities.	Absence of uncontrolled diseases. Quarantine greenhouse space available at different altitudes.

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Output 5 Germplasm available, restored, and safely duplicated.	2005 Number of germplasm requests received and fulfilled annually. Low cost rapid propagation system for cassava implemented with farmer association Users received germplasm and data. 2006-07 Cryo-conservation technology developed, tested and implemented for cassava 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. CIAT becomes partner to the Treaty.
Output 6 Designated Collections made socially relevant.	2006-07 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.	2005 NARS germplasm collections conserved. Number of trainees trained at CIAT. 2006-07 Methods for rapid multiplication of selected tropical fruit germplasm diffused to NARS. Number of universities and NARS using training materials.	Country questionnaires. Courses registered. Distribution and sales of training materials. Protocols published	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. Databases	NARS interested in conservation efforts. Farmers interested in conservation efforts.

OUTPUT 1. Improved characterization of the genetic diversity of wild and cultivated species and associated organisms

Activity 1.1 Characterization of genetic diversity

1.1.1 Diversity of microsatellite markers in common bean (*Phaseolus vulgaris* L.) mini core collection

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Introduction

Microsatellite markers have been selected as the marker system of choice because of their low cost, high efficiency, whole genome coverage, robustness and minimum DNA requirements. In addition, they are highly polymorphic, co-dominant, PCR based and easily detectable. Genotyping with microsatellites can occur on multiple platforms including slab or capillary polyacrylamide gels with automated (fluorescent) or semi-automated (high-throughput silver staining) detection technologies. In common bean there are a total of 235 previously published microsatellite markers of which 150 have been tested and 115 genetically mapped at CIAT. In this study we evaluate all of the mapped SSRs for their diversity value (D), polymorphism information content (PIC) and heterozygosity level on a mini-core collection. The most diverse microsatellites are being selected for genotyping in the Generation Challenge Program. Microsatellite markers will be useful for distinguishing races within the two major gene pools, for feeding into marker assisted selection programs and for tracing lineages and will supplement information collected from RAPD and AFLP studies that have defined the basic structure of diversity within the species.

Materials and Methods

Plant material: A total of 44 genotypes were used in this study, representing the parents of 25 mapping populations being studied for biotic and abiotic resistance and grain quality traits at CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia). The genotypes were grouped in 3 parental surveys that were carried out separately with common controls, namely DOR364, a Mesoamerican genotype; and G19833, an Andean genotype, included in each survey.

Microsatellite analysis: The genotypes were evaluated for allelic diversity at 130 microsatellite (57 gene-associated and 73 genomic) loci . Amplification used genomic DNA template that had been extracted based on the miniprep procedure from Afanador and Hadley (1993). Microsatellite amplification and detection conditions were as reported in previous annual reports. Markers that did not amplify were not considered further. To resolve allelic diversity as fully as possible, the PCR products for each survey

were separated by electrophoresis for 1.5 hours at 120 constant volts on silver-stained 4% polyacrylamide gels. Microsatellite alleles for the control genotypes (DOR364 and G19833) were sized by comparison to the 10 and 25 bp molecular weight standards (Promega). Alleles of the remaining genotypes were compared to the control bands for each microsatellites so that molecular weights (in nucleotides) could be determined across parental surveys. Null alleles were not used in diversity assessment.
Data Analysis: Polymorphism information content (PIC) was calculated according to Botstein et al.(1980) and refers to the relative value of each marker with respect to the amount of polymorphism it exhibits. The microsatellite alleles were also coded into a binary data matrix that was analyzed by multiple correspondence analysis (MCA), using the CORRESP procedure of SAS (SAS Institute, 1989).

Results and Discussion

Allele number and microsatellite diversity: The average number of alleles for the genomic microsatellites (8.14) was almost double the average number of alleles for the gene-based microsatellites (4.59) (Table 1). Similarly, the discrimination index was higher for the genomic microsatellites (D=0.595 on average) compared to the gene-based microsatellites (D=0.458 on average). The highest numbers of alleles among the genomic and gene-based markers were found for BM137 and BMd1 with 21 and 16 alleles, respectively. Coincidentally these two markers had the highest discrimination values (D=0.941 and D=0.927, respectively). A large number of gene-based markers were bi-allelic and effectively distinguished Andean and Mesoamerican gene pools but did not distinguish genotypes within each of these gene pools. Most of the genomic microsatellites were multi-allelic with more than 3 or 4 alleles each. Monomorphic markers across the 44 individuals tested included the gene-based microsatellites BMd35, BMd48, PVAAAT001, PVATCC001 and the genomic microsatellite BMd39.

Relationships between genotypes: The principal component analysis shown in Figure 1 identifies two principal clusters, one Andean and one Mesoamerican differentiated by one dimension. The tepary bean accession, used as an outgroup and included as a donor parent in congruity backcross introgression lines was differentiated from all common bean accessions on a second dimension. A third dimension distinguishes one Andean wild and one Mesoamerican wild P. vulgaris accession from the domesticated common bean genotypes. Interestingly greater diversity was found in the Andean gene pool with the microsatellites than in the Mesoamerican gene pool. This may be a reflection of the selection of parents since the Andean genotypes were more diverse morphologically than the Mesoamerican genotypes, even though the Mesoamerican gene pool is represented by a greater number of races (Durango, Guatemala, Jalisco and Mesoamerica) than the Andean gene pool (Nueva Granada and Peru). Variability within the Andean gene pool may reflect race structure with two subgroups identified however this would need to be confirmed with additional genotypes. One Andean genotype could be identified as a possible hybrid from Mesoamerican-Andean gene pool introgression. Compared to the other Mesoamerican genotypes the Guatemala race individuals were fairly distinct.

Ongoing activities

Ten panels of fluorescent microsatellite markers have been designed based on allele sizes found in this mini core. The panels are being tested for PCR multiplex capacity and fluorescence signal, each panel having from 6 to 10 markers for a total of 75 markers of which the best 50 markers will be identified and run on ABI377 and ABI3100 platforms. This work will allow us to optimize the choice of molecular markers for varietal identification, gene mapping and diversity studies.

Table 1. Number of alleles and polymorphism information content (PIC) of 124 common bean microsatellites (53 gene-based and 71 genomic) markers

Gene-based						Genomic					
Marker	No. Alleles	of PIC	Marker	No. Alleles	of PIC	Marker	No. Alleles	of PIC	Marker	No. Alleles	of PIC
BMd1	16	0.941	BMd45	2	0.416	AG1	7	0.483	BM181	8	0.571
BMd2	4	0.631	BMd46	3	0.379	BM3	1	0.325	BM183	11	0.839
BMd3	6	0.626	BMd47	3	0.573	BM6	1	0.044	BM184	8	0.684
BMd4	2	0.129	BMd49	1	0.201	BM48	1	0.087	BM185	10	0.833
BMd5	2	0.129	BMd50	2	0.334	BM53	20	0.927	BM187	16	0.854
BMd6	2	0.129	BMd51	3	0.407	BM68	3	0.493	BM188	14	0.880
BMd7	3	0.756	BMd53	5	0.574	BM98	4	0.601	BM189	6	0.647
BMd8	5	0.723	BMd55	2	0.088	BM114	12	0.818	BM195	1	0.044
BMd9	5	0.287	BMy1	11	0.823	BM137	21	0.937	BM197	4	0.557
BMd10	6	0.768	BMy2	7	0.577	BM138	7	0.779	BM199	17	0.913
BMd13	2	0.247	BMy4	7	0.729	BM139	13	0.632	BM200	15	0.893
BMd14	2	0.210	BMy5	4	0.481	BM140	7	0.459	BM201	11	0.820
BMd15	5	0.722	BMy6	9	0.510	BM141	16	0.813	BM202	6	0.796
BMd16	5	0.527	BMy8	18	0.943	BM142	4	0.537	BM205a	8	0.707
BMd17	6	0.676	BMy9	25	0.945	BM143	17	0.892	BM209	12	0.848
BMd18	4	0.774	BMy10	2	0.315	BM146	3	0.170	BM210	16	0.880
BMd19	4	0.285	BMy11	7	0.546	BM147	2	0.088	BM211	12	0.848
BMd20	8	0.799	BMy11b	6	0.829	BM148	1	0.044	BM212	7	0.517
BMd21	5	0.389	PVatcc002	2	0.044	BM149	6	0.320	BM213	3	0.394
BMd22	4	0.632	PVatcc003	2	0.501	BM151	7	0.823	BMd11	2	0.129
BMd23	2	0.129	PVatct001	2	0.206	BM152	13	0.897	BMd12	3	0.650
BMd25	2	0.439	PVccct001	2	0.501	BM153	12	0.876	BMd33	6	0.690
BMd26a	2	0.496	PVcct001	7	0.684	BM154	22	0.936	BMd36	10	0.762
BMd27	1	0.172	PVtttc001	5	0.638	BM155	4	0.387	BMd38	2	0.044
BMd28a	7	0.882				BM156	16	0.818	BMd40	6	0.801
BMd30	3	0.249				BM157	5	0.510	BMd41	6	0.716
BMd31	1	0.046				BM159	5	0.590	BMd42	8	0.764
BMd32	2	0.247				BM160	12	0.580	BMd43	3	0.414
BMd37	5	0.799				BM161	8	0.873	BMd44	3	0.210
						BM164	12	0.495	BMd56	4	0.534
						BM165	10	0.826	BMd57	2	0.044
Average (genic)		0.458	No. (genic)	4.59		BM166	2	0.044	GATS11	4	0.683
						BM167	9	0.693	GATS11B	2	0.385
Average (genomic)		0.595	No. (genomic)	8.14		BM170	12	0.877	GATS54	3	0.563
						BM172	11	0.580	GATS91	16	0.914
Average (total)		0.534	No. (total)	6.57		BM175	14	0.749			

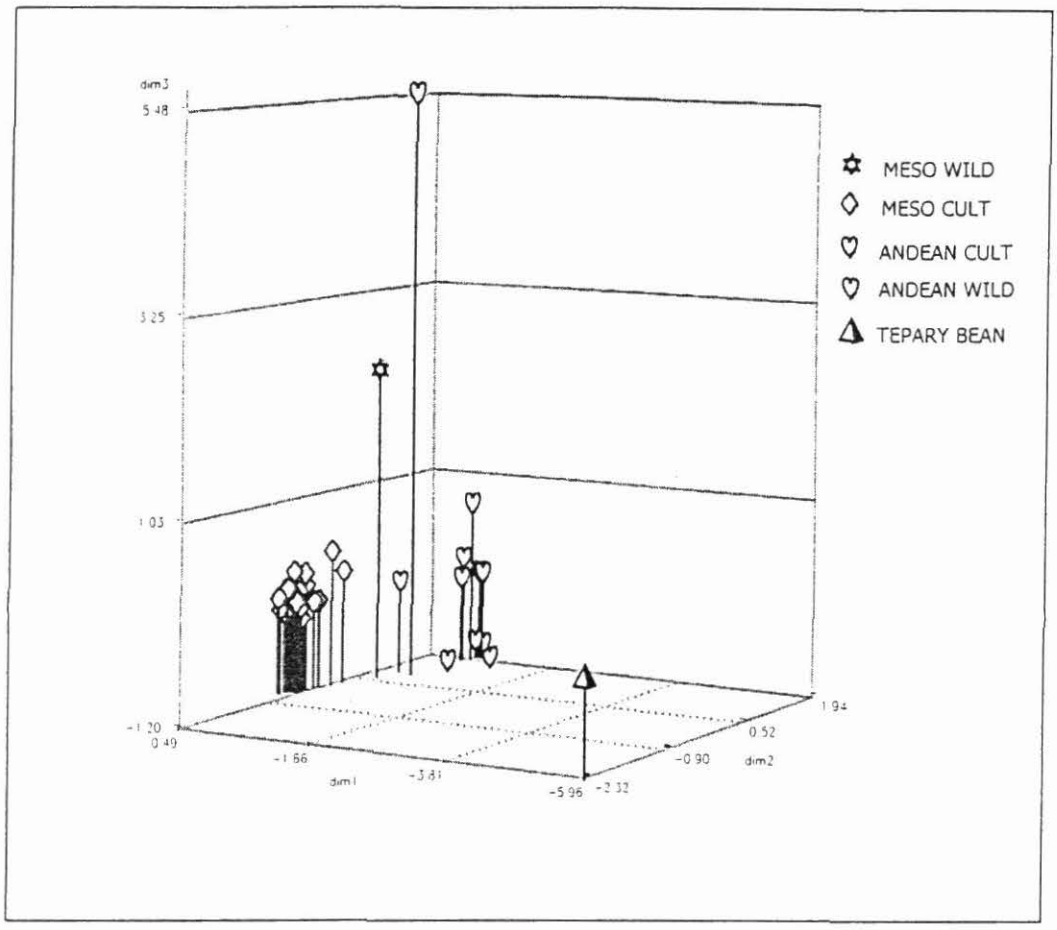

Figure 1. **UPGMA clustering based on analysis of a mini core collection of 44 common bean genotypes with 130 microsatellite markers and multiple correspondence analysis.**

1.1.2 Evaluation of a widely diverse set of Caribbean accessions of common bean

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SB-2 Project, CIAT

Introduction

Last year we evaluated a collection of 129 common bean accessions from the University of Puerto Rico, and the national collections of Haiti and Dominican Republic for diversity at microsatellite loci and found evidence for inter Andean x Mesoamerican gene pool in this secondary center for diversity. This year our goal was to evaluate a larger number of Caribbean genotypes that are held in the FAO-designated collection of common bean at CIAT for microsatellite diversity and compare to this first set. We focused especially a greater number of genotypes from Cuba (black and light red kidney)

and Jamaica (pink striped). We sampled from most Andean seed classes found in the Caribbean 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.

Methodology

A total of 182 entries of common bean genotypes from the FAO-designated collection at CIAT were genotyped, including 25 accessions from Cuba, 21 from the Dominican Republic, 108 from Haiti, 5 from Jamaica and 21 from Puerto Rico. DNA was extracted from young trifoliate leaves of five plants of every genotype using an ammonium acetate extraction technique and this template was used for PCR amplification of an initial set of 17 microsatellites (Table 1). Microsatellite PCR reactions and polyacrylamide gel electrophoresis were as described previously. Silver-stained gels were dried overnight and scanned for data analysis. As controls, ICA Pijao (Mesoamerican) and Calima (Andean) were analyzed in every gel.

Results and Discussion

Diversity values and allele number for each of the 17 microsatellite (11 genomic and 6 gene-based) markers is given in Table 1. 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. The total number of alleles evaluated across all 17 markers was 79 and on average each marker revealed 4.65 alleles each. The average diversity value was 0.582. The number of alleles was significantly higher for genomic microsatellites (5.55) than for gene-based microsatellites (3.0) based on unpaired t-test (at $P<0.05$). Interestingly the discrimination power (D) values were similar for both genomic and gene-based microsatellites (0.579 vs 0.588). The most polymorphic markers in terms of number of alleles or high D values were BM152 (13 alleles, 0.821 D value) and BM143 (10 alleles, 0.753 D value). BM152 and BM143 were also the most polymorphic markers for the previous set of Caribbean germplasm. The least polymorphic markers in terms of either low number of alleles (2 alleles) or low D values ($D<0.500$) were BM142, BM155, BMd26, BMc5 and GATs54 of which three 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 control Calima; and a Mesoamerican group that was similar to the control ICA Pijao (Figure 1). These two gene pools were related at a 0.20 Dice similarity value. There were a greater number of accessions and a higher overall diversity within the Mesoamerican group (98 accessions, 0.50 to 1.0 Dice similarity values) than within the Andean group (85 genotypes, 0.58 to 1.0 Dice similarity values) of this dendrogram. Among the Mesoamerican genotypes a group of Caribbean genotypes were similar to the black-seeded control ICA Pijao. In contrast, among the Andean genotypes only three were somewhat similar to the Calima. While some of the black seeded Mesoamerican genotypes from Cuba appear to be recent introduction that are closely related to ICA Pijao, a standard black seeded variety; the red mottled Andean

genotypes from Haiti and Dominican Republic were fairly distinct from this representative of the Nueva Granada race. Within each gene pool there is some evidence for subgroups but this will require analysis with additional markers to confirm. Some genotypes in both gene pools are intermediate and may represent a limited amount of introgression between the gene pools although the distinctiveness of the two groups is very defined.

Conclusions and ongoing activities

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 the genetic diversity of beans from the Caribbean. We will complete a set of 30 to 50 microsatellite markers on this germplasm to better understand its population structure and for use in association mapping studies. The microsatellite fingerprinting will also be useful for identifying potential duplicates in the FAO collection. By the end of the coming year we hope to have a better understanding of whether the large seeded Caribbean germplasm traces back to the Nueva Granada race and whether several races of Mesoamerican beans contributed genes to this germplasm.

Table 1. Diversity values and number of alleles identified by 17 microsatellite markers used in the analysis of Caribbean bean germplasm.

	Microsatellite marker	Map Position	Range in Allele sizes	No. of Alleles	Diversity Value (d)
Genomic	BMd12	B06	158-174	5	0.208
	BM140	B04	160-200	6	0.516
	BM142	B02	154-156	2	0.499
	BM143	B03	116-162	10	0.753
	BM152	B02	94-144	13	0.821
	BM155	B05	116-118	2	0.501
	BM170	B06	158-188	5	0.641
	BM183	B07	124-142	3	0.573
	BM184	B09	152-166	5	0.593
	BM189	B08	108-120	5	0.614
	BM205	B07	124-142	5	0.649
Genic	BMc5	B10	134-142	2	0.580
	BMd20	B05	120-130	4	0.649
	BMd26	B04	136-142	2	0.500
	BMy1	B04	152-172	5	0.705
	BMy2	B11	148-160	3	0.521
	GATs54	B03	116-118	2	0.571

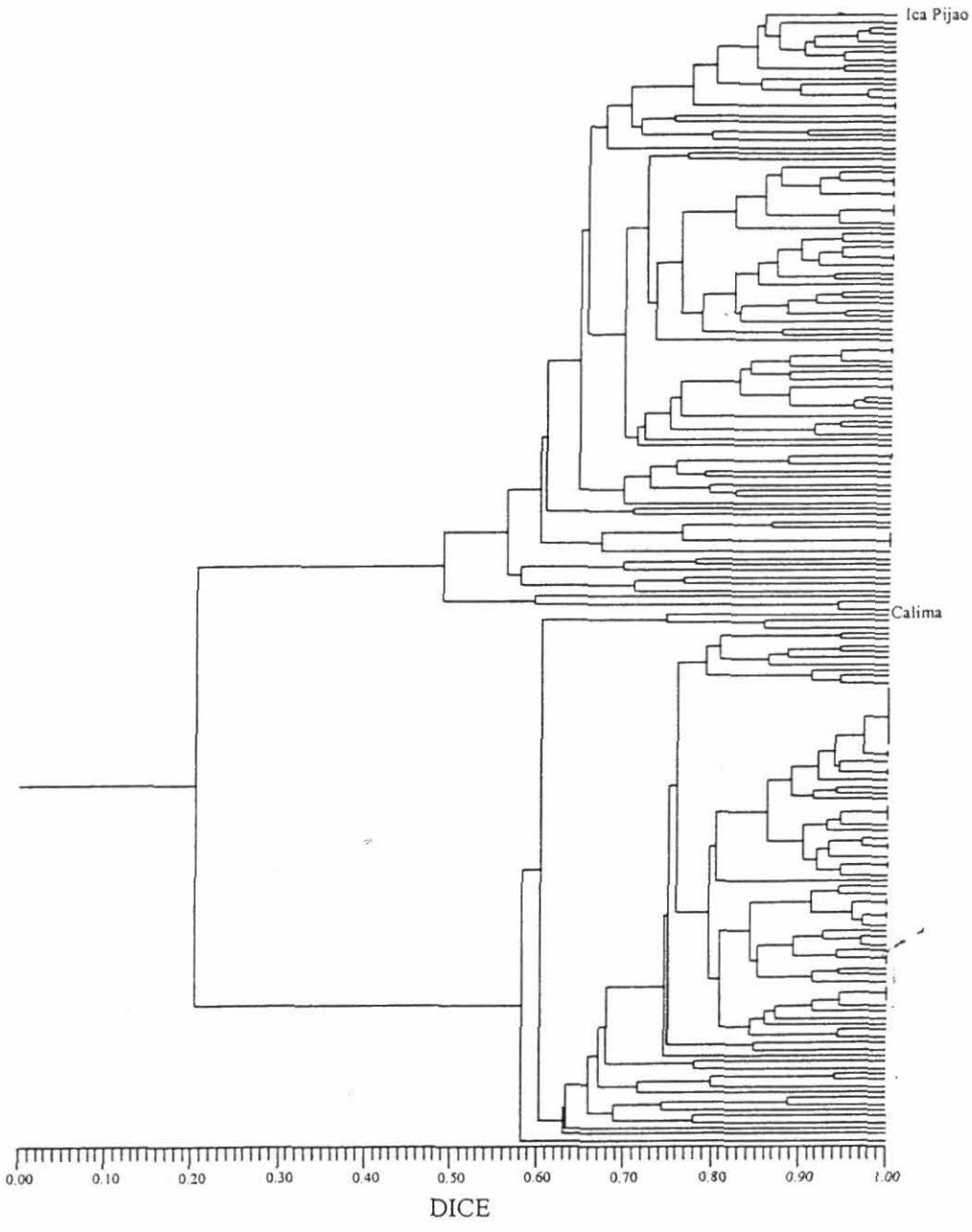

Figure 1. Dendrogram of relationships among Caribbean accessions of common bean uncovered by Microsatellite marker analysis.

1.1.3 Diversity in Colombian common bean germplasm

MW Blair, JM Diaz, L Diaz
SB-2 Project, CIAT

Introduction

North-West South America is a zone of germplasm exchange and gene flow between the Mesoamerican and Andean centers of diversity for common bean and therefore has a large amount of landrace diversity. With this in mind we began a marker diversity study using 91 Colombian genotypes from the CIAT core collection and 30 microsatellite marker to evaluate the ability of this type of marker to uncover this gene flow in this germplasm. Considering that Colombian germplasm is representative of diversity in Latin American beans more generally, this study was conducted in advance of the genotyping of the entire core collection with a set of fluorescent microsatellite markers as part of the Generation Challenge Program.

Materials and Methods

Plant material: A total of 91 genotypes were evaluated, including 74 Colombian landraces and the following CIAT advanced lines as checks: Mesoamericans = A686, BAT93, BAT304, BAT477, BAT1215; Andeans = ABA 2, AND1005, BAT1373, CAL149, DRK47, LRK31, PVA1111, PVA773. All these genotypes had been evaluated previously for phaseolin pattern. The genotypes Calima, DOR364, ICA Pijao and G19833 were used as common checks with previous studies.

Microsatellite analysis: The genotypes were evaluated for allelic diversity at 29 microsatellite (10 gene-based and 19 genomic) loci. Microsatellite amplification and detection conditions were as reported in previous annual reports. To resolve allelic diversity as fully as possible, the PCR products for each survey were separated by electrophoresis for 1.5 hours at 120 constant volts on silver-stained 4% polyacrylamide gels. Microsatellite alleles for the control genotypes (Calima, DOR364, ICA Pijao and G19833) were sized by comparison to the 10 and 25 bp molecular weight standards (Promega). Alleles of the remaining genotypes were compared to the control bands for each microsatellites so that molecular weights (in nucleotides) could be determined across parental surveys. Null alleles were confirmed by a second PCR amplification of the specific genotypes that did not amplify bands.

Data Analysis: Polymorphism information content (PIC) was calculated according to Botstein et al.(1980) and refers to the relative value of each marker with respect to the amount of polymorphism it exhibits. Genetic similarities between genotypes were determined with the Dice coefficient using NTSYS 2.02 (Rohlf, 1993). The similarity matrices were used to construct dendrograms with the same program. Multiple

correspondence analysis (MCA) was done using the CORRESP procedure of SAS (SAS Institute, 1989).

Results and Discussion

The microsatellites used in this study varied in the amount of polymorphism they uncovered (Table 1). High PIC markers included genomic microsatellites, BM143, BM152, BM160 and GATS91, all of which were genomic; while intermediate PIC markers included BM137, BM139, BM170, BM175, BM183 and BMy1, all except one of which, the last, were genomic. The gene-based markers had lower PIC values (0.582) and detected fewer alleles (5.1) than the genomic markers (PIC = 0.685, 10.1 alleles). Since many gene-based markers were bi-allelic, they were better at distinguishing the two gene pools better than the Mesoamerican genotypes that were more likely to share alleles across gene pool boundaries or have a large number of alleles in each group.

These trends were observed in both Andean and Mesoamerican subgroups, however different markers were more informative in one gene pool versus the other (data not shown). Interestingly, the Andean genotypes showed a larger number of alleles than the Mesoamerican genotypes for a majority of the genomic microsatellites (68.4%) but not for the gene-based microsatellites (30.0%). Gene-based microsatellites were more likely to have an even number of alleles for both gene pools (30.0%) compared to genomic microsatellites (21.0%). As a result the Andean genotypes were more diverse in terms number of alleles, 7.3 compared to 5.7 for Mesoamericans. This difference was more notable in the genomic microsatellites for which PIC values were higher in the Andean genotypes (0.619 vs. 0.609) than in the gene-based microsatellites for which PIC values were higher in the Mesoamerican genotypes (0.569 vs. 0.490). The difference in number of alleles between the gene pools may be related to the ascertainment bias resulting from the development of the genomic microsatellites from an Andean small insert library (Gaitan et al., 2002) compared to the gene-based microsatellites that were developed from sequenced genes from both gene pools (Blair et al., 2003). The differences in PIC reflect the distribution of diversity as well as the total number of alleles.

The number of genotypes that were heterozygous was low overall (2.43 %) showing that most of the genotypes, both the landraces and the advanced lines from CIAT are uniform genetically. The number of heterozygous genotypes for each marker ranged from 0% to 7.7% for BM140. The number of heterozygous loci was not associated with the PIC value of the marker. Certain genotypes had more heterozygous loci than others in the analysis; as expected, landraces had slightly more heterozygous loci (10.5%) than advanced lines and modern varieties (9.0%).

In the phylogenetic analysis, two clusters were amply evident and corresponded to the Andean and Mesoamerican gene pools (Figure 1). A bridge of accessions joined the two clusters and corresponded to intermediate individuals, mostly Andeans with gene introgression from the Mesoamerican cluster. The Andean gene pool was more diverse than the Mesoamerican cluster. Within the Andean genotypes, three sub clusters were observed while in the Mesoamerican cluster the genotypes tended to group together.

It was surprising to find greater than expected diversity within the Andean cluster which previously was thought to be highly similar based on RAPD studies (Beebe et al., 2001). This shows the usefulness of SSR analysis for landrace identification and grouping Further analysis of all genotypes with Andean phaseolin type is shown in the dendrogram in Figure 2, in which two clusters were evident, one with DOR364 (phaseolin type S) and ICA Pijao (S), the Mesoamerican controls and the other with the Andean control genotypes Calima and G19833. Two groups of intermediate genotypes include the genotypes G4691 (CH), G8157 (H), G19142B (CA), G7257 (T) on the Mesoamerican branch and three other genotypes on the Andean branch, including G21217 (H), G12692 (H) and G21242 (T). The remaining genotypes all grouped with the Andean cluster within which there was differentiation of three groups: one that was almost entirely made up of bush beans with type I growth habit and 'T' or 'CA' phaseolin; another that was made up almost entirely of climbing beans with type III or IV growth habit and 'H' or 'T' phaseolin and a third group that was intermediate. The first group probably represents the Nueva Granada race of Andean beans while the second group represents the Peru race of Andean beans. The third group probably represents inter-racial hybridization which has been exploited recently in our Andean breeding program to develop new genotypes of climbing and bush beans. Most genotypes were fairly distinct within the dendrogram showing a wealth of genetic diversity to exploit for plant breeding. One exception was the close similarity of Calima and G4494 which were identical. This was expected since one represents the germplasm accession and the other a working stock of the same variety. The results so far are positive in showing some race differentiation within the Andean gene pool and the presence of inter gene pool introgression which has been suggested as valuable for association mapping of traits (Amirul-Islam et al., 2004), therefore the present dataset of Colombian genotypes will be a useful starting point for further studies of microsatellite diversity in common bean.

Ongoing activities

Repeat the Phylogenetic and Multiple Correspondence Analysis with the complete dataset and define if race substructure is evident.
Add additional genotypes from the breeders' core of CORPOICA – Rionegro where genetic improvement for climbing beans is a priority. A total of 343 genotypes have been identified.
Evaluate similarity of Colombian germplasm with germplasm from other countries.
Begin phenotyping a sub-selection of the germplasm in a uniform trial to generate data for association studies.
Determine the level of inter gene pool and inter racial introgression in the landraces and advanced lines evaluated in this study and make recommendations for breeding activities

Table 1. Diversity assessment of 91 Colombian and CIAT genotypes with 29 microsatellite markers.

Marker Type	Microsatellite Marker	Map Position	Motif	Observed range	Expected size	No. of Alleles	% Het	Diversity Value (D)
Genomic	BM139	CR2	(CT)25	85-125	115	11	3.30	0.83
	BM140	CR4	(GA)30	160-198	190	8	7.69	0.79
	BM142	CR2	(GA)10A(GA)15	154-156	157	4	1.10	0.43
	BM143	CR3	(GA)35	113-179	143	25	5.49	0.94
	BM152	CR2	(GA)31	94-172	127	17	2.20	0.88
	BM155	CR5	(CA)8	116-118	114	3	0.00	0.30
	BM160	CR7	(GA)15(GAA)5	182-260	211	22	5.49	0.91
	BM164	CR2	(GT)9(GA)21	144-186	182	8	0.00	0.78
	BM167	CR2	(GA)19	149-250	165	14	0.00	0.85
	BM170	CR6	(CT)5CCTT(CT)12	156-180	179	10	4.40	0.71
	BM175	CR5	(AT)5(GA)19	156-186	170	15	2.20	0.89
	Bm183	CR7	(TC)14	133-167	149	10	4.40	0.55
	BM184	CR9	(AC)11	150-165	160	5	0.00	0.54
	BM189	CR8	(CT)13	106-118	114	6	0.00	0.70
	BM205	CR7	(GT)11	142-156	137	5	5.49	0.68
	BMd12	CR6	(AGC)7	162-168	167	5	0.00	0.55
	GATS11B	CR10	(CT)8	102-106	160	3	3.30	0.48
	GATs54	CR3	(GA)5AACAGAGT(GA)8	114-118	114	3	0.00	0.31
	GATS91	CR2	(GA)17	215-257	229	18	0.00	0.89
Gene-based	BMc5	CR10		134-142		2	2.20	0.48
	BMd10	CR1	(GA)8	137-143	139	4	0.00	0.46
	BMd15	CR4	(AG)6	152-204	166	6	6.59	0.62
	BMd17	CR2	(CGCCAC)6	98-116	116	3	1.10	0.42
	BMd20	CR5	(TA)4	119-131	123	7	2.20	0.61
	BMd26	CR4	(GAT)6	135-141	141	2	1.10	0.43
	BMy1	CR4	(CTT)3 (T)3 (CTT)6	152-173	152	12	7.69	0.87
	BMY2	CR11	(CT)11	150-166	157	7	2.20	0.66
	BMY4	CR1	(AG)8	162-172	164	5	0.00	0.71
	BMy6	CR4	(AT)4(T)2	135-139	163	3	2.20	0.55

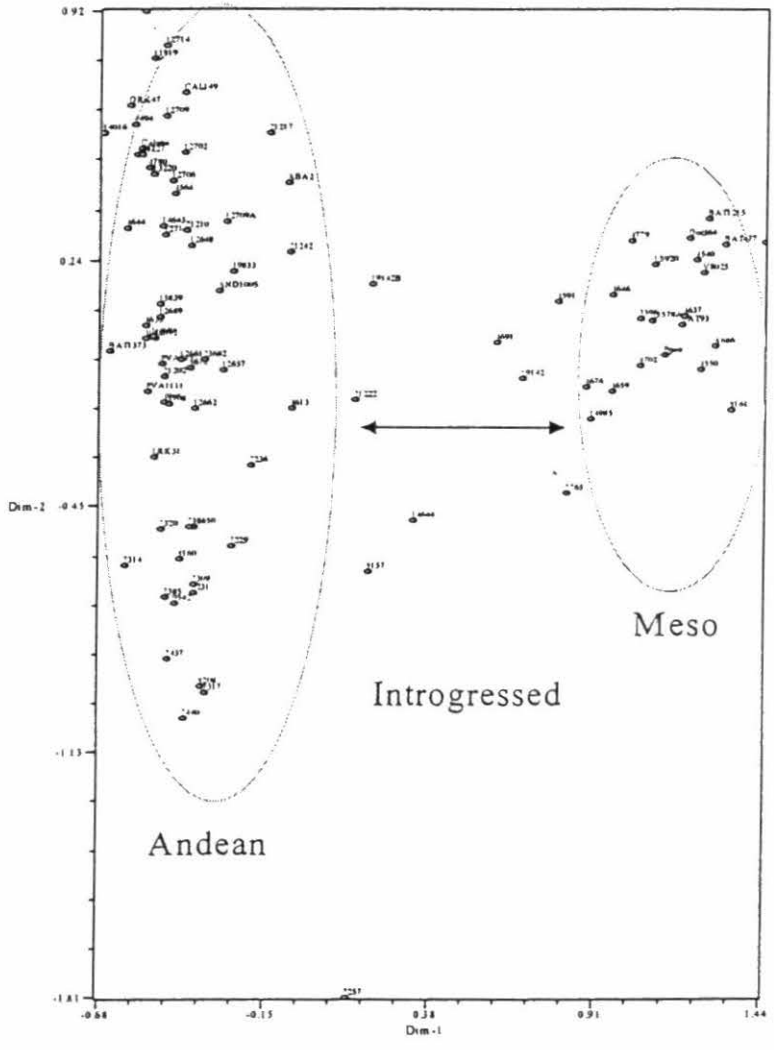

Figure 1. **Multiple correspondence analysis showing structure of Colombian germplasm from the CIAT core collection.**

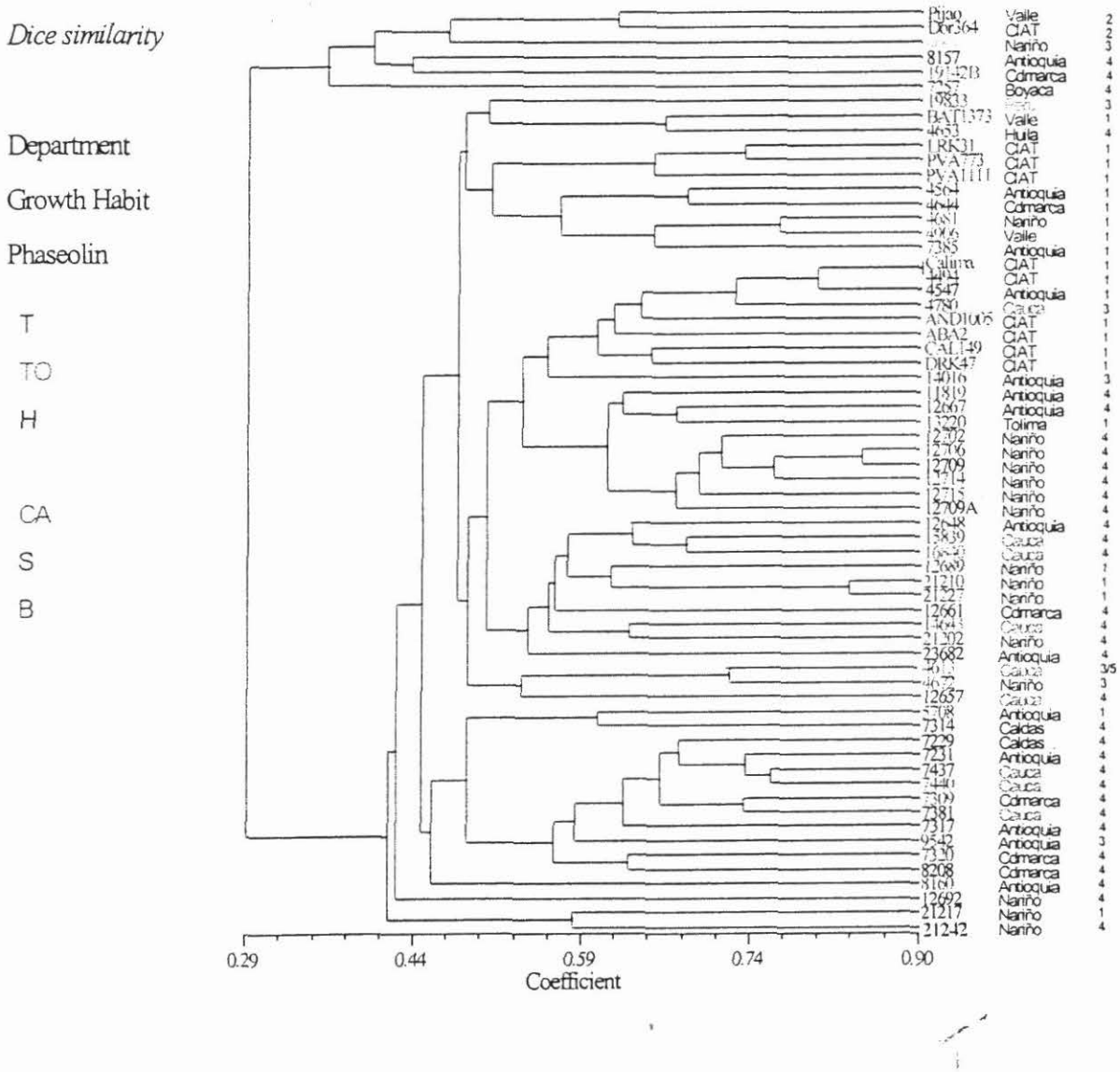

Figure 2. Dendrogram of relationships among *Colombian accessions of common* bean with Andean phaseolin uncovered by microsatellite marker analysis. Calima and G19833 were Andean controls and DOR364 and ICA Pijao were Mesoamerican controls.

1.1.4 Additional Diversity of Tepary beans evaluated

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1.SB-2, Project; 2. SB-1, Project; 3. IP-4 Project

Introduction

We have studied the genetic diversity within tepary bean (*Phaseolus acutifolius* A.Gray) and related species (*P. parvifolius*) with amplified fragment length polymorphism (AFLP) markers and found that similar to previous studies using isozyme markers and RFLPs (Scinkel and Gepts, 1988, 1989; Garvin and Weeden, 1994; Muñoz et al., 2002; Blair et al., 2002) diversity within the cultivated tepary bean is low but that intra- and inter-specific differences can be distinguished among the wild accessions of tepary bean and their wild relatives. In our previous evaluation we included relatively few accessions of the wild tepary beans from the *P. acutifolius* var. *tenuifolius* and *P. parvifolius* groups. Therefore our objective was to evaluate a larger number of these wild tepary beans and compare them to our previous evaluation. We also address the question of whether *P. latifolius* is a separate species from *P. acutifolius*, evaluating all accessions of this taxa that are stored in the CIAT collection. We also supplemented the AFLP study with a microsatellite evaluation of the same genotypes which will lead to a further analysis of whether microsatellite markers, given their higher rate of polymorphism than other markers systems, can uncover the pattern of domestication found in the cultivated tepary beans and how they are related to wild accessions. Tepary beans are of interest for dry land agricultural systems because tepary beans are the most drought-adapted species of the genus. They are also known to have high heat and salinity tolerance and good nutritional quality.

Materials and Methods

Plant material: The genotypes included a total of 38 wild tepary beans including 19 accessions of *P acutifolius* var. *tenuifolius*, 7 of *P. parvifolius*, 4 of *P. latifolius* and 9 of *P. acutifolius* var. *acutifolius* (Table 1) as well as the outgroup presented in the 2002 Annual Report (These included an outgroup of 9 genotypes from the *Phaseolus* genus including 2 *P. vulgaris* (common bean); 2 *P. lunatus* (lima bean); 1 *P. coccineus* (scarlet runner bean); 1 *P. polyanthus* (year bean); and 1 *P. glabellus* genotype). We also added the following wild species to the outgroup: G40513 and G40699 (*Phaseolus filiformis*), G40550 and G40685 (*P. angustissimus*) and G40675 (*P. carteri*). Tepary beans are almost exclusively self-pollinating, therefore the genotyping was based on bulk DNA from four plants per accession.

Table 1. Additional wild tepary bean genotypes included in diversity study.

Genotype	Species	Status	Origin	Collection
G40513	P.filiformis	Wild	Arizona	MEX
G40699	*P. filiformis*	Wild	Baja Calif. sur	USA
G40550	*P.angustissimus*	Wild	New Mexico	USA
G40685	*P.angustissimus*	Wild	New Mexico	USA
G40675	*P. carteri*	Wild	Baja Calif. sur	USA
G40 071	*P acutifolius var. tenuifolius*	Wild	Arizona	USA
G40 078	,,	Wild	Texas	USA
G40 087	,,	Wild	Durango	MEX
G40 093	,,	Wild	Durango	MEX
G40 095	,,	Wild	Durango	MEX
G40 101	,,	Wild	Durango	MEX
G40 104	,,	Wild	Jalisco	MEX
G40 105	,,	Wild	Jalisco	MEX
G40 108	,,	Wild	Durango	MEX
G40 114	,,	Wild	Arizona	USA
G40 178	,,	Wild	Chihuahua	MEX
G40 210	,,	Wild	Arizona, Sta. Cruz	USA
G40 211	,,	Wild	Arizona, Sta. Cruz	USA
G40 217	,,	Wild	Arizona, Pima	USA
G40 233	,,	Wild	Arizona	USA
G40 239	,,	Wild	Durango	MEX
G40 249	,,	Wild	Chihuahua	MEX
G40 251	,,	Wild	Chihuahua	MEX
G40 253	,,	Wild	Chihuahua	MEX
G40 273	,,	Wild	Chihuahua	MEX
G40080	*P.parvifolius*	Wild	Sinaloa	MEX
G40102	,,	Wild	Durango	MEX
G40181A	,,	Wild	Arizona	USA
G40182	,,	Wild	Oaxaca	MEX
G40195	,,	Wild	Texas	USA
G40240	,,	Wild	Durango	MEX
G40241	,,	Wild	Durango	MEX
G40 177B	*P.latifolius*	Wild	Arizona	USA
G40 177C	,,	Wild	Arizona	USA
G40 177D	,,	Wild	Arizona	USA
G40 177E	,,	Wild	Arizona	USA
G40 089	*P. acutifolius var. acutifolius*	Wild	Durango	MEX
G40 096	,,	Wild	Durango	MEX
G40 103	,,	Wild	Sinaloa	MEX
G40 106	,,	Wild	Jalisco	MEX
G40 188	,,	Wild	Michoacán	MEX
G40 197	,,	Wild	Texas	USA
G40 199	,,	Wild	Zacatecas	MEX
G40 236	,,	Wild	Durango	MEX
G40 242	,,	Wild	Durango	MEX
G40 244	,,	Wild	Zacatecas	MEX
G40 286	,,	Wild	Arizona	USA

AFLP analysis: Amplicon-template preparation, pre-amplification, and selective amplification were described in 2002 Annual report. We used the same EcoRI (E) -MseI (M) adapters and primer combinations with 3 selective nucleotides as we used in the previous study (E-ACC/M-CTA and E-AAG /M-CTT). PCR products were run on 4% silver-stained polyacrylamide gels for 1, 1.5 and 2 hours as described previously.

Microsatellite analysis: The genotypes were evaluated for allelic diversity with 15 microsatellite (7 gene-associated and 8 genomic) markers that had previously been confirmed to amplify well in *P. acutifolius* and related species. Microsatellite amplification and detection conditions were as reported in previous annual reports. To resolve allelic diversity as fully as possible, the PCR products for each survey were separated by electrophoresis for 1.5 hours at 120 constant volts on silver-stained 4% polyacrylamide gels. Null alleles were not considered for the analysis.

Data Analysis: Genetic similarities between genotypes were determined with the Dice coefficient using NTSYS 2.02 (Rohlf, 1993) for both microsatellites (considering all alleles) and AFLPs (considering both monomorphic and polymorphic bands). In the AFLP analysis, primer combinations were compared with Mantel "Z" statistic for the correlation of genetic distance matrices. The similarity matrices were used to construct dendrograms with the same program. Principal component analysis was done with the software package SAS (SAS Institute, 1989).

Results and Discussion

AFLP results: Both AFLP combinations used in this study had a good polymorphism rate, clear amplification profile and well-distributed range in PCR product sizes. The AFLP combinations produced a total of 297 bands. The E-AAG/M-CTT combination produced 146 bands while the E-ACC.M-CTA combination produced 151 bands. The correlation between the two genetic matrices for each AFLP{ combination was 0.90. The use of two AFLP primer combinations seems to have sampled different parts of the bean genome and gave us a more accurate picture of the relationships within and between species. A total of 26 genotypes (*P. lunatus* =3, *P.coccineus*=1,*P. polyanthus*=1, *P.filiformis*=2, *P.carteri*=1, *P.vulgaris*=2, *P.glabellus*=1, *P.acutifolius*=2, *P.a* var. *acut*=6, *P.a* var *tenuifolius*= 10, *P. latifolius*=3, *P.parvifolius*=5) were analyzed by AFLP.

Data from the two primer combinations were combined for the phylogenetic analysis. Figure 1 shows the dendrogram derived from the full AFLP dataset. The analysis of the combined dataset coincided with the established taxonomic relationships for the group of species analyzed, with *P. glabellus* and *P. lunatus* as the most distant group from *P. acutifolius* followed by *P. vulgaris*, *P. coccineus* and *P. polyanthus* as another distant group. The level of similarity was around 23 to 25% between the three groupings. A fourth cluster of species from the Rugosi section (*P. filiformis* and *P. carteri*) was somewhat closer to the Acutifolii section as defined by Freytag and Debouck (2003) with a similarity level of 29%. The Phaseoli and Coccinei sections shared approximately 50% similarity. Within the Coccinei section *P. coccineus* and *P. polyanthus* shared 65% similarity, while within the Rugosi section *P. filiformis* and *P. carteri* shared 46%

similarity. In both *P. vulgaris* and *P. lunatus* the distinction between Andean and Mesoamerican gene pools was clear. The level of similarity between gene pools was slightly higher between Andean and Mesoamerican in *P. vulgaris* (85%) than in *P. lunatus* (82%). Within the *P. acutifolius – latifolius - parvifolius – tenuifolius* spectrum, all the accessions shared up to approximately 68% similarity. In the dendogram, a total of four groups could be distinguished within this spectrum: 1) cultivated *P. acutifolius* 2) wild *P. acutifolius* var. *tenuifolius* 3) *P. latifolius* and wild *P. acutifolius* var. *acutifolius*; and 4) *P. parvifolius*.

Microsatellite results: A larger number of genotypes were evaluated with the microsatellite markers and overall this was an easier marker system to work with. Here too there was good separation of the species and the groups of wild tepary beans. In general the level of similarity was lower with microsatellites than with AFLP analysis as seen in the dendrogram (Figure 1). The placement of *P. glabellus* and *P. coccineus* needs to be confirmed.

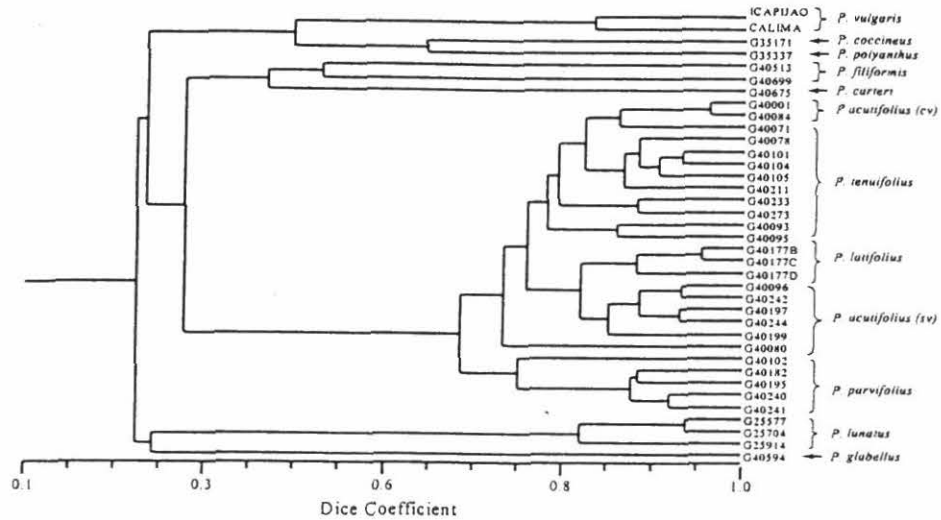
Conclusions

As mentioned above, the AFLP and microsatellite markers accurately displayed the genetic structure of the *Phaseolus* genus, where tepary beans are in the tertiary gene pool of common (*P. vulgaris* L.) and scarlet runner (*P. coccineus*) beans but are fairly distant from other *Phaseolus* species such as lima beans (P. lunatus). The relationships within the *P. acutifolius – latifolius - parvifolius* clade has been controversial. The marker data presented here suggest that the *P. acutifolius* and *P. parvifolius* may or may not deserve to be different species but that *P. acutifolius* and *P. latifolius* definitely do not. Accessions of *P. latifolius* clearly belong to the group of wild *P. acutifolius* var. *acutifolius*. The AFLP results also suggest that cultivated forms of tepary bean were domesticated from wild genotypes of *P. a.* var. *tenuifolius* not from *P. a.* var. *acutifolius*.

Ongoing activities

AFLPs were found to be evolutionarily conserved markers and served to reference different species relative to each other Combine datasets from the two evaluations of tepary bean (group I and group II).

a) AFLP markers



b) microsatellite

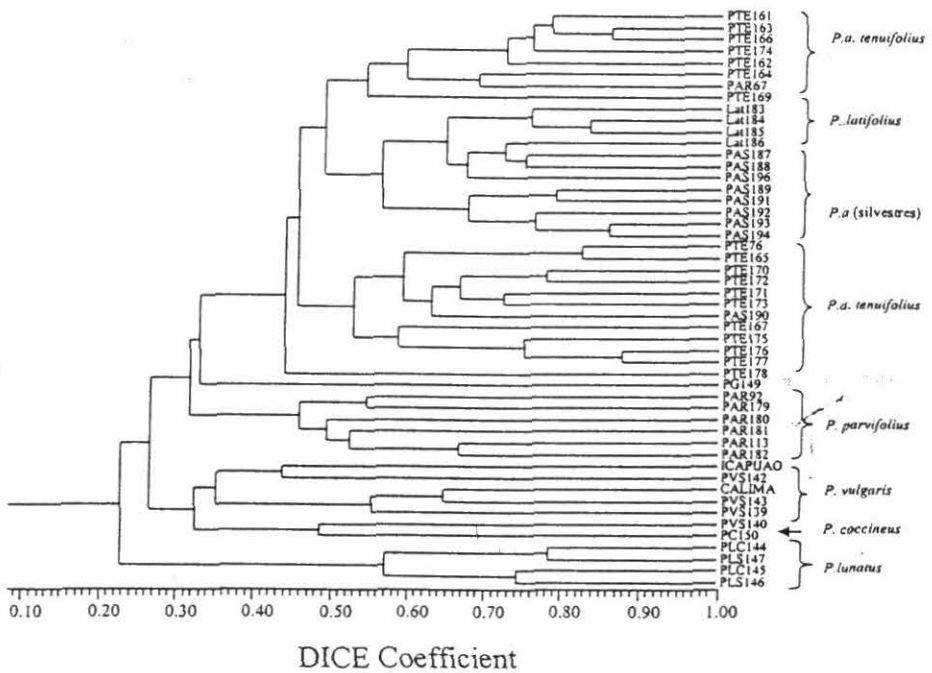


Figure 1. Dendrogram of relationships among tepary bean germplasm uncovered by AFLP and microsatellite marker analysis.

Conclusions

As mentioned above, the AFLP and microsatellite markers accurately displayed the genetic structure of the *Phaseolus* genus, where tepary beans are in the tertiary gene pool of common (*P. vulgaris* L.) and scarlet runner (*P. coccineus*) beans but are fairly distant from other *Phaseolus* species such as lima beans (P. lunatus). The relationships within the *P. acutifolius – latifolius - parvifolius* clade has been controversial. The marker data presented here suggest that the *P. acutifolius* and *P. parvifolius* may or may not deserve to be different species but that *P. acutifolius* and *P. latifolius* definitely do not. Accessions of *P. latifolius* clearly belong to the group of wild *P. acutifolius* var. *acutifolius*. The AFLP results also suggest that cultivated forms of tepary bean were domesticated from wild genotypes of *P. a.* var. *tenuifolius* not from *P. a.* var. *acutifolius*.

Ongoing activities

AFLPs were found to be evolutionarily conserved markers and served to reference different species relative to each other Combine datasets from the two evaluations of tepary bean (group I and group II)

References

Blair MW, Muñoz LC, Debouck D (2002) Tepary Beans (*P. acutifolius*) : Molecular analysis of a forgotten genetic resource for dry land agriculture. Grain Legumes 36: 25-26.

Freytag and Debouck 2003. Taxonomy, distribution, and ecology of the genus Phaseolus (Leguminosae–Papilionoideae) in North America, Mexico and Central America. Sida, Botanical Miscellany No. 23

Garvin, F.D and N. Weeden. 1994. Isozyme evidence supporting a simple geographic origin for domesticated tepary bean. Crop Sci. 34:1390-1395.

Muñoz LC, Blair MW, Debouck D (2002) Genetic diversity of the CIAT tepary bean (*Phaseolus acutifolius A.* Gray) collection measured with amplified fragment length polymorphism markers. Annual Report of the Bean Improvement Cooperative. 45: 234-35

Schinkel. C., and P. Gepts. 1989. Allozyme variability in the tepary bean, Phaseolus acutifolius A. Gray. Plant breeding 102:182-195.

Schinkel. C., and P. Gepts. 1988. Phaseoline diversity in the tepary bean, Phaseolus acutifolius A. Gray. Plant breeding 101:292-301.

1.1.5 Assessing genetic diversity in *Phaseolus vulgaris* germplasm through single nucleotide polymorphisms

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Introduction

Single nucleotide polymorphisms (SNPs) are biallelic markers which together with insertions/deletions are the most abundant sources of polymorphisms in human genome (Brookes, 1999). The potential of these markers has been proposed for association studies (Rafalski, 2002). Several studies related to SNP identification in plants such as maize (Rafalksi *et al.*, 2001), barley (Paris *et al.*, 2001) and soybean (Zhu *et al.*, 2003) have been initiated. SNP discovery in common bean was previously reported by Gaitán and Tohme (2002) who found 223 SNPs in 20964pb of *P. vulgaris* genome after sequencing PCR products of ten Andean and Mesoamerican bean genotypes.

Materials and Methods

Bean genotypes that are being used in this study include most of the wild *P. vulgaris* core collection selected by Tohme *et. al.* (1996) and analyzed by these authors using AFLPs. Also a set of approximately 200 Andean and Mesoamerican varieties belonging to the known bean market classes is been assayed with SNPs. These include both landraces of some American countries and bred lines with known responses to biotic and/or abiotic stresses.

Three to five seeds of each genotype were germinated in the laboratory. Seeds were placed in sterile paper towels soaked in 10mM CaSO4 solution and kept in a dark chamber at RT. After five to 10 days, first young leaves were collected in 2ml microcentrifuge tubes and stored at -80°C until use. After grinding in liquid nitrogen, genomic DNA extraction was carried out using a CTAB-Chloroform protocol according to the modifications made by Afanador *et al.* 1993.

SNP detection was being carried out using the single base extension (SBE) methodology described by Chen et al. (2000) standardized by Cregan (USDA Beltsville Agricultural Research Center) (http://bldg6.arsusda.gov/~pooley/soy/cregan/snp.html) and implemented at CIAT by Gaitán-Solís (this issue).

Briefly, PCR products containing the SNP were obtained from amplification of genomic DNA. Excess dNTPs and primers of the PCR product were removed with shrimp alkaline phosphatase and exonuclease I. Then the amplified fragment was annealed with an SBE primer with a 20-22 oligonucleotide (ZIP code or tag) attached to its 5´end. The single base extension is carried out using ddNTPs (one labeled with biotin) and thermosequenase so that the 3´end of the SBE primer anneals to the base that immediately precedes the SNP. Streptavidin-phycoerythrin is conjugated to the biotin

labeled oligonucleotide, which is hybridized to polystyrene microspheres (5.6μM diameter) bounded to a ssDNA sequence complementary to the ZIP code. The reaction is read in a flow cytometer (Luminex 100), which detects each microsphere by its unique fluorescent signal and the presence or absence of the SNP (streptavidin-SBE product). Data were analyzed with Masterplex GT (Miraibio Inc.) package in which the mean fluorescence intensity emitted by each of the samples is analyzed and used to define the SNP alleles belonging to each genotype.

Results

One third of the genotypes (56 cultivated and 29 wild genotypes) selected for this project were run with 16 single nucleotide polymorphisms using single base extension method described before. Heterozygote individuals were identified with 2 out to 16 SNPs in the subset of cultivated germplasm, and 6 out 16 SNPs in the subset of wild germplasm.

Although results are preliminar, 14 haplotypes have been identified in this small subset of samples (Table 1).

Table 1. Identification of SNP haplotypes in *P. vulgaris* germplasm.

Haplotype Identification	SNP Identification 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	G/C	C	C	T	G	C	T	A	T	T	G	G	A	T	G	C
2	G	A	C	A	G	A	G	G/A	G	C	G	C	C	G	A	C
3	G	A	C	A	G/T	C	G	G/A	G	C	G	C	C	G	A	C
4	G	A	C	A	T	A	G	G/A	G	C	G	C	C	G	A	C
5	G	A	T	A	G	A	G	G	G	C	G	C	C	G	A	C
6	G	A	T	A	G	A/C	G/T	G	G	C	G	C	C	G	G	C
7	G	A	T	A	G	C	G	G/A	G	C	G	G	C	T	A	C
8	G	A	T	A	T	A	G	A	G	C	G	C	C	G	A	C
9	G	A	T	A	T	A	G	G/A	G	C	G	C	C	G	G	C
10	G	A	T	A	T	A/C	G	G	G	C	G	C	C	G	A	C
11	G	A	T	A	T	C	G	G	G	C	G	C	C	G	A/G	C
12	G	A	T	A	T	C	G	G	G	C	G	C	C	T	A	C
13	G	C	C	A/T	G	C	T	G/A	T	C	G/A	G	A	T	G	T
14	G	C	T	A	T	A	G	G/A	G	C	G	C	C	G	A	C

SNP haplotypes can provide a higher level of organization of genetic variation with little added cost over individual SNPs (Judson et al., 2002). Also, the identification of SNP haplotypes may be an important tool since some of them are being associated with human diseases like rheumatoid arthritis, diabetes mellitus and affective disorders such as depression (van West et al., 2004). Hopefully the same approach can be used after SNP genotyping in common bean.

On-going activities

- Continue the characterization of genetic diversity in *P. vulgaris* with single nucleotide polymorphisms.

- Genetic mapping of SNP markers in *P.vulgaris* mapping populations.

Acknowledgements

We appreciate the invaluable collaboration of Orlando Toro from CIAT Genetic Resources Unit for providing the seeds of the wild and weedy germplasm.

References

Afanador Kafury, Lucia; Haley, Scott D; Kelly, James D; Adoption of a "mini-prep" DNA extraction method for RAPD marker analysis in common bean (Phaseolus vulgaris L.). In: Bean Improvement Cooperative. Annual Report (USA). Mar, 1993. v. 36 p. 10-11

Brookes, A. J. (1999) The essence of SNPs, Gene, 234: 177-86.

Chen, J., MA. Iannone, M. Li, J.D. Taylor, P. Rivers, A.J. Nelsen, K.A. Slentz-Kesler, A. Roses, and M.P. Weiner. 2000. A Microsphere-Based Assay for Multiplexed Single Nucleotide Polymorphism Analysis Using Single Base Chain Extension. Genome Res. 10: 549-557.

Gaitán, E. and J. Tohme. 2002. Discovery of SNPs in *Phaseolus vulgaris*. SB-2 Project, Annual Report 2002, CIAT. 105-106.

Judson R., B. Salisbury, J. Schneider, A. Windermuth and JC Stephens. 2002. How many SNPs does a genome-wide haplotype map require? Pharmacogenomics, 3(3): 279-391.

Paris, M., R. Lance and M.G.K. Jones. 2001. Single nucleotide primer extensions to type SNPs in barley. Proceedings of the 10th Australian Barley Technical Symposium. (www.regional.org.au/au/abts/2001/t3/paris.htm)

Rafalski A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Current Opinion in Plant Biology, 5:94-100.

Rafalski, A., Ching, A., Bhattramakki, D., Morgante, M., Dolan, M., Register, J. C., Smith, O. S., and Tingey, S. 2001. SNP Markers in Maize: Discovery and Applications, Plant and Animal Genome IX onference, W149.

Zhu, Y.-L, Q.-J. Song, D.L. Hyten, C.P. Van Tassell, L.K. Matukumalli, D.R. Grimm, S.M. Hyatt, E.W. Fickus, N.D. Young, and P.B. Cregan. 2003. Single nucleotide polymorphisms (SNPs) in soybean. Genetics 163: 1123-1134.

van West, D., J Del-Favero, Y Aulchenko, P Oswald, D Souery, T Forsgren, S Sluijs, S Bel-Kacem, R Adolfsson, J Mendlewicz, C Van Duijn, D Deboutte, C Van Broeckhoven and S Claes. 2004. A major SNP haplotype of the arginine vasopressin 1B receptor protects against recurrent major depresion. Mol Psychiatry, 9(3):287-292.

1.1.6 Gene flow studies in the bean plant model

Special Project, supported by BMZ, Germany

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Field Work

The field work in December 2003 and January 2004 was aimed at obtaining an additional harvest of intermediate forms in places of Costa Rica where gene flow has been observed in previous years. This work results in the disclosure of two more cases of wild- weed-crop complexes in the Central Valley (San Andrés León Cortés area), and twenty-two populations of wild common bean distributed in four watersheds (Virilla, Candelaria, Pirrís, and Reventazón). Interestingly, the field work has also revealed a new *Phaseolus* species for that country.

1.1.7 Studies of gene flow under field station conditions

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University of Costa Rica

In this part, gene flow has been studied on station in Costa Rica, namely at the stations of the university, Alajuela and Fraijanes. Hybridization is estimated by recording dominant marker genes expressed in F1 (purple flowers, purple hypocotyl and nodes; works by Bassett, Leakey) after letting insects to pollinate flowers randomly between donor and receiver selfed bean lines. The same design has been used over three years. In contrast to experiments conducted at Quircot in farmers' fields (see below), the percentage of outcrossing in field trials conducted systematically in Alajuela is low (0.17%). This figure is obtained when the number of hybrid seeds is computed against the total of harvested seeds in the experimental plots (and similar to reports by Alan & Moh, Ferreira, etc). If we compute the number of plants where a hybridization event has occurred, then the rate of outcrossing can go as high as 8.3 %. Discrepancies in outcrossing rates reported in the literature can be due to the way the computing of hybrid seeds has been done.

As indicated above, we were interested in testing outcrossing rates at the observation site of Quircot, where wild common beans still exist. Although the installation of plants with recessive genes proved to be much more difficult than expected, and similarly it was very difficult to obtain synchrony between the recessive material and the wild form, our preliminary results looked most interesting (Table 1). For that purpose, we planted twenty pots with two plants of each landrace per pot. Pots were installed next to natural stands of wild common bean (average distance: 2 meters). Pods were harvested at physiological maturity and treshed. Seeds were planted in pots in the mesh-house in order to check whether they resulted from cross pollinations or not, with parents on the side as checks. As compared to the trials on the experimental stations, this testing is more difficult as the

landraces do not express all marker genes in the F1 generation. It was thus necessary to conduct the experiment for another generation, in order to check the possible segregation. Although the trial has been conducted on few landraces, the average percent outcrossing is higher as compared to the trials conducted on the stations at Alajuela or Fraijanes. One possible explanation might be in the extended flowering period between the landraces and the sympatric wild common bean populations. Other trials conducted in Quircot with white seeded commercial varieties with short flowering periods and very limited synchrony with the wild form resulted in no outcrossing. This result would thus strengthen the hypotesis that in order to measure significant gene flow extended synchrony of flowering between the cultivated materials and the wild common bean must happen.

Table 1. Outcrossing estimates for a trial planted at Quircot, Cartago, where potted landraces were installed next to natural stands of wild comon bean.

Landrace identification (provision. no.)	Harvested plants	Plants with cross pollination	Seedlings from outcrossing	Total of planted seeds	% Outcrossing
10	18	0	0	468	0
19	14	1	2	294	7,14
32	18	1	5	378	5,56
33	17	1	5	374	5,88
46	29	2	9	580	6,9

References

Alan, J. & Moh, C. 1966. Determinación del porcentaje de cruzamiento natural en el frijol común (*Phaseolus vulgaris* L.) en Alajuela, Costa Rica. Turrialba 16 (2): 156-158.

Araya Villalobos, R., W.G. González Ugalde, F. Camacho Chacón, P. Sánchez Trejos & D.G. Debouck. 2001. Observations on the geographic distribution, ecology and conservation status of several *Phaseolus* bean species in Costa Rica. Genet. Resources & Crop Evol. 48: 221-232.

Bassett, M.J. 1996. List of genes - *Phaseolus vulgaris* L. Annu. Rept. Bean Improvement Coop.(USA) 39: 1-19.

Brunner, B.R.& J.S. Beaver. 1989. Estimation of outcrossing of the common bean in Puerto Rico. HortSci. 24: 669-671.

Ferreira, J.J., E. Alvarez, M.A. Fueyo, A. Roca & R. Giraldez. 2000. Determination of the outcrossing rate of *Phaseolus vulgaris* L. using seed protein markers. Euphytica 113: 259-263.

Ibarra-Pérez, F.J., N.C. Ellstrand, J.G. Waines. 1996. Multiple paternity in common bean (*Phaseolus vulgaris* L., Fabaceae). Amer. J. Bot. 83: 749-758.

Leakey, C.L.A. 1988. Genotypic and phenotypic markers in common bean. *In:* P. Gepts (ed.) Genetic resources of *Phaseolus* beans. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 245-327.

Wells, W.C., W.H. Isom & J.G. Waines. 1988. Outcrossing rates of six common bean lines. Crop Sci. 28: 177-178.

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

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Introduction

We present here evidence on gene flow events among biological forms of common bean in Costa Rica, in addition to our previous work (González-Torres et al. 2003, González-Torres et al. 2004). The analysis was carried out on six natural populations of common bean, using two seed sets collected in the Central Valley over different years: a) 1987, 1998, 2003, and b) 2004. After an in-depth characterization of wild forms and a couple of landraces still found in the Central Valley, we focus on weedy or intermediate forms, the characteristics, namely 100-seed weight, of which are intermediate between the two biological forms.

Figure 1 graphically shows three results found in the first seed set: a) Repeated events of gene flow of wild pollen towards the cultivated forms. This is so far the dominant direction evidenced in our work. Individual 2 there has small seeds as does the wild form, and two micro sats alleles obtained from the cultivated form through gene flow. Individual 10 shows a 'wild' cp DNA haplotype in contrast with some 'cultivated' characteristics (a bigger seed for instance, as compared to the wild forms) and one infrequent SSR allele (gray wave). b) Events of Chloroplast capture (obtained through repeated outcrossing, resulting in individuals with the nuclear genome of a particular biological form with cytoplasmic genome of the other). Individual 1 displays mainly 'wild' characteristics; however it has its chloroplast haplotype and two SSR alleles typical of 'cultivated' forms. Individual 4 has hybrid isozyme patterns and one hybrid SSR locus. Individuals 5 and 6 have all characteristics as found in wild forms, but their chloroplast haplotype is that of 'cultivated' form.

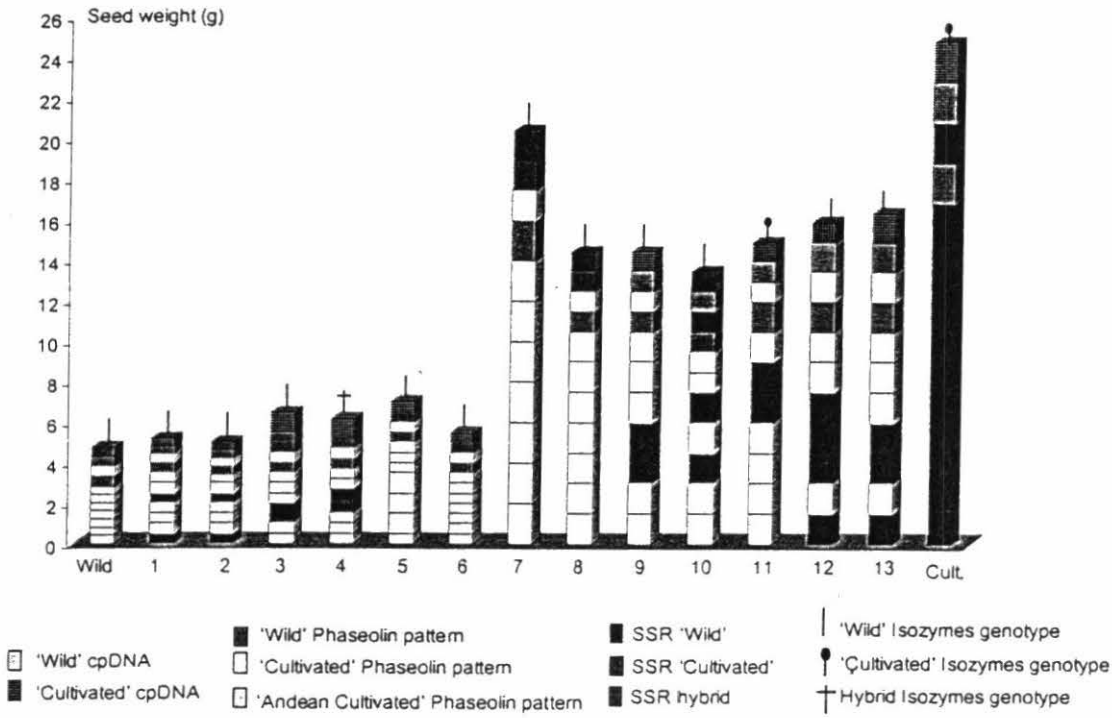

Figure 1. **Graphical representation of weedy materials with the different markers used to indicate evidence of gene flow. The gray boxes are common SSR loci among biological forms.**

c) Outcrossing between Andean and Mesoamerican genepools: in Figure 1, individual 12 displays chloroplast haplotype typical of 'Mesoamerican cultivated form', and a phaseolin type as found in Andean materials. In contrast, its SSR alleles are of mixed nature, 'wild' and 'cultivated'. This situation could be understood along the introduction of Andean materials into Costa Rica, as to avoid pathogen damage and to have a more attractive seed type (color and size) for sale on market.

The results obtained in the characterization of the populations are summarized in Table 1.

Table 1. Morphological, biochemical and molecular markers used and No. individuals analyzed for each parameter. The underlined fonts refer to 'wild' characteristics, while the italic fonts refer to 'cultivated' characteristics.

Biological form	Seed average weight (g)	Phaseolin type	Isozymes		Microsatellites		Chloroplast haplotype
			Pattern[1]	Allele[2]	Primer	Allele	
Wild	6 (2.5-7) N=443	"S-4" "S" "M1" "S-3" N=392	DIA -1 N=229	PRX 100 N=197	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 GATS91 N=134	160 80 164 110 165 147 138 122 224	H N=210
Weedy	13 (8-21.3) N=226	"C" *"CH"* "H" "S" *"X-7"* "S-4" N=196	DIA-1 *DIA-2* *DIA-4* N=157	PRX 100 *PRX 98* N=182	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 GATS91 N=226	160, *177* 80 164, *185* 110 165, *189* 147, *150* 138, *148* 122, *136* 224, 243	G, H J, K, L N=170
Cultivated	23 (22-46) N=198	"S" "T" *"X-7"* "CH" N=198	*DIA -2* *DIA -4* N=64	*PRX 98* N=29	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 GATS91 N=58	*180* 80 *183* 110 *189* *150* *148* *136* 243	*J, K, L* N=53

The discrimination among some chloroplast haplotypes (namely, 'H' or 'G'), key as to determine the direction of gene flow in the individuals of the first set, requires the sequencing of rps14-psaB gene fragment, and then a SNPs analysis using a fluorescent method.

The figure 2 shows the presence of Adenine nucleotide (highest columns) or Thiamine (shortest columns) in the gene fragment NdhA intron of chloroplast indicating the haplotype identification of wild type or cultivated type, respectively. The identification of haplotype 'G' in some evaluated individuals suggests the introduction of cultivated materials from México and Guatemala to Costa Rica and crossing of these materials with local wild materials, since this haplotype seems not original in Costa Rica (Chacón 2001).

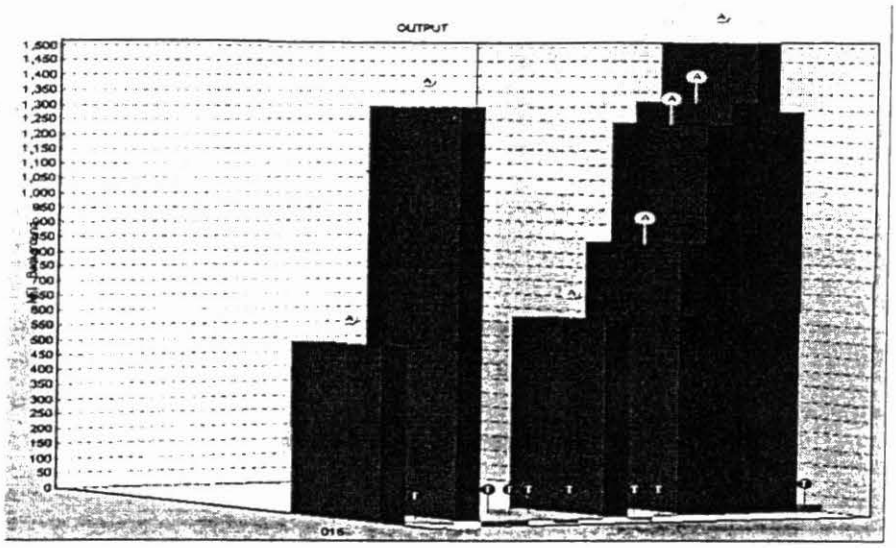

Figure 2. Result of the SNPs analysis used to determine chloroplast haplotypes in a selection of individuals.

The SNPs analysis indicates evidence of chloroplast capture of haplotype 'G' into wild materials. This result suggests a complex and different model of outcrossing in natural populations of local materials of Costa Rica with cultivated materials from Guatemala. In addition, we have seen many individuals with haplotypes 'J' and 'L', usually found in landraces of countries near Costa Rica, that are used by Costa Rican farmers.

On the other hand, out of the collection realized during 2004, we selected 657 seeds (600 seeds of 'wild' type; 27 seeds of 'landraces', and 30 seeds of 'weedy' types). In addition, we germinated 894 seeds from previous years for further analysis (Table 2).

Table 2. Individual analyzed collection realized during 2004

Number of seeds	Number of plants collected	Extraction of DNA	Determination of Chloroplast haplotype
1551	1176	1176	345 individuals have chloroplast haplotype 'J' or 'L' 644 individuals show chloroplast haplotype 'F', 'G', or 'H' 38 individuals have a possible chloroplast haplotype 'A', 'B', 'C' or 'D' (to verify) 149 individuals have still an indeterminate chloroplast haplotype

References

Chacón-Sánchez, M.I. 2001.PhD Thesis,Univ. of Reading, United Kingdom.

González-Torres, R.I., E. Gaitán, R. Araya, O. Toro, J. Tohme & D.G Debouck. 2004. Annu. Rept. Bean Improvement Coop. (USA) 47:167-168.

González-Torres, R.I., E. Gaitán, M.C. Duque, O. Toro, J. Tohme & D. Debouck. 2003. Annu.Rept.BIC. (USA) 46:1-2

1.1.9 Phylogenetic relationships in the genus *Manihot* Mill. (*Euphorbiaceae*): Biogeography and comparative ecology of the species from the Andes and the Amazon Regions

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Introduction

The genus *Manihot* Mill. (Euphorbiaceae) comprises 98 species of herbs, shrubs and vines, which grow in semi-arid zones of tropical regions, from southern North America to Argentina, and the West Indies. The geographical range of the genus extends to Africa and Asia, where cassava (*Manihot esculenta* Crantz subsp. *esculenta*) grows as a cultivated plant (Rogers & Appan 1973). Genetic variability in cassava has been decreasing as a consequence of the artificial selection exerted by man (Chavarriaga-Aguirre *et al.* 1999), resulting in plants vulnerable to tropical diseases or severe climatic conditions, like drought (Tanksley & McCouch 1997). Hybridization with wild *Manihot* species has been proposed as a mechanism for recovering such variability, because alleles lost during domestication are present in its wild ancestors (Nassar 2000).

Evolutionary relationships of the genus are still unclear. Rogers and Appan (1973) proposed that *Manihot aesculifolia*, from Central America, was cassava's closest wild relative. Molecular techniques such as RFLP (Fregene *et al.* 1994), AFLP (Roa *et al.* 1997) and microsatellites (Olsen & Schaal 2001), have suggested that this plant is most closely related with some populations of *M. esculenta* subsp. *flabellifolia*, from the Brazilian Cerrado (Allem 1994). Phylogeography studies confirmed this result, although little is known about the populations from northern South America (Olsen & Schaal 1999). Preliminary phylogenetic hypotheses have shown low levels of genetic variation in the genus (Schaal *et al.* 1994), and also a geographical structure that separates a South American and a Central American clade.

A molecular phylogeny of *Manihot*, based on chloroplast and nuclear markers will be built. Based on this phylogeny a biogeographic pattern of the genus will be proposed. Intraspecific relationships and phylogeography of *M. esculenta* populations from northern

South America will also be inferred. The evolution of ecological characters (by means of the independent contrasts method) (Harvey & Pagel 1991), will serve as a basis for studying the adaptation process of the species to their actual habitats.

Methodology

Thirty-one wild *Manihot* species were sampled from the Ciat *in vitro* collection, 70% of them being putative Brazilian accessions. No collection data is available for the majority of them. DNA was extracted from fresh leaves using the method of Dellaporta (1983). PCR amplifications were done using primer sequences previously reported (parentheses). The chloroplast regions *rpL16* (Small *et al.* 1998), *rps2* (DePamphilis *et. al.* 1997), *trnL-trnF* (Taberlet *et. al.*,1991), *atpB-rbcL* (Chiang *et. al.* 1998) and *accD-psal* (Demesure *et. al.* 1995); and the nuclear *G3pdh* (Olsen & Schaal 1999) were amplified. PCR products were cleaned, and then sequenced using *BigDye Terminators* technology (Applied Byosystems, Inc.). Sequencing reaction products were purified by ethanol precipitation and run on an ABI Prism 377 automated sequencer (PE Applied Biosystems). Sequences were aligned and run in PAUP* v. 4.0b10 (Swofford 2000). Preliminary Maximum Parsimony analyses were done for each data set independently, by means of a heuristic search, using unordered characters with equal weights. Gaps were treated as extra characters. All the trees were un-rooted because no outgroup taxa is available. Support for each branch was estimated by means of a 50% bootstrap analysis with 100 replicates.

Results

Almost no variation has been found in the five-chloroplast regions analyzed. In the case of the *rpL16* data set, 1151 characters were included and just 4 were parsimony informative (0.3% variability). A similar situation occurred with the *rps2* data set, with just one parsimony informative character among 513 analyzed (0.1% variability). In the case of *trnL*, variation was a little higher, with 8 parsimony informative characters among 1021 (0.78% variability). One most parsimonious tree was found (tree length=15 steps, CI=0.73), and just one clade had a significant bootstrap support (Fig. 1). No variation was found for the *atpB-rbcL* data set, and there were just two variable characters, none of them informative. 889 characters were included in the *accD-psal* analysis, and two of three variable characters were parsimony informative (0.3% variability).

At the nuclear level, *G3pdh* gave more promising results. 4.8% variability was found, corresponding to 34 parsimony informative characters among 704 characters included in the analysis. 1 most parsimonious tree was found (tree length=83 steps, CI=0.78). In general, bootstrap support for the different clades is low (Fig. 2).

Traditional taxonomic sections *Brevipetiolatae*, *Carthaginensis*, *Glaziovianae*, *Graciles*, *Manihot*, *Parvibracteatae*, *Peruvianae* and *Quinquelobae*, are paraphyletic (Fig. 2). There is no congruence between chloroplast and nuclear phylogenies.

Conclusions

The chloroplast regions analyzed are not sufficient to resolve the phylogenetic relationships in the genus *Manihot*. The evidence of an extremely conserved chloroplast genome might be a reflection of the recent speciation in the genus, as some investigators have proposed (Rogers & Appan 1973; Schaal *et al.* 1999). On the contrary, nuclear *G3pdh* is more variable, although some clades are still unresolved. Overall incongruities between chloroplast and nuclear phylogenies might be an artifact of the extremely low variation in the first data set.

No geographical structure has been observed, mainly because of the limited sampling of non-Brazilian species. Paraphyly found in some sections of the genus demands the addition of more taxa, and also a revision of the classification of Ciat's *in vitro* collection of wild *Manihot* species.

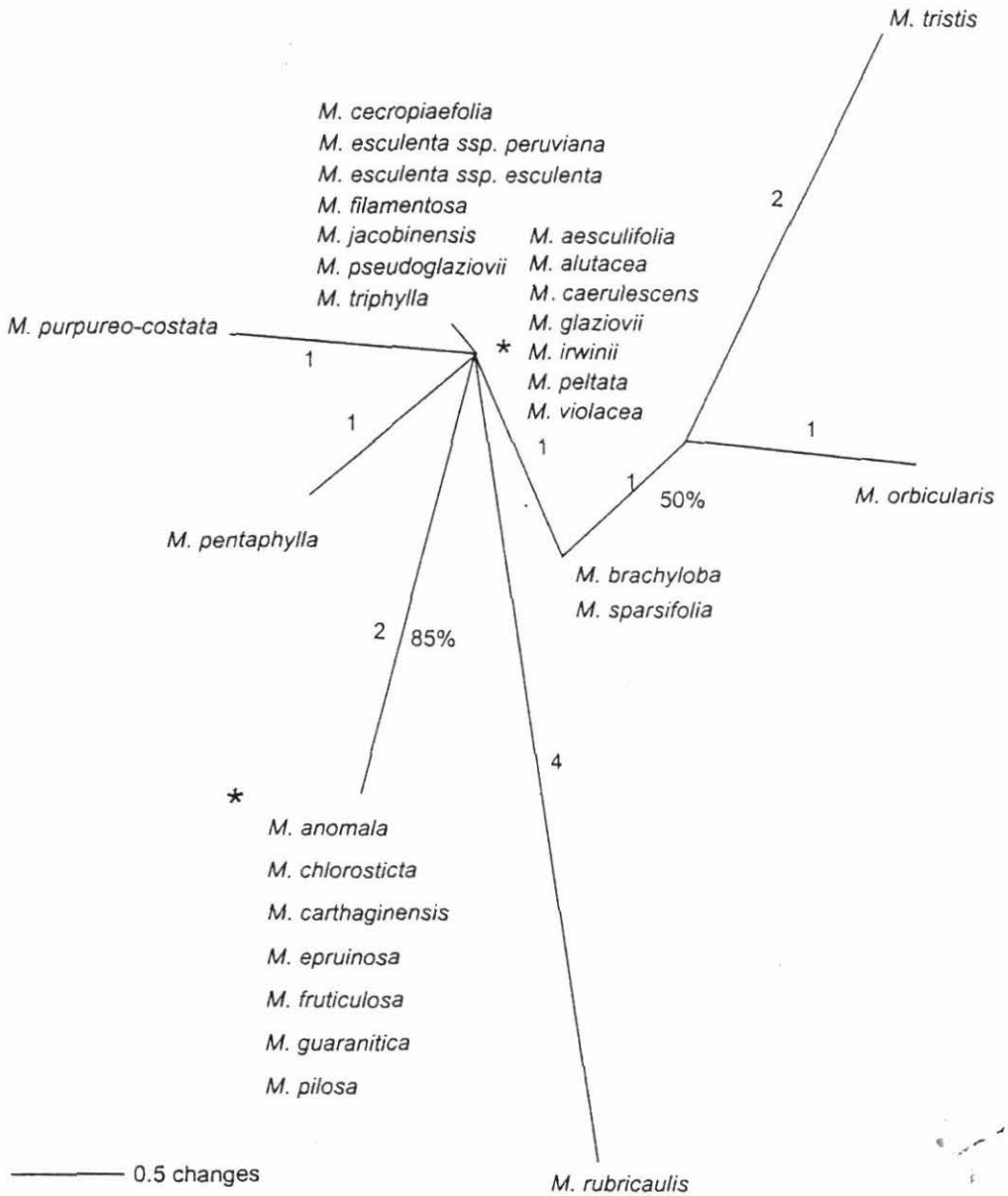

Fig. 1. *trnL-trnF* un-rooted phylogram obtained by Maximum Parsimony. Bootstrap values are shown next to branches with more than 50% support. Number of changes is also shown. *Zero branch length

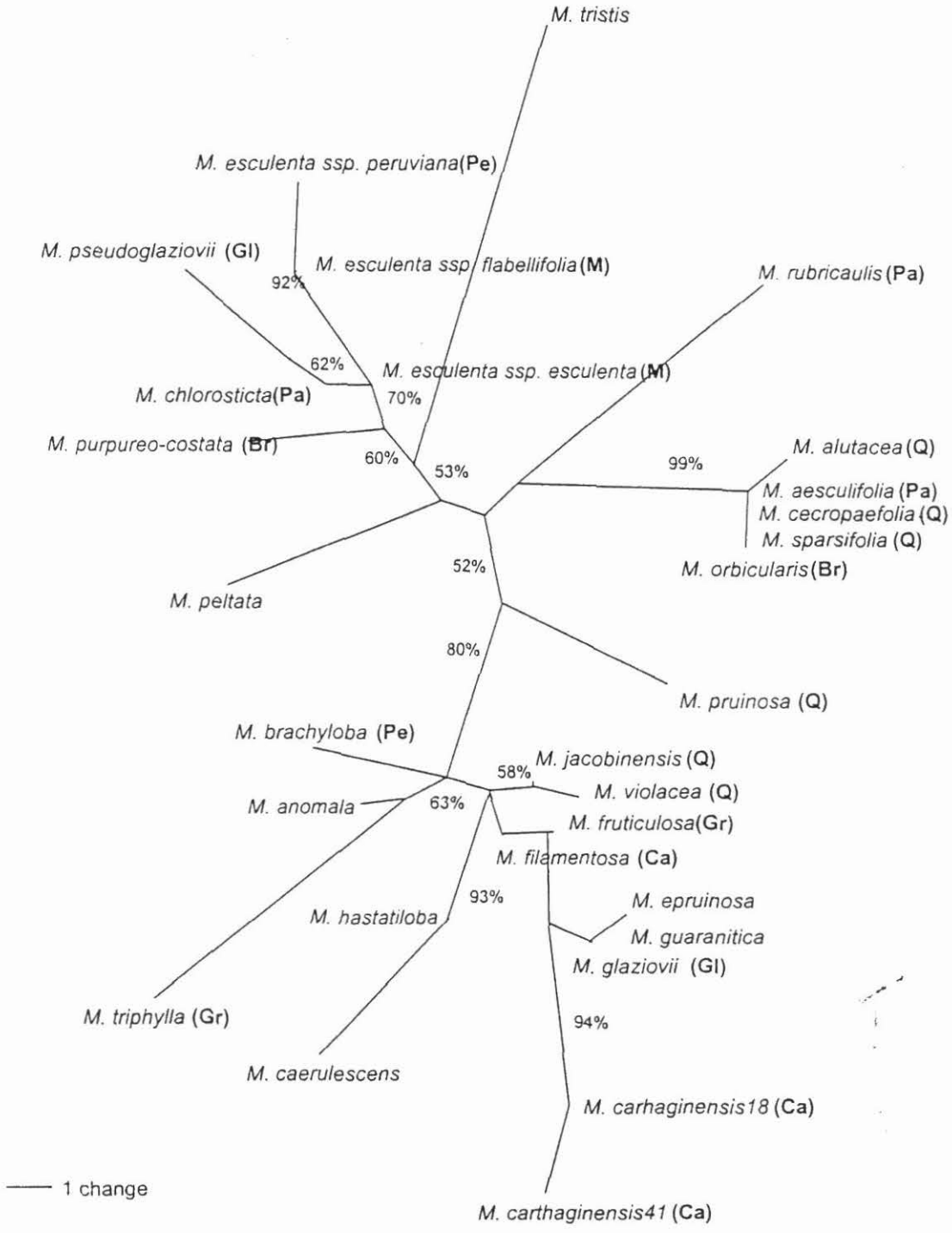

Fig2. ***G3pdh* unrooted phylogram obtained by maximum parsimony. Bootstrap values are shown next to branches with more than 50% support. Paraphyletic sections are abbreviated in parenthesis (Be= *Brevipetiolatae*, Ca=*Carthaginensis*, Gl=*Glaziovianae*, Gr=*Graciles*, M=*Manihot*, Pa=*Parvibracteatae*, Pe=*Peruvianae*, Q=*Quinquelobae*)**

Future plans

Expansion of sampling by future additions of wild *Manihot* species from Central America (Ciat Herbarium), Guyana (*Manihot grahamii*), Ecuador (*M. leptophylla*) and Brazil (*M. esculenta* subsp. *flabellifolia*); as well as two species of the outgroup genus *Cnidoscolus*
Continue the exploration of new chloroplast and nuclear regions, potentially variable at the interspecific level

Clarification of the biogeographic pattern in *Manihot*
Mapping of ecological characters on the phylogeny and independent contrast analysis
Establishment of sampling of *Manihot esculenta* populations from northern South America

Phylogeography analyses

References

Allem AC. 1994. The origin of *Manihot esculenta* Crantz (Euphorbiaceae). *Genetic Resources and Crop Evolution* 41: 133-150.

Chavarriaga-Aguirre P, MM Maya, J Thome, MC Duque, C Iglesias, MW Bonierbale, S Kresovich & G Kochert. 1999. Using microsatellites, isozymes and AFLPs to evaluate genetic diversity and redundancy in the cassava core collection and to asses the usefulness of DNA-based markers to maintain germplasm collections. *Molecular Breedings* 5: 263-273.

Chiang TY, BA Schaal & CI Peng. 1998. Universal primers for amplification and sequencing a noncoding spacer between the *atpB* and *rbcL* genes of chloroplast DNA. *Bot. Bull. Acad. Sin.* 39: 245-250.

Dellaporta SL, J Wood, JR Hicks. 1983. A plant DNA minipreparation: version II. *Plant. Mol. Biol. Rep.* 1:19.

Demesure B, N Sodzi & RJ Petit. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* 4: 129-131.

DePamphilis CW, ND Young & AD Wolfe. 1997. Evolution of plastid gene *rps2* in a lineage of hemiparasitic and holoparasitic plants: Many losses of photosynthesis and complex patterns of rate variation. *Proc. Natl. Acad. Sci.* USA. 94: 7367–7372.

Fregene MA, J Vargas, J Ikea, F Angel, J Tohme, RA Asiedu, MO Akoroda & WM Roca. 1994. Variability of chloroplats DNA and nuclear ribosomal DNA in cassava (*Manihot esculenta* Crantz) and its wild relatives. *Theor. Appl. Genet.* 89: 719-727.

Harvey PH & MD Pagel. 1991. *The Comparative Method in Evolutionary Biology*. 1st ed. Oxford University Press. New York.

Nassar NMA. 2000. Wild cassava, Manihot spp.: biology and potentialities for genetic improvement. *Genet. Mol. Biol.* 23: 201-212.

Olsen KM & BA Schaal. 1999. Evidence on the origin of cassava: Phylogeography of *Manihot esculenta*. *Proc. Natl. Acad. Sci. USA*. 96: 5586-5591.

Olsen KM & BA Schaal. 2001. Microsatellite variation in cassava (*Manihot esculenta*, Euphorbiaceae) and its wild relatives: further evidence for a southern amazonian origin of domestication. *Am. J. Bot.* 88: 131-142.

Roa AC, MM Maya, MC Duque, J Thome, AC Allem & MW Bonierbale. 1997. AFLP analysis of relationships among cassava and other *Manihot* species. *Theor. Appl. Genet.* 95: 741-750.

Rogers DJ & SG Appan. 1973. Manihot, Manihotoides (Euphorbiaceae). In: *Flora Neotropica* (Monograph No. 13). Hafner Press, New York.

Small RL, JA Ryburn, RC Cronn, T Seelanan & JF Wendel. 1998. The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogenetic reconstruction in a recently diverged plant group. *Am. J. Bot.* 85: 1301–1315.

Schaal B, P Olson, T Prinzie, LJCB Carvalho, NJ Tonukari & D Hayworth. 1994. Phylogenetic analysis of the genus *Manihot* based on molecular markers. Cassava *Biotechnology Network Proceedings (CIAT)*. Pp. 62-70.

Swofford DL. 2000. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4.0b4a Sinauer Associates, Sunderland, Massachusetts.

Taberlet P, L Gielly, G Pautou & J Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* 17: 1105–1109.

Tanksley SD & SR McCouch. 1997. Seed Banks and Molecular Maps: Unlocking Genetic Potential from the Wild. *Science* 277: 1063-1066.

1.1.10 Generation Challenge Program: Assembling Germplasm and Molecular Markers Sets for Analysis of Structural Diversity in Cassava

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CIAT

Funding: Generation Challenge Program

Important Outputs

1) Selection of a set of 3000 cassava accessions for structural diversity characterization using SSR markers
2) Selection of 36 SSR markers for molecular analysis of the germplasm set.

Introduction

The objective of sub-programme 1 of the GCP is the selection of a representative sub-set of germplasm and the molecular analysis of structural diversity to identify population structures as a guide for future association mapping studies. At a meeting to select marker systems for target GCP crops held at the Plant and Animal (PAG) genome 2004 it

was decided that 3000 cassava accessions, represented by 1500 accessions from CIAT's world germplasm collection, 1000 accessions from Africa (IITA) and 500 accessions from EMBRAPA will be selected for the study. DNA from these accessions will be extracted at each institution and sent to CIAT for re-distribution to all three participating institutions. Molecular markers for analysis of structural diversity will be 36 SSR markers, 2 each from the 18 linkage groups of the cassava map, that gave clear and reproducible allele patterns and high PIC will be used. to assess the in (CNPMF, Salvador, Bahia, Brazil). IITA and CIAT will analyze 14 and 16 SSR markers respectively while CNPMF will analyze 6 SSR markers, in the 3000 accessions. CIAT will sub contract CNPMF.

Following the PAG meeting and further discussions with SP1 colleagues, a pilot study was proposed to analyze, a sub set, 500 genotypes, of the larger selection with SSR and DArT markers to fine-tune final selection criteria for the larger set of germplasm. The pilot study was also to compare the power of DArT and SSR markers to detect underlying genetic diversity structure. The DArT analysis was to be carried out by a young female national program scientist from Thailand at CAMBIA, Canberra, Australia in collaboration with IPGRI, CIAT. Selections for the pilot study were in the same proportions as the full set of 3000 genotypes, in other words170, 80, and 250 accessions from IITA, CNPMF and CIAT respectively. The comparison of SSR and DArT analysis of the 436 accessions was presented as one of the 'success story' at the GCP annual meeting in Brisbane. Result of the pilot study is also reported in Activity 8.17 of this report. We describe here selection of the set of 3000 accessions, selection of 36 SSR markers and work carried out so far on SSR analysis of genetic diversity in the selected germplasm.

Methodology

The selection of a set of 3000 cassava accession was based on a selection criteria that emphasizes a very broad genetic diversity and key agronomic traits such as Drought tolerance, resistance to major pests and diseases, adaptation to different ecologies, etc. The complete set of criteria used to select the germplasm set is listed in Table 1.

Table 1. Description of selection criteria for assembly of 3000 cassava accessions for SSR analysis

Selection Criteria	
1	CIAT and IITA Core Collections
2	CIAT and IITA Elite Clones
3	Officially released varieties from CIAT, IITA, and CNPMF
4	Drought tolerance
5	Tolerance to all major cassava pests and diseases worldwide
6	Good culinary quality
7	Tolerance to acid soils and low phosphorus soils
8	High beta-carotene varieties (yellow varieties)
9	Low and high level of amylose and amylopectin
10	Low and high level of carotene, Iron, Zinc and HCN in the root
11	High level of protein in the root
12	Good adaptation to different tropical and sub-tropical agro-ecologies of South and Central, America, Southeast Asia and sub-Saharan Africa
13	Dwarf accessions
14	Wild accessions (no more than 5% of total number)

For selection of molecular marker sets, criteria was a marker system with:

1. High level of information or polymorphism information content (PIC) per locus
2. Easily assayed in most cassava research labs around the world, for example PCR-based
3. Have been used previously in analyzing cassava diversity and can resolve close relationships in cassava germplasm
4. Amenable to automation

The marker system that best fits the above criteria in cassava is by far simple sequence repeat (SSR) markers. More than 600 of these markers exist for cassava of which about 200 are mapped, 67 of these markers have also been used to assess diversity in a sub-set of 300 genotypes from all over the world, in other words PIC values exist for them. The cassava team also agreed to do a pilot study to compare another marker system, DArTs with SSRs in assessing structural diversity using a random sub-set of 426 accessions from the larger collection of 3000 accessions. The result of this study is reported in Activity 8.7 of this report.

Leaf tissue from the CIAT selection was obtained from tissue culture plantlets from the genetic resources unit (GRU) or plants maintained in the field or screen house. DNA isolation from the selected germplasm was by a Dellaporta (1983) mini prep extraction method. DNA extracted from selections at CIAT, IITA and CNPMF was shipped to CIAT for re-distribution to all three participating institutions and also to CAMBIA for DArT analysis. CIAT as lead institute will collate and analyze the molecular data from all markers and all accessions, as well as compile passport data, including the local names, source (Country/State/Province/Region/Village), geographical position (Longitude, Latitude, Altitude) and the main agronomic traits, from all accessions into a data base that is accessible to the entire cassava research community.

Results

A total of 3000 accessions were selected for SSR analysis: 1500 at CIAT, 1000 at IITA and 500 at CNPMF, an excel file of the selection can be viewed at www.ciat.cgiar.org/molcas. Due to a delay in obtaining a permit from the Brazilian GR council to access materials from CNPMF, in the best of circumstances it could take 4-5 months to obtain a permit, another list of 500 accessions from CNPMF held in the CIAT world cassava collection was made for immediate access and molecular analysis. This new Brazilian germplasm set has 200 in common with the one selected at CNPMF. An agreement has been reached with CNPMF to analyze the CIAT selection with the participation of a CNPMF scientist and later analyze the 300 outstanding CNPMF accessions in Brazil.

DNA samples from 170 accessions were received from IITA, Ibadan of which 155 had sufficient quantity and quality for molecular analysis, and DNA from 281 genotypes were prepared at CIAT for the pilot study (Fig 1). An aliquot of all 426 DNA samples were sent to IITA Nairobi (ILRI-Bioscience facility) where molecular analysis will be conducted. No DNA samples were sent to CNPMF because an agreement on access to CNPMF's germplasm, a pre-requisite for release of funds from CIAT for CNPMF's sub-contract, could not be reached due to delays in obtaining a permit from the Brazilian genetic resources (GR) council. SSR analysis of the 436 accessions was carried out only in CIAT due to technical problems with the genotyping facility at IITA-Nairobi.

All DNA samples from the complete set of 1000 accessions selected at IITA, Ibadan have been received at CIAT. At CIAT, DNA isolation is been conducted as materials are received from the genetic resource unit (GRU), to date DNA isolation has been completed for roughly half of the 2000 selection: 1500 from CIAT and 500 from CNPMF. DNA extraction is expected to be complete by the second week in October.

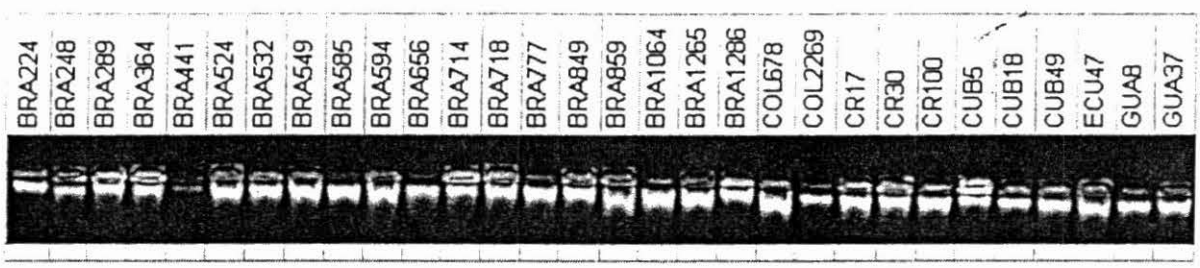


Figure 1. Agarose gel showing quality of some DNA samples from CIAT germplasm

In previous studies of genetic diversity of cassava accessions from 14 countries in Africa and the Neotropics, 36 markers, 2 from every one of the 18 linkage groups that represent the 18 haploid chromosomes of cassava, with high PIC and that give very reproducible patterns were used. We proposed to use these 36 SSR markers (see Table 2.) with broad coverage of the cassava genome for structural diversity analysis of 3000 cassava accessions. These markers were used in genotyping 426 accessions of

the pilot study and they revealed a structure in the accessions based upon region of origin and other unknown factors.

Conclusion and Perspectives

Selection of a set of 3000 cassava accessions and 36 SSR markers for structural diversity characterization of cassava has been completed. Also completed was a pilot study to characterize 426 accessions with the 36 SSR and DArT markers. Ongoing activities include completion of DNA isolation and SSR analysis of the rest of the germplasm data set.

References

Dellaporta SL. Wood J. Hicks JR 1983. A plant DNA minipreparation: versión II. Plant Mol Biol. Rep 1: 19-21.

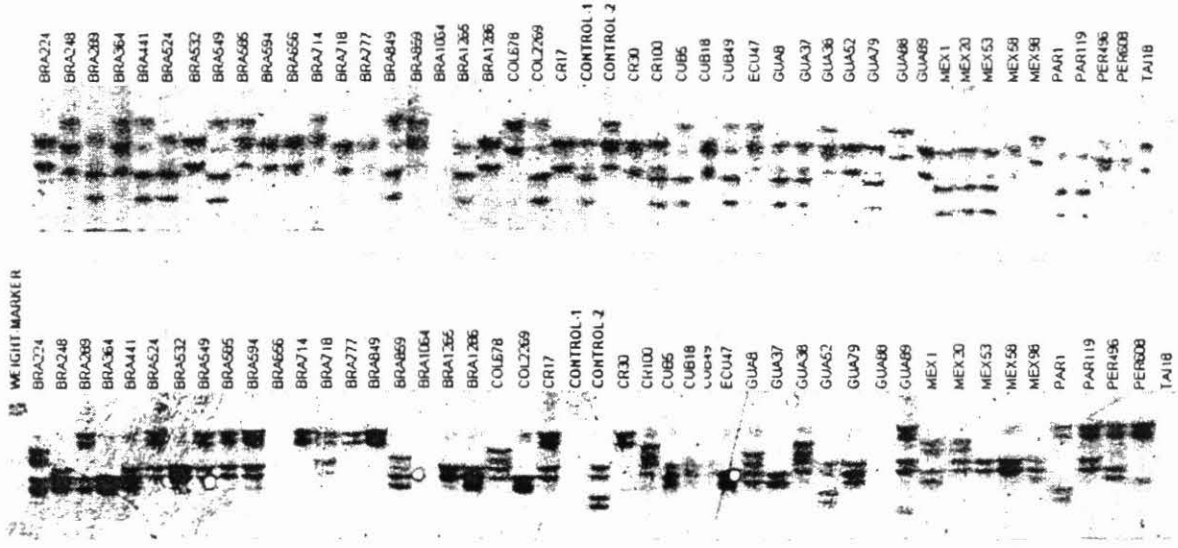
Mba R. E.C., P. Stephenson, K. Edwards, S. Melzer, J. Nkumbira, U. Gullberg, K. Apel, M. Gale, J. Tohme, and M. Fregene (2001) Simple Sequence Repeat (SSR).Markers Survey of the Cassava (*Manihot esculenta* Crantz) Genome: Towards a SSR-Based Molecular Genetic Map of Cassava. Theor and Appl Genet 102:21-31

Table 2. SSR markers selected to study structural diversity in Cassava

Place of Evaluation	SSR locus	Type of repeat	Left primer (Reverse)	Right primer (Forward)	Product Size (bp)	T * (°C)	$MgCl_2$ (mM)^	Thermocycler Program
IITA	SSRY4	$(GA)_{16}$	ATAGAGCAGAAGTGCAGGCG	CTAACGCACACGACTACGGA	287	45	1.5	MICROBC1
IITA	SSRY9	$(GT)_{15}$	ACAATTCATCATGAGTCATCAACT	CCGTTATTGTTCCTGGTCCT	278	55	1.5	MICROBC1
CIAT	SSRY12	$(CA)_{19}$	AACTGTCAAACCATTCTACTTGC	GCCAGCAAGGTTTGCTACAT	266	55	1.5	MICROBC1
CIAT	SSRY19	$(CT)_8(CA)_{18}$	TGTAAGGCATTCCAAGAATTATCA	TCTCCTGTGAAAAGTGCATGA	214	55	1.5	MICROBC1
CIAT	SSRY20	$(GT)_{14}$	CATTGGACTTCCTACAAATATGAA	TGATGGAAAGTGGTTATGTCCTT	143	55	1.5	MICROBC1
CIAT	SSRY21	$(GA)_{26}$	CCTGCCACAATATTGAAATGG	CAACAATTGGACTAAGCAGCA	192	55	1.5	MICROBC1
EMBRAPA	SSRY34	$(GGC)_5GGTGGC(GGT)_2$	TTCCAGACCTGTTCCACCAT	ATTGCAGGGATTATTGCTCG	279	55	1.5	MICROBC1
EMBRAPA	SSRY38	$(CA)_{17}$	GGCTGTTCGTGATCCTTATTAAC	GTAGTTGAGAAAACTTTGCATGAG	122	55	1.5	MICROBC1 or NEWBC1
CIAT	SSRY51	$(CT)11CG(CT)_{11}(CA)_{18}$	AGGTTGGATGCTTGAAGGAA	GGATGCAGGAGTGCTCAACT	298	55	1.5	MICROBC1 or YUCADIV
EMBRAPA	SSRY52	$(GT)_{19}$	GCCAGCAAGGTTTGCTACAT	AACTGTCAAACCATTCTACTTGC	266	55	1.5	MICROBC1 or YUCADIV
CIAT	SSRY59	$(CA)_{20}$	GCAATGCAGTGAACCATCTTT	CGTTTGTCCTTTCTGATGTTC	158	55	1.5	MICROBC1
EMBRAPA	SSRY63	$(GA)_{16}$	TCAGAATCATCTACCTTGGCA	AAGACAATCATTTTGTGCTCCA	290	55	1.5	MICROBC1 or YUCADIV
EMBRAPA	SSRY64	$(CT)_{13}CG(CT)_6$	CGACAAGTCGTATATGTAGTATTC	GCAGAGGTGGCTAACGAGAC	194	55	1.5	MICROBC1 or YUCADIV
EMBRAPA	SSRY69	$(CT)_{18}ATT(AT)_2(N)_7(CTTT)2$	CGATCTCAGTCGATACCCAAG	CACTCCGTTGCAGGCATTA	239	55	1.5	NEWBC1
CIAT	SSRY82	$(GA)_{24}$	TGTGACAATTTTCAGATAGCTTCA	CACCATCGGCATTAAACTTTG	211	55	1.5	MICROBC1 or YUCADIV
CIAT	SSRY100	$(CT)_{17}TT(CT)_7$	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	210	55	1.5	NEWBC1
IITA	SSRY102	$(GT)_{11}$	TTGGCTGCTTTCACTAATGC	TTGAACACGTTGAACAACCA	179	55	1.5	NEWBC1
IITA	SSRY103	$(GA)_{22}$	TGAGAAGGAAACTGCTTGCAC	CAGCAAGACCATCACCAGTTT	272	55	1.5	NEWBC1
IITA	SSRY105	$(GT)_6GC(GT)_2(GA)_{16}$	CAAACATCTGCACTTTTGGC	TCGAGTGGCTTCTGGTCTTC	225	55	1	NEWBC1
CIAT	SSRY106	$(CT)_{24}$	GGAAACTGCTTGCACAAAGA	CAGCAAGACCATCACCAGTTT	270	55	1.5	NEWBC1
CIAT	SSRY108	$(CT)_{24}CCT$	ACGCTATGATGTCCAAAGGC	CATGCCACATAGTTCGTGCT	203	55	1.5	MICROBC1 or YUCADIV
CIAT	SSRY110	$(GT)_{12}$	TTGAGTGGTGAATGCGAAAG	AGTGCCACCTTGAAAGAGCA	247	55	1.5	NEWBC1
IITA	SSRY135	$(CT)_{16}$	CCAGAAACTGAAATGCATCG	AACATGTGCGACAGTGATTG	253	45	1.5	YUCADIV
IITA	SSRY147		GTACATCACCACCAACGGGC	AGAGCGGTGGGGCGAAGAGC	113	45	1.5	YUCADIV
IITA	SSRY148		GGCTTCATCATGGAAAAACC	CAATGCTTTACGGAAGAGCC	114	45	1.5	YUCADIV
CIAT	SSRY151		AGTGGAAATAAGCCATGTGATG	CCCATAATTGATGCCAGGTT	182	45	1.5	NEWBC1
CIAT	SSRY155		CGTTGATAAAGTGGAAAGAGCA	ACTCCACTCCCGATGCTCGC	158	55	1	YUCADIV
IITA	SSRY161	$(CT)_{11}TT(CT)_{21}(CA)_{19}$	AAGGAACACCTCTCCTAGAATCA	CCAGCTGTATGTTGAGTGAGC	220	55	1.5	YUCADIV
CIAT	SSRY164	$(GA)_{29}$	TCAAACAAGAATTAGCAGAACTG G	TGAGATTTCGTAATATTCATTTCAC TT	187	45	1.5	NEWBC1
CIAT	SSRY169	$(GA)_{19}(A)_3(GAA)_2$	ACAGCTCTAAAAACTGCAGCC	AACGTAGGCCCTAACTAACCC	100	55	1	YUCADIV
CIAT	SSRY171	$(TA)_5CATA(GATA)_8GC(GA)_{23}$	ACTGTGCCAAAATAGCCAAATAGT	TCATGAGTGTGGGATGTTTTTATG	291	55	1.5	NEWBC1
IITA	SSRY177	$(CCT)_6CT(N)_{65}(CT)_4AT(CT)_{18}$	ACCACAAACATAGGCACGAG	CACCCAATTCACCAATTACCA	268	45	1.5	YUCADIV
IITA	SSRY179	$(GA)_{28}$	CAGGCTCAGGTGAAGTAAAGG	GCGAAAGTAAGTCTACAACTTTTCT AA	226	55	1.5	MICROBC1
IITA	SSRY180	$(GA)_{16}(G)_4(GA)_5$	CCTTGGCAGAGATGAATTAGAG	GGGGCATTCTACATGATCAATAA	163	55	1.5	MICROBC1
IITA	SSRY181	$(GA)_{22}(G)_3C(GA)_3GGAA(GA)_4$	GGTAGATCTGGATCGAGGAGG	CAATCGAAACCGACGATACA	199	55	1.5	YUCADIV

*: Annealing Temperature. ^: $MgCl_2$ final concentration per PCR reaction (final volumen in the PCR reaction: 25µl

A



B

Figure 2. PAGE gel of PCR amplification of cassava accessions with SSR markers: SSRY135(A) and SSRY4 (B).

1.1.11 Generation Challenge Program: Comparison of Simple Sequence Repeats (SSR) and Diversity Array Technology (DArT) Markers for Structural Characterization of Diversity in Cassava

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Funding: Generation Challenge Program (GCP)

Important Outputs

1) Structural characterization of genetic diversity in cassava with 251 polymorphic dominant DArT markers compared to that with 36 SSR co-dominant markers
2) A clear trade-off between number of loci and amount of information provided by each locus

Introduction

At the heart of the Generation Challenge Programme (GCP) is a vision to harness advances in molecular biology and the rich natural variation found in crop genetic resources to create a new generation of hardy crops for small farmers. Characterizing structural and functional diversity of 11 mandate crops: Barley, Maize, Rice, Sorghum, Wheat, Chickpea, Cowpea, Common Bean, Cassava, Potato and *Musa*, is the entry point of the GCP. SP1 is the subprogram in charge of ensuring a scientifically sound scheme to put germplasm collections to work for the discovery of new genes and alleles that will contribute to solve the important challenges of modern agriculture. By examining the genetic structure of a large and representative sample of a collection revealed with molecular markers, SP1 proposes to re-sample the original germplasm and select a subset that will be subject to fine phenotyping and association studies.

For many reasons up to date, the markers of choice for germplasm characterization have been microsatellites (SSR). SSR are abundant in most genomes, highly polymorphic and easily assayed. SSR marker mutations are formed by slipped strand mis-pairing. A newer marker tool, Diversity Array Technology (DArT) is a DNA hybridization-based system based on single nucleotide polymorphisms, insertion-deletions and DNA methylation changes. DArT offers the highest throughput available up to date at a fraction of the cost of SSR markers and allows for whole genome scanning in a speedy manner.

A pilot experiment was designed to test the usefulness of DArT markers as an alternative to SSR for detecting structural variation in a more cost-effective way. A randomly selected

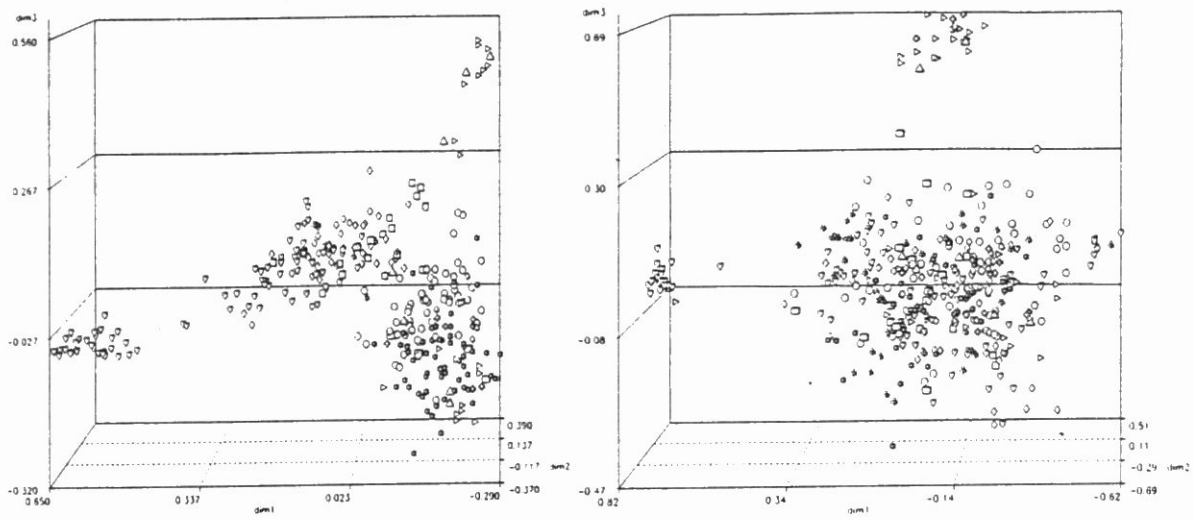
set of 436 accessions of cassava (*Manihot esculenta* Crantz) were analyzed with DArT and SSR markers and results compared. The hypothesis is whether DArT markers are more adept at uncovering genetic diversity structure.

Methodology

Plant material for the pilot study included accessions from the International Institute of Tropical Agriculture (IITA) in Nigeria, principally local varieties from West Africa and elite IITA varieties, and from CIAT, selected at random from South and Central American varieties held at the world germplasm collection. DArT analysis was conducted at the Center for the Application of Molecular Biology to Agriculture (CAMBIA), Canberra, Australia in collaboration with IPGRI and CIAT. A National Program Scientist, Ms Prapit Wongtiem, of the Field Crop Research Station, Rayong, Thailand participated in the DArT analysis. A cassava DArT array of approximately 1000 polymorphic clones constructed earlier (Ling et al. 2004) was the source of markers. The cassava DArT chip was developed using DNA from accessions originating from 17 different countries, improved varieties from CIAT and IITA, and wild accessions of *Manihot esculenta* sub spp flabellifolia, *M. carthaginensis* and *M. walkerae*. At CIAT, the same accessions were analyzed with 36 microsatellites selected from 18 linkage groups of the cassava genetic map. Data analysis was conducted at CIAT. Cluster analysis of the DArT and SSR data were performed using principal coordinate analysis of a similarity matrix derived by the Jaccard method using NTSYS-PC (Rohlf 1993).

Results

A total of 251 loci were sampled with DArT markers compared to 36 loci for SSR markers. Results of cluster analysis by PCoA gave similar outcomes of distinct clusters, but there was a clear separation of the Latin American and African accessions with SSR markers (Fig 1). Three clusters common in both markers were a group of genotypes from Guatemala, a sub-set of accessions from Nigeria, and a third large conglomeration of genotypes from the rest of the world, a total of 20 countries. SSR markers separated this third clusters into two according to geographic origin of the germplasm. These results agree with a previous attempt to elucidate the organization of genetic diversity in cassava using 67 SSR markers, that study revealed a high level of genetic differentiation between a group of genotypes from Guatemala and a separation between African and Latin American accessions.



A B

Figure 1. Principal coordinates analysis (PcoA) of SSR markers (A) and DArT markers (B). The African accessions are represented in red while the Latin American accessions are in blue color. Three distinct groups can be seen with both markers but there a separation of the Latin American and African accessions is evident with SSR markers.

Possible sources of the observed structure could be founder effects (geographic dispersal to the old world), selection (especially for diseases prevalent in Africa), small effective sample sizes (as in the case of spread of cassava to Africa from Latin America), migration (introgression from wild relatives), independent domestication events especially for the Guatemalan accessions, and mutations. The possibilities of introgression from wild relatives into accessions from Guatemala is quite high, the geographical origin of these Guatemalan accessions overlaps with that of 2 *Manihot* species unique to Central America. It is also remotely possible that the accessions from Guatemala represent a second center of domestication, similar to other crops like common beans (*Phaseolus vulgaris*) and pepper (*Capsicum* spp.) that were independently domesticated in Central and South America. The separation between the African and Latin American accessions could be due to selection and/or small effective sample sizes, suggesting that Africa could yet benefit from introgression of germplasm from Latin America. The cluster made up of some Nigerian accessions was also observed in an earlier study (Raji 2002, unpublished data), these accessions are from the Northern part of the country and it is not clear if this is due to small effective sample sizes and selection for tolerance to drought prevalent in the Northern part of the country, this again suggests a need to broaden the germplasm base in this part of the country.

A large number of alleles were detected by each SSR loci (an average of 10 alleles per locus) compared to DArT markers (2 per loci) although DArT markers sampled many

more loci (251) of the genome compared to SSR (36) in this study. This suggests a trade-off between information and number of loci. Because DArT loci can be significantly increased up to 1000 with minimal additional costs for molecular characterization it can be speculated that the level of resolution obtained with DArT could be increased to a similar level as SSR markers. Furthermore, given that the investment (labor and consumables) for development of both marker systems is approximately similar, even if both technologies provided a similar level of resolution, DArT would appear as an attractive marker alternative. This is so because of the cost per assay (lots of data points in one single assay vs two data points per assay with SSR), which would make DArT especially interesting for orphan crop species that do not count on existing marker systems.

Conclusion and perspectives

In conclusion, diversity estimated with 36 co-dominant SSR markers is more efficient than 251 DArT dominant markers. These results reveal a trade-off between amount of information and number of loci provided by each locus, in this study DArT sampled a larger number of loci of the genome, but they are dominant markers and consequently have less information compared to co-dominant SSR markers. But the hypothesis that DArT markers are more useful than SSR markers in detecting structural variation cannot be accepted, more conclusive evidence will have to await analysis using a denser DArT array and a larger data set of accessions from each country and region.

References

Ling Xia, Kaiman Peng, Yang Shiying, Peter Wenzl, M Carmen de Vicente, Martin Fregene, Andrzej Kilian (2004) DArT for High-Throughput Genotyping of Cassava (*Manihot esculenta*) and its Wild Relatives. Theor Appl Genet (in review)

1.1.12 Analysis of genetic diversity in a cassava germplasm collection from Cuba using SSR markers

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1.INIVIT-Cuba; 2. CIAT

Funding: The Cassava Biotechnology Network (CBN)

Important Outputs

Assessment of genetic diversity of a collection of Cuban cassava land races and detection of a structure in this collection

Introduction

The assessment of genetic diversity of cassava germplasm from Cuba using 36 SSR markers was started last year. The study, concluded this year, seeks to understand the organization of diversity and genetic differentiation, with respect to germplasm from the rest of the world, of local cassava varieties from Caribbean island in light of evidence of a possible second center of diversity of cassava in Central America. A second objective was to provide cassava breeders in Cuba information to better exploit genetic diversity in their cassava collection. The study was carried out as collaboration with INIVIT, Cuba, with funding from the cassava biotechnology network (CBN)

Methodology

Plant material was 94 accessions selected from a collection of cassava held at INIVIT in Cuba, selection criteria were 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, 20 from South America, and 13 improved genotypes from CIAT, were included. These genotypes a representative of a larger set of germplasm from these countries based upon previous SSR studies (Fregene et al 2003), were included for estimation of genetic differentiation. DNA from all accessions was obtained using the Dellaporta et al. method (1983). Concentration and quality of the DNA was checked by flourometer and agarose gel electrophoresis respectively. The DNA samples were diluted to a working concentration of 10ng/ul for subsequent PCR amplification.

PCR amplification, gel analysis and date collection of the DNA samples with 36 SSR markers were as described earlier (CIAT 2003; Mba et al. 2003). The raw SSR data was used to calculate estimates of genetic diversity and differentiation using the computer package GENSURVEY (Vekeman et al 1997). Genetic differentiation was estimated using the statistic F_{ST} (theta) and G_{ST} (Nei 1978) using the FSTAT computer program (Goudet 1990). Confidence intervals of F_{ST} and G_{ST} were calculated by jackknifing (200

replications) or by bootstrapping (1000 bootstraps). Pair-wise values of F_{ST} between countries were used in drawing a dendogram by the UPGMA method and the program NTSYS-PC (Rohlf 1993). Other analyses conducted with the SSR data include calculation 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**) and cluster analysis of the genetic distance matrix using principal coordinate analysis (PCoA) and multiple correspondence analysis (MCA), using the computer package NTSYS-PC (Rohlf 1993).

Results

The evaluation of 36 SSR markers in 142 accessions yielded a high level of polymorphism, with the exception of 2 (SSRY127 and SSRY132) that were monomorphic. Number of alleles for the polymorphic markers ranged from 2 to 10 for each SSR loci. Seventeen alleles unique to accessions from Cuba were identified in the following SSR markers: SSRY 4 (0.04), SSRY 20 (0.006), SSRY 38 (0.005), SSRY 59 (0.006 y 0.079), SSRY 63 (0.033), SSRY 69 (0.023), SSRY 100 (0.011), SSRY 103 (0.052, 0.012, 0.012 and 0.006), SSRY135 (0.005), SSRY 151 (0.05), SSRY 171 (0.012 and 0.036) and SSRY 177 (0.014). Unique alleles were also found in some genotypes from Colombia (6), Nigeria (3), Tanzania (3) and Guatemala (1). Average gene diversity was high, with an average of 0.6292 ± 0.0120, for all the samples analyzed but highest for those from Cuba and Tanzania (Table 1).

Table 1.Genetic diversity within samples of cassava accessions from 5 countries and standard deviation for *jackknifing* over loci (200 replicaciones). H_t, H_s, D_{st}, y G_{st} are given over loci and samples (country).

Country sample	n	#loc	#loc_P	PLP	K	K_P	HO_p	HE_p	HEc_p
CUBA	86	34	34	100.0	5.8	5.8	0.6016	0.6314	0.6351
GUATEMALA	10	34	34	100.0	4.2	4.2	0.5556	0.6063	0.6385
COLOMBIA	11	34	33	97.1	4.5	4.6	0.5675	0.6087	0.6396
NIGERIA	16	34	33	97.1	4.5	4.6	0.5885	0.5949	0.6136
TANZANIA	10	34	31	91.2	4.2	4.5	0.6459	0.5869	0.6190
Average				97.06	4.64	4.72	0.5918	0.6057	0.6292
Stand. Dev.				3.60	0.65	0.61	0.0351	0.0169	0.0120

	Ht	Hs	Dst	Gst
Average	0.6538	0.6057	0.0482	0.0740
Stand. Dev.	0.1770	0.1682	0.0253	0.0377
95% CI	0.5780	0.5341	0.0383	0.0618
95% CI	0.7137	0.6663	0.0585	0.0878

n:	Number of individuals
#loc:	Number of loci
#loc_P:	Number of polymorphic loci
PLP:	Percentage of polymorphic loci
K:	Number of alleles per locus
K_P:	Number of alleles per polymorphic loci
HO_p:	Observed Heterozygosity
HE_p:	Expected Heterozygosity
Hec_p:	Expected Heterozygosity corrected for small sample sizes (Nei, 1978)
Ht:	Total Genetic diversity
Hs:	Average genetic diversity within populations
Dst:	Average genetic diversity between populations
Gst:	Coefficient of genetic differentiation.

Estimates of genetic differentiation between the country samples ranged from 0.04 to 0.06 with the highest being between Cuban and Guatemalan/Tanzanian accessions (Fig1). Cluster analysis by principal coordinate analysis and PCA gave basically the same results. A pattern of clustering was observed between the African and Latin American accessions and within the Cuban accessions (Fig 2).

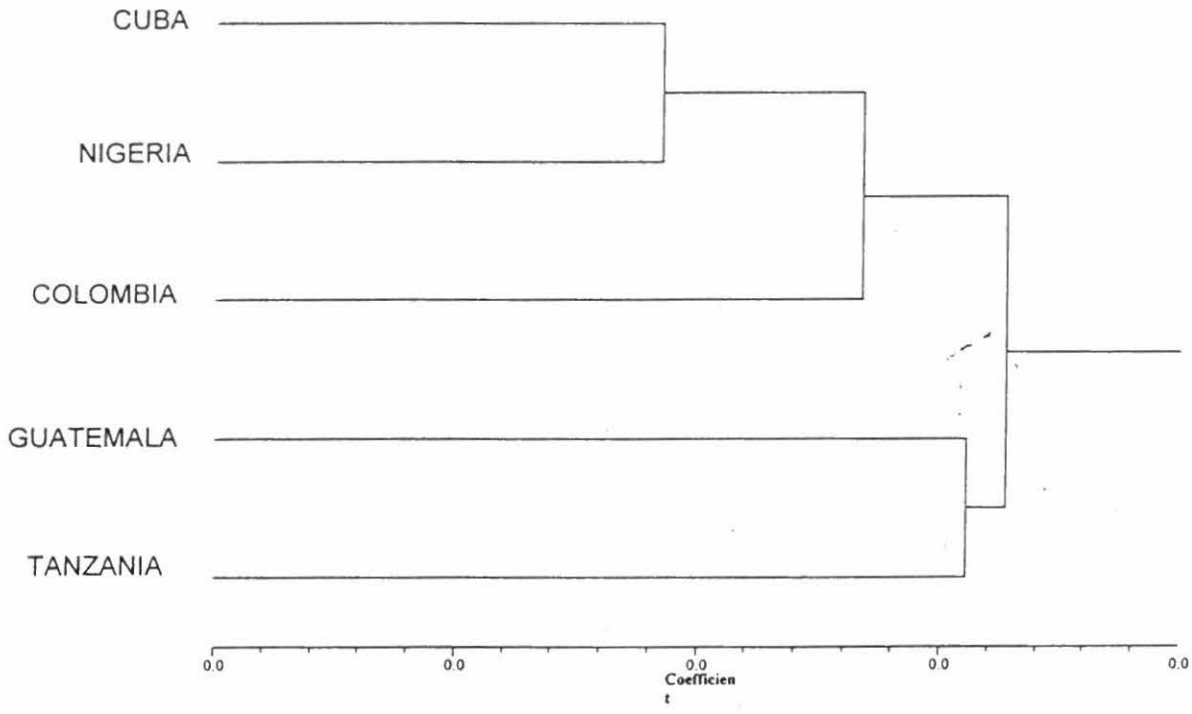

Figura 1. UPGMA dendogram of pair-wise comparison of the fixation index (F_{ST}) between samples of cassava from Cuba, Nigeria, Colombia, Guatemala and Tanzania.

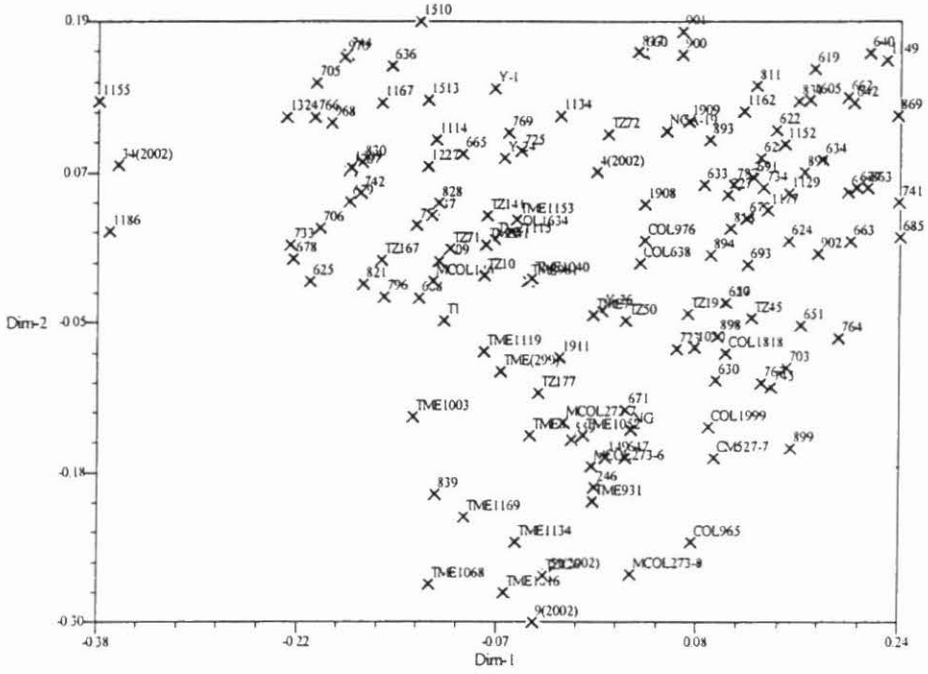

Figura 2. **Principal coordinate analysis of cassava accessions from Cuba and reference accessions from Nigeria, Colombia, Guatemala and Tanzania.**

Conclusion and Perspectives

Genetic Diversity of cassava in the Caribbean island of Cuba follows diversity found for cassava in the rest of the world, a high genetic diversity and low genetic differentiation. However a structure was observed within the Cuban collection using cluster analysis. This structure is the basis of future work on linkage disequilibrium mapping of dry matter content using candidate starch biosynthesis genes.

References

Fregene M, Suárez M, Mkumbira J, Kulembeka H, Ndedya E, Kulaya A, Mitchel S, Gullberg U, Dixon AGO, Dean R, Kresovich S (2003) Simple sequence repeats marker diversity in cassava landraces: genetic diversity and differenciation in an asexually propagated crop. Theoretical and Applied Genetics, accepted.

Mba REC, Stephenson P, Edwards K, Melzer S, Mkumbira J, Gullberg U, Apel K, Gale; M, Tohme J, Fregene MA (2001) Simple Sequence Repeat (SSR) Markers Survey of the cassava (*Manihot esculenta* Crantz) Genome: Towards an SSSR-Bassed Molecular Genetic Map of Cassava. Theoretical and Applied Genetics. 102: 21-31.

Minch (1993), http://www.lotka.stanford.edu/microsat.html

Dellaporta SL, Wood J, Hicks JR (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21.

Goudert J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. J Hered 86:485-486. Kimura M, Crow JT (1964) The number of alleles that can be maintained in a finite population. Genetics 49:725738.

Nei M (1978) Estimation of average heterozygosity and genetics distances from a small number of individuals. Genetics 89:583-590.

Rohlf DJ (1993) NTSYS-PC numerical taxonomy and multivariate analysis system. Version 1.8 Exeter Publ., Setauket, New York, USA.

Vekemans X, Lefebvre C (1997) On the evolution of heavy maetal tolerant populations in *Armeria maritima*: evidence from allozyme variation and reproductive barriers. J Evol Biol 10:175-199.

Wright S (1951) The genetic structure of populations. Ann Eugen 15:323-354.

1.1.13 Studies in Market Preferences of Cassava Cultivars in Malawi using SSR Markers

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1.SLU, Uppsala, Sweden ; 2 WAU, Wageningen, Netherlands; 3. IPICs, Sweden; CIAT

Funding: SAREC, IPICs, Uppsala, Sweden

Important Outputs:

1) Identification of 54 unique genotypes that form 3 broad clusters that might represent market classes of cassava in Northern and Southern Malawi

Introduction

Cassava is the second most important staple crop after maize in Malawi. Two other factors have emerged in Africa that will further increase the role of cassava as a staple crop: 1) The increasingly unpredictable rain pattern causing large fluctuations in maize harvests and 2) a dramatical increase in prices of chemical fertilizers and hybrid maize seeds make maize growing a less viable, or even impossible, alternative, for the smallholder farmer. As in many parts of Africa, the growing of cassava has increased considerably over the past years in Malawi. From 1992 to 1996 cassava production in Malawi is officially reported to have increased from about 100 000 tons/yr to more than 500 000 tons/yr. Since the cassava mosaic and mealy bug outbreak in the 1980s there has been a rapid shift in cassava cultivars and there is an obvious need for new cassava varieties with resistance to the major pests and diseases. Major disease outbreaks, like the one caused by the mealy bug in the eighties in Malawi or the cassava mosaic virus outbreak in Uganda, could be less devastating if resistant cassava material was available and accepted by the farmers within their farming systems. We conceive that the methods for provision of new cultivars to farmers can be considerably improved. A project to identify, evaluate and discover traits

that make certain local varieties popular amongst consumers was developed and funded by SAREC. We describe here Molecular marker technology for tracking gene-flows in trading of cassava cultivars and to explore how molecular markers can be used to identify cultivar preferences in urban cassava markets

Methodology

The study was conducted in two geographic areas, in the north and the south of Malawi, respectively. In the north, Nkhata-Bay close to Lake Malawi and has a fairly long dry period, mean daily temperature (DMT) during the growing season, above 20°C, and relatively low population density. The area in the south, Mulanje, is less dry and more densely populated. In each area, 5 farmers who are recognized by other farmers as cassava farming enthusiasts were recruited into the study. Using a combination of interviews, cassava cultivars were collected from markets in the above areas and planted at the Namiganzi farm center, Malawi. A total of 54 cultivars were collected. The cultivars were subjected to molecular analysis using 36 SSR markers for cultivar identification. SSR marker and data analysis were as described earlier Fregene et al. (2003).

Results

The 54 cultivars collected from Malawi revealed a fairly high amount of SSR allele diversity (Fig 1). The total number of alleles per locus ranged between 3 and 7. The fairly large number of alleles per locus enabled the unique identification of every single cultivar collected. Analysis of genetic relationships between the clones revealed that they formed 3 loose clusters that appear to represent market classes of cassava in Malawi (Fig 2). Detailed and conclusive interpretation of the above results will have to wait for agronomic data of the cultivars from the replicated trial at the Namiganzi farm center to be collected later in the year and additional information collected during the interviews.

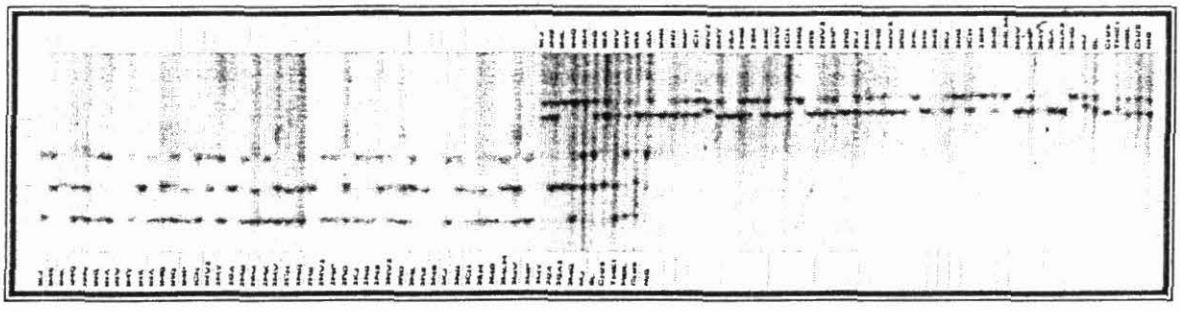

Figure 1. Silver stained PAGE gel electrophoresis of PCR amplification of the cassava genotypes collected in Malawi using the primers SSRY51 and SSRY63

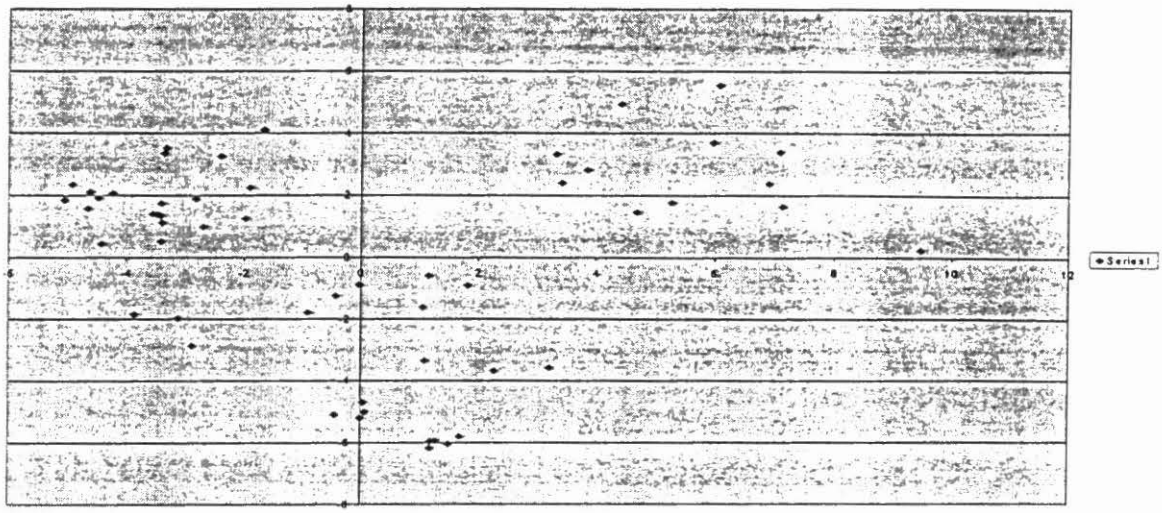

Figure 2. Principal component analysis (PCA) of pairwise genetic distances based on proportion of shared alleles (PSA) between the cassava accessions from Malawi

Conclusions and Future Perspective

An attempt has been made to use molecular markers in the identification of cultivar and therefore their preferences in urban cassava markets in Malawi. Using 36 SSR markers, 54 unique genotypes could be identified from 54 cassava cultivars collected from markets in Northern and Southern Malawi. The cultivars also formed 3 clusters that might represent market classes. Data from agronomic trial is being awaited to obtain conclusive evidence of this and also to identify what makes these cultivars preferred as a first step in making cassava breeding projects more relevant to end users.

References

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.

Activities related with the maintenance of the germplasm bank of cassava and other *Manihot* species. Basic genetic studies.

CIAT has been trusted with the maintenance of the cassava world germplasm bank, which includes more than 6000 accessions of *Manihot esculenta* and other *Manihot* species. In the following pages a summary of activities related to the germplasm bank and other basic genetic studies will be described. It is important to emphasize that all these activities are also reported as part of the joint work between IP3 and SB2 projects. The inclusion of this Output in the IP3 Annual Report is just to provide a whole picture of research and results around cassava at CIAT.

1.1.14 Maintenance of Manihot germplasm bank in the field

Introduction

The Genetic Resources Unit is officially in charge of the maintenance of the cassava germplasm bank, both *in vitro* and in the field. However, for practical reasons, the field operations are coordinated by IP3 project. Since year 2000 an extensive activity to clean up from frogskin disease, the germplasm bank has been carried out. Plots from the germplasm bank maintained in the field, because of its very nature, could not be eliminated even if frogskin disease appeared in some of the plants. Eventually the incidence of the disease increased to unacceptable levels.

In order to reduce the costs of maintenance of the germplasm collection and because of the problems associated with frog skin disease it was decided that the collection will be moved from the field and be maintained using the "bonsai system" under greenhouse conditions. This is an activity described in more detail in the respective report from the Germplasm Collections and will not be further discussed herein.

Because the Germplasm Collection is no longer in the field during this period, a set of clones has been regenerated in order to produce enough roots for the evaluation of different traits, particularly novel starch quality traits. IP3 project is trying to produce plants from as many as 2000 clones from the germplasm collection. These plants are produced from stocks that have been certified to be frog skin free. In addition the core collection (about 600 clones) has been shipped to Thailand so there is a duplicate of this important group of clones. The core collection will be phenotypically characterized in Thailand and also in Colombia. This evaluation will allow a measurement of the relative stability or sensitivity to genotype by environment interaction of the morphological descriptors used in the Genetic Resources Unit for cassava.

Specific Objectives:

a) To grow plantlets from the in vitro core collection.
b) To grow plantlet from in vitro plants of the germplasm bank for starch quality evaluations.

Results

We have begun a systematic characterization of the starch properties in the roots of the accessions from the germplasm bank. Every year up to 2000 accessions are evaluated. So far approximately 4000 clones have been characterized in the last few years and a group of about 2000 clones will be evaluated next year. To do this evaluation plants from these clones have been gradually recovered from the *in vitro* collection. The results of this evaluation will be published as soon as the data set is completed and an agreement has

been reached with the company financing this research. To produce the required plants the following steps need to be taken.

Regeneration of each accession from the in vitro collection.
From each accession, a plant from the *in vitro* collection was regenerated and indexed to certify it is free of diseases. Plants passing this first test are then hardened in conditions that do not allow for the presence of white flies, and therefore, minimizes the possibility of acquiring the frogskin disease agent again.

Because of the higher incidence of frogskin disease at CIAT plants that are certified to be disease free, or those developed from botanical were planted outside CIAT in isolated plots (CEUNP). Only virus-free plants were planted in those isolated plots. In the meantime, plantings at CIAT were reduced as a higher proportion of the cassava germplasm is being certified to be disease-free. In short the outside plantings were certified to be *"clean"*, whereas the plantings at CIAT were not. This situation was maintained until the middle of 2001, when materials not certified to be disease free moved out of CIAT, and those that are *clean*, came back to the station.

In addition of maintaining an ideal reservoir for the agent of the frogskin disease in the germplasm bank, there is a second factor that facilitated the spread of the disease. In effect, the white flies problem has increased considerably during the last few years. A major factor for this increment has been the continuous planting of cassava year round. The insects, therefore, had an ideal condition for maintaining high population densities. Between June 1 and June 30, 2003, there was no cassava plant in the field at CIAT's station in Palmira. It is expected that this measure will reduce population densities for the insect, and in turn, will reduce to a minimum the already inefficient transmission of the frogskin disease agent to healthy plants.

A common procedure to harvest cassava is to first take the stakes (vegetative *"seed"*) out of the field, and then harvest the roots. In fact this practice prevents the elimination of stakes from diseased plants, because when the roots are evaluated for symptoms, the stakes from each plant has already been mixed with other stakes from different plants. Starting in this year, the harvest protocol has been changed slightly. The whole plant is first taken out of the ground, so before taking the stakes the roots can be inspected to make sure they are asymptomatic. Stakes are taken only from plants that do not show the symptoms. This practice will reduce to a very minimum the "seed" transmission of the disease to only two possible cases: **a)** when the worker fails to recognize the symptoms; or **b)** when the plant has been infected late in the season and, therefore, it does not show the symptoms but the disease will be transmitted through its stakes.

All the activities were carried out as expected. A large proportion of accessions from the germplasm bank was evaluated for frogskin disease and, if clean, planted in isolated conditions. Sequential plantings were performed as the plants were certified to be disease-free. Therefore, harvest of these plants was also done sequentially. The levels of frogskin

were very low, as expected. However, given the results from the previous year, when higher than acceptable levels of frogskin disease were observed, it has been decided not to plant the entire germplasm bank in the field, until the vector(s) and pathogen(s) are clearly determined. At the end of October a total of 228 genotypes from the core collection have been hardened in screen house conditions (Figure 1), with a total of 975 plants (average of 4.3 plants/genotype). Vitroplants came in batches and that is why the four plantlets at the right bottom of Figure 1 show different developments.

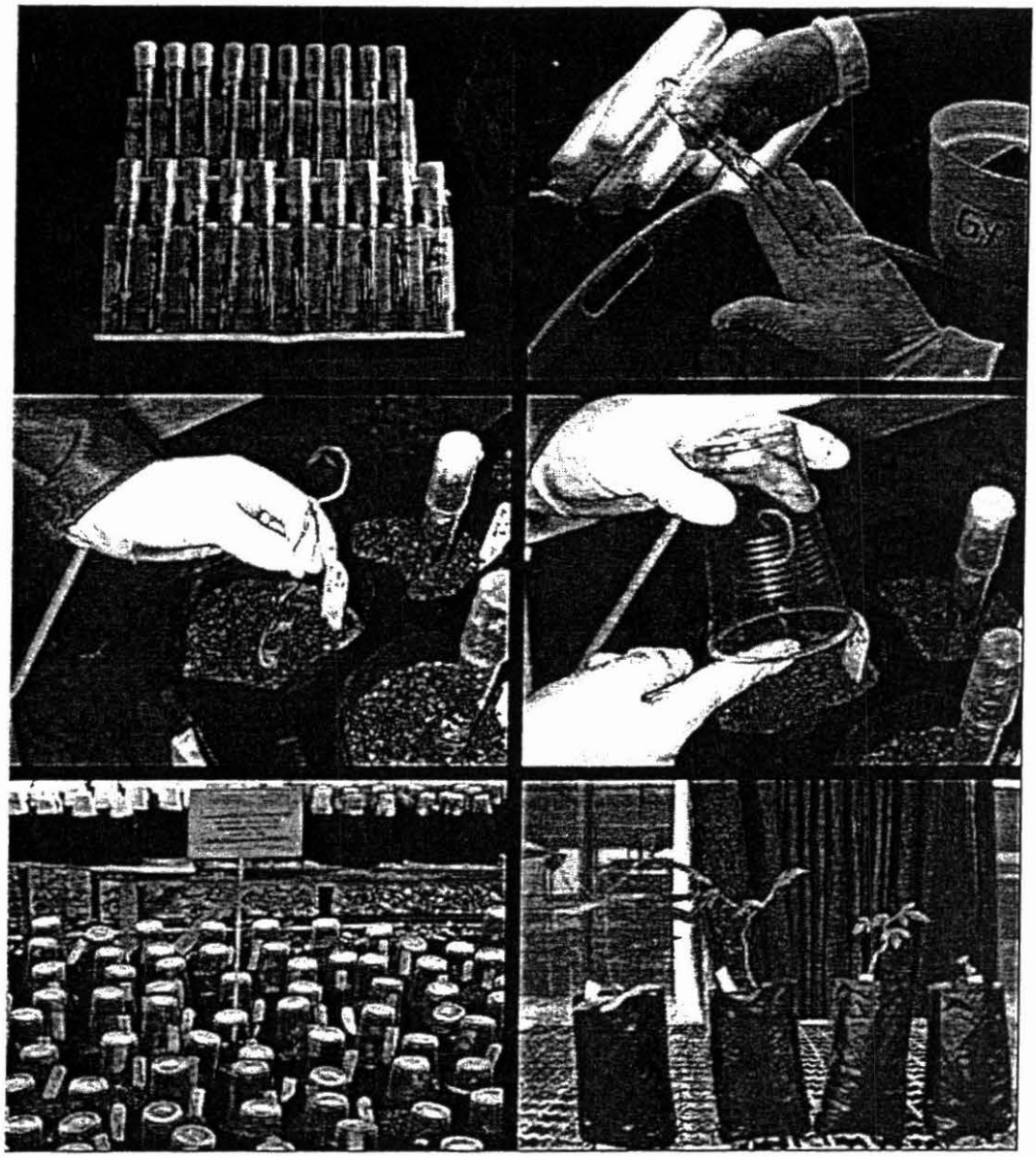

Figure 1. Illustration of the process to harden vitro plants followed for the recovery of genotypes from the core collection for their evaluation in the field.

1.1.15 Evaluation of M. esculenta and related species from the germplasm collection for useful traits, particularly for higher protein content in the roots

Introduction

Many of the activities related to the evaluation of the introgression of useful genetic variability from wild relatives of cassava is described in Output 12 and also in Output 1. In this activity, however, a particular action will be described because of its relevance. In a previous Annual Report (2002) it was reported that a few clones more commonly from Central America had been found to have high levels of crude protein in the roots. These clones were properly identified and were recovered from the germplasm collection to be evaluated again to confirm their protein content in the roots. One important feature that will be evaluated is the stability of that trait through multi-location evaluations and also the effect of the age of the plant.

Specific Objectives

a) To grow plants recovered from the *in vitro* collection and measure protein content in their roots.

Results

Plants from 21 clones were hardened and grown in the field. At eight months of age one root was taken from the plants in the field and flour obtained from them. N content was measured at CIAT's analytical laboratory and multiplied by the standard 6.25 factor to obtain % of crude protein content in the roots. Figure 2 shows the results from these measurements (at eight months of age) as well as the original data from a 1999 evaluation on 10-month old plants.

The results presented in Figure 2 are preliminary. The fact that the quantifications were made at two different laboratories should not have an effect based on the results presented in Output 1. The major difference between the two evaluations is the age of the plants. These plants will be kept in the field for few more months and toots taken at 10 months of age for yet another quantification of protein content. In spite of the obvious disagreement between the two analyses depicted in Figure 2, it is obvious that at least five clones showed again protein levels above 5%, which is about twice as much as the content traditionally considered for cassava. These plants are in the field to be crossed and self-pollinated, and constitute a very valuable and promising germplasm.

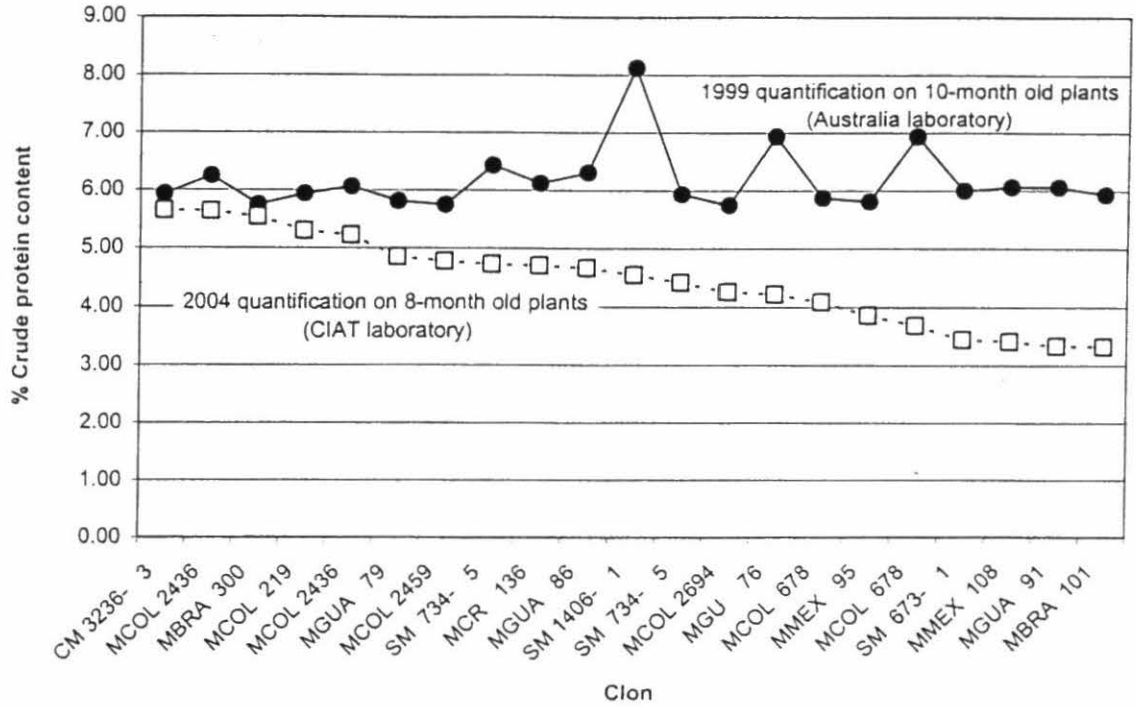

Figure.2. Results of crude protein content in roots from 21 cassava clones evaluated in 1999 at 10 months of age (quantification made at White's Analytical Laboratory, Adelaide University) and again in 2004 at 8 months of age (quantification at CIAT's Analytical Laboratory).

1.1.16 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

Domestic rice and weedy rice are predominantly self-pollinated, although a high out-crossing rate between domestic rice and weedy rice has been reported (Langevin et al., 1990). Most studies suggest that the hybridization rate in commercial rice oscillates between 0 and 6.8 %. The out crossing between rice and weedy rice can be highly variable and usually in a similar range or lower as that between commercial rice varieties (OECD, 1999 cited by Gealy et al., 2003). Rice pollination is susceptible to genetic and environmental factors, such as temperature, humidity, genotype, flower morphology, stigma receptivity, pollen viability, pollen germination and the development of pollen tube (Jensen and Salisbury1988). Feral characteristics such as dormancy, shattering and vigorous growth make weedy rice highly competitive whit respect to domestic rice and a

potential candidate as gene receptor from the cultivated species. According to Gealy et al. (2003) gene flow in principle is simple, however, the parameters that determine the establishment of a new trait in a weedy population are complex and once hybrids are formed, traits may introgressed into the weedy rice population within only a few generations. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in tropical America, and its effect(s) on the population genetic structure of the recipient species. Last year, we reported preliminary results indicating higher crossing rates in manual-made crosses under greenhouse conditions respect to the natural flow in the field. Hybridization rates in manual crosses were higher (at least 2 fold) when transgenic or non-transgenic rice was used as the male parent (pollen donor) and weedy rice as the female parent (pollen recipient). A lower hybridization rate was noted in the reciprocal crosses (using weedy rice as the pollen donor), probably suggesting a preferential gene flow rate from rice into the weedy rice. Similar results were obtained when tracing the presence of anthocyanins (dominant trait) from the variety Purple as a morphological marker. Because of the different hybridization rates obtained between field and greenhouse assays, additional crosses were made this year with plants grown in the greenhouse in order to determine the reproducibility of results. Additionally, this year we report the agronomic performance and molecular characterization of 16 manual-made hybrids.

Materials and Methods

Manual crosses. Crosses were made following procedures as described by Sarkarung (1996) with some modifications (Jaime Carabalí, personal communication, CIAT Project IP4). Six weedy 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 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.

Seed germination and seedling growth. The harvested seeds were disinfected with hypochlorite (bleach 10%) and cultured on ¼ MS medium under dim light for 8 days. After germination, seedlings were transferred to direct light. After 5 days in water, the plants were transplanted in pots in the greenhouse.

Agronomic performance of hybrids plants. Plants were grown to maturity and evaluated by different common agronomic traits (IRRI, 1996) under greenhouse conditions. The evaluations were conducted using 10 to 15 progeny plants per each hybrid generated between transgenic, non-transgenic, or Purple variety and weedy rice.

Molecular characterization. DNA was extracted from young leaves according to McCouch et al. (1998). Two or three polymorphic microsatellite markers clearly distinguishing weedy rice, the transgenic line or non-transgenic variety, and the Purple variety were used to determine the hybrid nature of the materials recovered from each cross. All the plants were analyzed by *gus* expression in the root and leaves. The *gus* and *hpt* genes were detected using PCR and Southern blot analyses (Sambrook et al., 1989).

Results and Discussion

Likewise last year results, in 2004 general higher hybridization rates were observed when rice was used as male parent (pollen donor) and weedy rice as a female parent (pollen recipient) (Table 1A and 1B). A lower hybridization rate was noted in the reciprocal crosses (using weedy rice as a pollen donor), which was highly significant in crosses with the variety Purple (1/4 lower). No significant differences were noted when using the transgenic line either as female (6.9% to 19.5%) or male (5.0% to 11.2%) parent. This trend was maintain when averaging values obtained over the two-year period (2003 and 2004)(Table 1C). About 4 fold higher hybridization rates were obtained when using Purple as male and about 2 fold when using the non-transgenic line as male parent (Table 1C), probably suggesting a preferential gene flow rate from rice into the weedy rice. The transgenic line produced fewer amounts of pollen and with a reduced viability respect to the non-transgenic counterpart, which may explain the different behavior respect to the other rice lines (Table 1C)..

Similarly to 2003, hybridization rates as high as 43% from a single manual cross (Table 1B) were confirmed by microsatellite analysis. These rates are similar to those observed between commercial rice and sterile male rice that reaches 34 % (Gealy et al., 2003), and significantly higher than those found between the same weedy types and rice in the field which ranged from 0 to 0.3 % (Fory et al., 2004, herein this SB2 Annual Report). For over two years the weedy rice biotype 1-3-4 consistently showed the lowest hybridization rate in manual crosses from 5.7% (Table 1A) to 6.6% (Table 1B) with a mean of 6.9% (Table 1C), and the biotype 1-21-3 showed the highest hybridization rate from 19.7% (Table 1A) to 22.9% (Table 1B) with a mean of 21.8% (Table 1C). It is important to note that under field conditions, the highest hybridization rate between weedy rice and rice (the transgenic line and Purple variety) was also obtained with the weedy rice biotype 1-21-3 (0.3%) (Fory et al., 2004, herein this SB2 Annual Report). These results suggest there is different combinatory ability among weedy rice biotypes.

Matsui and Kagata (2003) have reported a correlation between the morphological traits of the floral organs and self-pollination after evaluating 12 rice varieties. This study showed that plants with small basal pores may be more likely to be cross pollinated than those with large basal pores which are more prone to self-cross. Differences in flower structure between weedy types may determine their disposition to cross pollination as well.

Sixty three percent of hybrids generated between rice and weedy rice flowered earlier than at least one of the parents, of these about half flowered earlier than both parents (the transgenic and non transgenic materials) (Table 2). None of the hybrids flowered later than any of the parents (Table 2). Although hybrids with the transgenic line showed more tillers than any other hybrids, only one cross (4-12-2/Transgenic line) showed a significant difference with the weedy rice. The other hybrids representing 94 % of total did not show significant difference in tiller capacity respect to both parents (Table 2). The non-transgenic and transgenic lines (controls) were 85-87 cm in height, whereas weedy rice was taller (from 85 to 102 cm tall). Fifty percent of hybrids were as tall as the parents. About 43 % hybrids were significantly different in height respect at least to one parent. Of these, 86 % hybrids were taller than the transgenic line, and 17 % hybrids were shorter than the non-transgenic Cica 8 variety. Future work includes evaluating the fitness performance of the hybrids in advanced generations in the field.

Table 1A.- Hybridization rates with manual crosses between six red rice biotypes, the F_3BC_1 transgenic Cica 8 A3-49-60-12-3/Cica 8 line and the Purple variety grown in the field or greenhouse [1] in 2003.

Weedy rice	Transgenic Line		Non-transgenic		Purple Variety		
	Male	Female	Male	Female	Male	Female	Total
1-3-4	0.0	0.0	17.1	Nd	10.8	0.8	5.7
1-21-3	12.4	15.0	58.8	4.8	7.5	Nd	19.7
4-12-2	5.0	3.8	37.2	Nd	0.5	0.3	9.4
5-36-4	1.2	8.2	41.7	Nd	0.0	0.0	10.2
5-38-5	0.0	0.0	51.9	29.7	3.5	2.4	14.6
5-48-2	11.6	14.8	19.9	15.1	0.7	0.0	10.3
Promedio	5.0	6.9	39.8	16.5	5.0	0.9	12.4

[1]A 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 variety. NT: Non-transgenic Cica 8. Nd: Not determined

Table 1B.- Hybridization rates with manual crosses between six red rice biotypes, the F_3BC_1 transgenic Cica 8 A3-49-60-12-3/Cica 8 line and the Purple variety grown in the field or greenhouse [1] in 2004.

Weedy rice	Transgenic Line		Non-transgenic		Purple Variety		
	Male	Female	Male	Female	Male	Female	Total
1-3-4	5.1	2.4	16.4	7.8	4.8	3.1	6.6
1-21-3	Nd	24.3	ND	21.6	42.9	3.0	22.9
4-12-2	15.4	10.9	21.3	40.0	7.5	1.2	16.1
5-36-4	2.6	24.3	24.6	11.4	13.1	3.4	13.2
5-38-5	21.8	28.6	15.2	4.3	26.4	0.4	16.1
5-48-2	Nd	26.6	16.3	15.2	5.7	14.8	13.1
Promedio	11.2	19.5	18.8	16.7	16.7	4.3	14.5

[1]A 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 variety. NT: Non-transgenic Cica 8. Nd: Not determined

Table 1C.- Average hybridization rates with manual crosses between six red rice biotypes, the F_3BC_1 transgenic Cica 8 A3-49-60-12-3/Cica 8 line and the Purple variety grown in the field or greenhouse [1] in 2003 and 2004.

Weedy rice	Transgenic Line		Non-transgenic		Purple Variety		
	Male	Female	Male	Female	Male	Female	Total
1-3-4	5.1	2.4	16.4	7.8	7.8	2.0	6.9
1-21-3	12.4	19.6	58.8	13.2	25.2	1.5	21.8
4-12-2	10.2	7.3	29.2	40.0	4.0	0.8	15.3
5-36-4	1.9	16.2	33.1	11.4	6.6	1.7	11.8
5-38-5	21.8	28.6	33.5	17.0	15.0	1.4	19.6
5-48-2	11.6	20.7	18.1	15.1	3.2	7.4	12.7
Promedio	10.5	15.8	31.5	17.4	10.9	2.5	14.7

[1]A 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 variety. NT: Non-transgenic Cica 8. Nd: Not determined

Table 2.- Agronomic performance of hybrids obtained b manual crosses between six red rice biotypes, the F_3BC_1 transgenic Cica 8 A3-49-60-12-3/Cica 8 line and the- Purple variety grown in greenhouse.

Crosses between weedy rice (WR) and rice		Days to 50 % plants flowering	Number of Tillers	Plant Height
Female parent	**Male Parent**			
WR 1-3-4	Cica 8 non transgenic	103 defg**	9 cd NS	106 bcd *NT
WR 1-21-3	Cica 8 non transgenic	91 h **	11 abcd NS	89 fghij NS
WR 4-12-2	Cica 8 non transgenic	108 bcdef NS	9 cd NS	88 ghij NS
WR 5-48-2	Cica 8 non transgenic	99 g **	9 cd NS	103 bcdef *NT
WR 5-36-4	Cica 8 non transgenic	105 defg *NT	13 abc NS	103 bcdef **
WR 5-38-5	Cica 8 non transgenic	104 defg *NT	13 abcd NS	103 bcdefg*NT
Cica 8 non transgenic	WR 1-21-3	92 h **	12 abcd NS	84 ijk NS
Cica 8 non transgenic	WR 5-38-5	103 defg*NT	9 bcd NS	105 bcde *NT
Cica 8 non transgenic	WR 5-48-2	103 defg*NT	15 abc NS	103 bcd*NT
WR 1-21-3	Transgenic line	92 h **	17 a NS	78 jk NS
WR 5-48-2	Transgenic line	104 defg *T	16 ab NS	92 defghij NS
WR 4-12-2	Transgenic line	109 abcde NS	17 a *WR	91 efghij NS
Transgenic line	WR 1-21-3	90 hi **	15 abc NS	72 k *WR
WR 1-3-4	Purple variety	98 g **	9 bcd NS	110 a * P
WR 1-21-3	Purple variety	85 i **	13 abcd NS	89 fghij NS
WR 5-38-5	Purple variety	108 bcdef NS	13 abc NS	95 cdefghi NS
Weedy Rice	1-21-3	104 defg	11 abcd	91 defghij
	1-3-4	109 abc	12 abcd	102 bcdefgh
	4-12-2	113 a	10 bcd	85 ijk
	5-36-4	102 fg	13 abc	86ijk
	5-38-5	107 bcdef	12 abcd	96 cdefghi
	5-48-2	106 cdef	11 abcd	97 bcdefghi
Transgenic line	*A3-49-60-12-3 /Cica 8-2*	112 abc	13 abc	85 ijk
Non transgenic varieties	Cica 8	112 abc	13 abcd	86 ijk
	Purple	107 bcdef	15 abc	88 hij

Different letter denote statistical significance (P < 0.05). Ryan - Einot - Gabriel - Welsch. Multiple Range Test. ** Crosses showing significant differences from both parents. * Crosses showing significant differences with only one parent (T transgenic line, NT non transgenic and P, Purple variety, WR weedy rice). NS = there are not significant differences with parents.

References

Gealy, D., Mitten, J. and Rutger, N. 2003. Gene flow between red rice (*Oryza sativa*) and herbicide resistant rice (*O. sativa*): Implications for weed management. Weed Technology. 2003. 17: 627-645.

IRRI (International Rice Research Institute). 1996. Standard evaluation system for rice. Manila, Philippines. 52 p.

Jensen, W. and Salisbury, F. (1988). Botánica. 2a edición. McGraw-Hill México. 762p.

Langevin, S., Cley, K. and Grace, J. (1990). The incidence and effects of hybridization between cultivated rice and its related weed red rice (*Oryza sativa* L.). Evolution. 44:1000-1008.

Sarkarung, S. 1996. Un Método Simplificado Para Cruzamientos *En*: Arroz. Manual Técnico. Fondo Latinoamericano de Arroz de Riego (FLAR). CIAT. 35p.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: A laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

McCouch SR, Kochert G, Yu H, Wan Z, Khush G, Coffman W and Tanksley. 1988. Molecular mapping of rice chromosomes. Theoretical and Applied Genetics 76: 815-829

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

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

Introduction

The presence of weedy rice in paddy fields is frequently the result of the predominant use of farmer-saved seed instead of a certified seed source, and of direct seeding system for rice cultivation by farmers. The problem is exacerbated by the lack of crop rotation and the common practice of several crop cycles per year. In contrast to temperate regions where weedy rice is mainly composed of *Oryza sativa f. spontanea* (red rice), in tropical America, the weedy rice complex is broadly diverse and apparently composed of numerous *Oryza* species, usually with feral traits (taller, awned seeds with red pericarp, and shattering), varying degree of sexual compatibility (Oka and Chang, 1961), and flowering overlapping with the crop in different environments. There are indications that under temperate conditions, genes placed in rice may be quickly transferred into weedy rice (Langevin et al. 1990). Gene introgression not only presupposes physical proximity of the crop to its wild/weedy relative(s) and overlapping flowering so pollination can affect gene transfer, but also genetic compatibility of the crop and its immediate wild/ weedy relative(s) as well as the fitness of the derived hybrid. This work is part of a project

directed to analyze the gene the 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 two experimental field designs to trace gene flow from transgenic and non-transgenic (Purple line) rice into weedy rice. The current report summarizes the evaluation of gene flow using the multiple square plot design and the progress attained establishing the use of molecular markers to assess and trace gene flow from transgenic and non-transgenic rice into weedy rice at large scale under controlled experimental field conditions.

Materials and Methods

Experimental field designs to trace gene flow from transgenic and non-transgenic rice into weedy rice. Six weedy rice accessions representing the diversity of types found in the plots of Colombian farmers were selected to conduct gene flow analysis and identify indicators for easy tracing and monitoring of genetic introgression in the crop-weedy rice complex. The selection included weedy types susceptible to the rice hoja blanca virus (RHBV), with high flowering synchrony with rice, and easily distinguishable using specific microsatellite markers detecting polymorphisms for various SSR specific alleles. Gene flow was assessed using a transgenic line (A3-49-60-12-3/Cica 8-2) as pollen donor. This line contains the RHBV-N protein gene that confers resistance to RHBV, and the *gus* and *hph* (hygromycin resistance) marker genes. A non-transgenic rice variety locally known as Purple, characterized by having purple leaves, tillers, and grain apiculus, and the dominant inheritance of anthocyanins was used as control. Multiple-square assay design was used, which consisted of randomized plots with rice (purple variety or transgenic line) planted intermingled with 20% weedy rice in square plots, simulating farmers field conditions and reflecting the economic threshold level for weedy rice infestation in Colombia (Pineda et al., 2003 SB2 Annual Report 2003).

Tracing gene flow. Gene flow was either traced at the phenotypic level by detecting *gus* expression or by evaluating the presence of anthocyanin in plant tissues and putative hybrids. After the phenotypic identification, putative hybrids were confirmed by microsatellite markers. Parallel, DNA samples of various plants were directly sampled and processed by bulk-analysis without previously knowing the phenotypic profile. PCR analysis was conducted to trace the presence of transgenes (*gus*, 35 S promoter, *hph* and N-protein) and SSR analysis to confirm the hybrid nature of recovered F_1 plants. One thousand plants per plot and four replicates per each treatment (total of at least 4,000 plants per combination of parents) were analyzed.

Results and Discussion

Hybridization rates of 0.0 to 0.3 % (mean 0.16-0.17%) were obtained when the transgenic marker genes were used to trace gene flow in 6,209 progeny derived from crosses between weedy rice and the transgenic line (Table 1). Hybrids plants expressed the *gus* gene and displayed the region of the promoter 35S CaMV, *hph* and N-protein genes. Microsatellite

markers also confirmed the hybrid nature of these plants (Table 1). In contrast, when scoring the hybridization rate using the phenotypic trait alone (i.e. anthocyanin color in flower/stem/leaves) some putative hybrids identified by the presence of anthocynins in the tillers were not true hybrids when analyzed with microsatellites. Furthermore, when plants were analyzed by bulk DNA without taking into account their anthocynin profile, a higher outcrossing rate was estimated respect to that based on the presence of the purple color. These results suggest that the scoring of phenotypic trait alone could over- or sub-estimate the level of hybridization rate. In rice, different genes regulate the anthocyanin pigmentation in various tissues. There are three genes that are involved in the expression of anthocyanin, gene C (chromogen production), gene A (activator) and gene P (pigmentation expression) (Nagao, 1951 and Takahashi 1957 cited by Sakamoto et al., 2001). The analysis by microsatellites indicates that the hybridization rate between transgenic rice and weedy rice is similar to that with the Purple variety (mean 0.18%). Outcrossing rates < 1% from transgenic/non-transgenic rice to weedy rice/rice varieties had recently been reported by other authors under temperate conditions (Noldin et al. 2001; Zhang et al. 2003 and Messeguer et al. 2004). But the cumulative hybridization rate (in consecutive years/period) may be higher under tropical conditions as compared with temperate conditions because of the lack of crop rotation and several crop cycles per year. Gene introgression is dynamic over time and hybrid fitness analysis between rice/ weedy rice needs to be evaluated at the landscape level in the farmers field. Towards that end our group will examine the gene the flow/introgression dynamics in the crop/wild/weedy rice complexes using microsatellites complemented with organelle (maternal inheritance) polymorphism as a tool to trace the direction of gene flow.

An optimized methodology using bulk DNA and PCR-based analysis allows the analysis of large number of seed samples with high precision to identify hybrid candidates. This methodology is useful for tracking and monitoring gene flow at large scale in farmers' fields and in crop-to-wild contact zones. The scoring of the phenotypic trait alone (i.e. gus expression, non-transgenic anthocyanin vegetative tissues) could either under or overestimate the hybridization rate. Similar results had been found in other works scoring herbicide resistance. Because of these potential errors, it is advisable that phenotypic data from putative out-crossing events in a particular crop/year be confirmed with molecular techniques that can specifically identify the original parents or trait/gene(s). When handling thousands of samples, bulk DNA could first be assayed for the presence of the transgene(s) allowing the detection of 1 hybrid in 200 plant-bulks and the putative bulk then split in smaller samples allowing the detection of 1 hybrid in 40 plant-bulks with microsatellite. SSR are valuable genetic markers because they are simple co-dominant, which allow detection of high levels of allelic diversity, and are easily and economically assayed by PCR. The analysis using microsatellites will give a better understanding of the gene flow/introgression dynamics in crop/wild/weedy complexes and of the potential impact on biodiversity.

Conclusions

Preliminary results suggest a natural gene flow rate from transgenic/non-transgenic rice to weedy rice of about 0.0 % to 0.3% in the field, which is significantly lower respect to manual crossing (up to 43%). No differences were found in the hybridization rates between weedy rice and transgenic or non-transgenic rice. The scoring of the phenotypic trait alone (i.e. *gus* expression, non-transgenic anthocyanin vegetative tissues) could either under or overestimate the hybridization rate. Microsatellites allowed the detection of some false negative/positive hybrid plants.

Future plans

In order to complete the gene flow analysis we have planted 30,000 plants under field conditions and aim to analyze 40,000 plants before December 2004. The evaluation of the first 20.000 plants is currently in progress. In the second generation of plants derived from the confirmed hybrids, we will be evaluating for resistance to RHBV and fitness performance of the corresponding hybrids.

Table 1. Hybridization rates between weedy rice biotypes[1] and the F_3BC_1 transgenic A3-49-60-12-3/Cica 8 line under field conditions.

Weedy rice[1]	plants	% plants w/ Gus expression	% plants with transgenes	% hybrids identified by SSR
1-21-3/T (I)	1,583	0.31	0.30	0.30
4-12-2/T (V)	1,611	0.00	0.00	0.00
5-48-2/T (R)	1,393	0.24	0.21	0.21
5-38-5/T (V)	1,622	0.14	0.12	0.12
Total/ Mean	6,209	0.17	0.16	0.16

[1]Weedy rice accessions similar to *Oryza rufipogon* (R), weedy rice similar to commercial varieties (V), and weedy rice with intermediate traits between commercial varieties and wild species (I) were included in the gene flow assay.

Table 2. Hybridization rates between weedy rice biotypes[1] and the Purple variety (P) under field conditions.

Weedy rice	plants	plants first identified by purple color stem [2]		plants Analyzed h without considering purple color Stem[3]
		% Hybrids by stem color	% Hybrids by SSR	% Hybrids by SSR
1-3-4/ Purple (R)	2,801	0.04	0.04	Nd
5-36-4/ Purple (I)	2,725	0.07	0.02	Nd
1-21-3/ Purple (I)	3,930	0.27	0.05	0.00
4-12-2/ Purple (V)	4,000	0.07	0.00	0.00
5-48-2/ Purple (R)	1,407	0.00	0.00	0.28
5-38-5/ Purple (V)	3,071	0.06	0.06	0.43
Total	17,934	0.10	0.03	0.18

[1]Weedy rice accessions similar to *Oryza rufipogon* (R), weedy rice similar to commercial varieties (V), and weedy rice with intermediate traits between commercial varieties and wild species (I) were included in the gene flow assay. [2]The number of plants analyzed considering the purple stem color ranged from 1.407 to 3.071 per treatment. [3]The number of plants analyzed by micosatellite ranged from 806 to 1053 plants per treatment. A total of 3757 plants were analyzed by microsatellite (Nd = treatment not determined).

References

Langevin S.A., Clay K. and Grace J. B. 1990. The incidence and effects of hybridization between cultivated rice and its related weed red rice (*Oryza sativa* L.). Evolution 44: 1000-1008.

Messeguer J., Marfa V., Catala M.M., Guiderdoni E. and Mele E. 2004. A Field study of pollen-mediated gene flow from Mediterranean GM rice to conventional rice and the red rice weed. Molecular Breeding 13: 103-112.

Noldin J., Yokoyama S., Antunes P. and Luzzardi R. 2001. Taxa e sentido do cruzamento natural entre o arroz tranasgenico resistente ao herbicida glufosinato e o arroz vermelho. *En* II Congresso Brasileiro de Arroz Irrigado; XXIV Reuniao da Cultura do Arroz Irrigado. ANAIS. Instituto Rio Grandense do Arroz, IRGA p 497-499.

Oka H.I. and Chang W.T. 1961. Hybrid swarms between wild and cultivated rice species, *Oryza perennis* and *O. sativa*. Evolution 15: 418-430.

Zhang N., Lincombe S. and Oard J. 2003. Out-crossing frequency and genetic analysis of hybrids between transgenic glufosinate herbicide-resistant rice and the weed, red rice. Euphytica 130: 35-45.

Sakamoto, W., Ohmori, T. Kageyama, K., Miyazaki, C., Saito, A., Murata, M., Noda, K. and Maekawa, M. 2001. The purple leaf (Pl) locus of rice: the Pl^{w} Allele has a complex organization and includes two genes encoding basic helix –loop –helix proteins involved in anthocyanin biosynthesis. Plant Cell Physiol. 42: 982-991.

1.1.18 Diversity Array Techology (DArT) for potato diversity analysis

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Introduction

Genetic resources are at the base of generation of new varieties and are a reservoir of genetic stability and adaptability. It is essential to know about diversity in order to preserve and use it for improvement of actual potato cultivars. Today, the most promising technology allowing higher through output and larger genome coverage for diversity studies is Diversity Array Technology (DArT) (Jaccoud et al. 2001). DArT is a technology employing microarrays based on the generation of DNA segments 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 could 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. One of the barriers of this technology reside in the reduction of genome complexity to obtain high polymorphic genome libraries, this year some polymorphism enrichment strategies were tested to obtain potato polymorphic genome libraries.

Materials and methods

In the past year (Posso et al, 2003) ninety-six potato genotypes from the CIP potato germplasm collection were collected and DNA extracted. Six of them were used to make diversity panels. Two libraries were elaborated by using two digestion enzymes. This year those libraries were evaluated for polymorphism and other three libraries were also built.

Generation of diversity panels. Genomic libraries change depending on the digestion enzymes used to make it. To facilitate identification of genomic libraries a number was assigned to each one. Library 1, is the product of *Mse*I digestion of six genomic DNA genotypes; Library 2, was elaborated using the same six genotypes digested with *Pst*I enzyme; Library3 result from two potato genotypes digested by *Pst*I enzyme; Library 4, employed *Pst*I and *Apo*I enzymes for double digestion of six genomic DNA genotypes, and Library 5 used *Pst*I and *Taq*I enzymes for double digestion of the same six potatoes genotypes.

The protocol of libraries 3, 4 and 5 was partially the same reported for *Mse*I and *Pst*I libraries in the past year (Posso et al. 2003.). For library 3, the protocol differs in that only two genotypes were used to generate genomic library, and it includes a step of nylon membranes hybridization between those genotypes. For libraries 4 and 5, the protocol includes an additional cutter enzyme used to a second DNA digestion before PCR amplification and cloning.

Generation of probe representation. Two types of representation were elaborated. For the first one, the same protocol for the elaboration of libraries was used (Posso et al, 2003). The other representation was made by PCR amplification using a primer with an additional nucleotide, this kind of representation was hybridized to library 1.

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) (in demostration at CIAT), TIGR MIDAS v. 2.17 for windows and TIGR Multi Experiment Viewer v. 2.2 for windows.

Net intensity spots values were normalized by using a loess regression that adjusts spots signal values according to the spot position on the slide and between signal channels. Normalizations between slides were made using TIGR MEV. Polymorphic sequences between two genotypes were considered as those with log2 cy3/cy5>= 2 and log2 cy3/cy5 <= -2 and polymorphic sequences between six genotypes were found using a program in house made that use SAS language.

Results

The construction of five libraries was justified in order to optimize genome complexity reduction and to obtain more polymorphic sequences.

Table 1: Libraries polymorphism

Library	Enzyme	No. of clones in library evaluated	Polymorphism in library (%).
1	*Mse*I	768	0.00
2	*Pst*I	384	0.00
3	*Pst*I	768	0.00
4	*Pst*I/*Taq*I	768	0,39
5	*Pst*I/*Apo*I	5760	3,33

No polymorphic clones were detected into libraries made with only one digestion enzyme, when they were hybridized with representations of two genotypes (**Table1**). This result shows that potato genome complexity was not sufficiently reduced to pick polymorphic sequences between genotypes when one enzyme is used for DNA digestion. These results were low compared with those of Jaccoud et al, (2001) and Lezar S et al. (2004), who found 2.89% and 2.35% polymorphic sequenecs in libraries of rice and eucalyptus generated with one enzyme digestion and hybridized with representations of the same kind. This difference could indicate a higher complexity of potato genome, with a genome size of 800 Mb (Edwige Isidore et al. 2003), compared with those of rice, with genome size of 430 Mb, and eucalyptus.

On the other hand, the low polymorphism is the result of highly repetitive DNA content of plant genomes that undoubtedly results in cross-hybridization of DNA fragments to printed probes. It has been demonstrated that small regions of similarity can lead to cross-hybridization on oligonucleotide microarrays (Kane et al, 2000). Cross-hybridization obscures polymorphic sequences and makes it difficult to find of polymorphism into genomic libraries.

Cross hybridization into library1was revealed by using representations resulting from PCR amplifications with primers including an additional nucleotide. When panels of library1 were hybridized with six representations of that kind, 29 polymorphic sequences were found, see **Table 2.**

Table 2: Polymorphic clones evidenced on library 1 by representation amplified using primers +T.

Couple of representation hybridized (CIP number)	No. of polymorphic clones
Genotypes CHS625 and PS-3	1
Genotypes 703508 and 704746	27
Genotype 705428 and 700347	1

The polymorphism rate varies more than 3 fold among libraries (0.00% to 3.33%, **Table 1**). This result shows that polymorphism enrichment strategy contributes to reduce potato genome complexity and it showed to be necessary to evidence polymorphic clones into potato libraries.

The polymorphism enrichment protocol that arose more polymorphism was the double digestion one. No polymorphic sequences were found by using paired hybridization protocol, library 3, and rates of 0.39 and 3.33 were found on libraries 4 and 5 that were built using double digestion (**Table 1**). The best results were obtained when using *Apo*I as the second digestion enzyme, library 5. Differences on polymorphic rates among double digested libraries were also observed by Killian et al (2003) on cassava genomic libraries and Wenzel et al (2004) on barely genomic libraries with rates varying from 9,07% to 14,16% and 2,9% to 10.4%, respectively. Here again low polymorphism rates obtained in our experiment could be caused by potato genome complexity and cross-hybridization. Those results encourage the use of different strategies to increase polymorphism. The use of a third digestion enzyme in combination of primers with selective nucleotide could be useful for this purpose.

Diversity panels of library 5 were also hybridized with all genotypes used for library construction and a part of cloning vector sequence, used here as a reference. A total of 194 polymorphic clones between six potato genotypes were found using a threshold value of 0.3 as a difference in relative intensity between two intensity classes (See **Figure 1** for examples of signal ratio distributions). Contrary to that was expected only a few polymorphic clones found in hybridization of library 5 panels with two genotypes were

also found in hybridizations using cloning vector as a reference. We suspect that cross-hybridization could be responsible for this result.

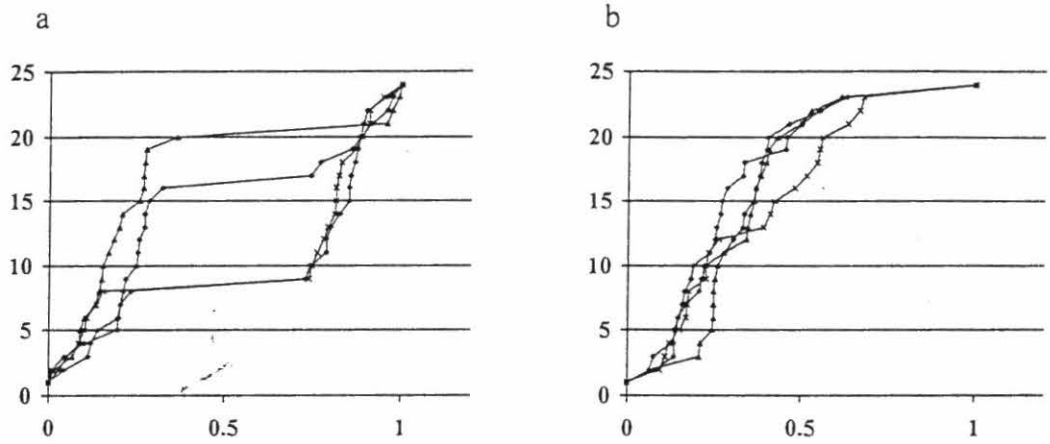

Figure 1: Examples of signal ratio distribution of log2 transformed hybridization data among 6 potato genotypes. a Distribution of relative log2 cy3/cy5 of four polymorphic clones that show bimodal distribution across slides. B Non polymorphic clones show a unimodal distribution.

Conclusions

The complexity of potato genome combined with cross-hybridization among high similar sequences makes it difficult to obtain high polymorphic genome libraries. Double digestion helps to reduce potato genome complexity and allows to find polymorphic clones into potato genome libraries. The use of selective nucleotide for PCR amplification is a good strategy to find polymorphic clones on a library that is hidden by cross hybridization.

Ongoing work

Continue to develop a program to analysis polymorphism among potato genotypes when diversity arrays are hybridized with a representation of a genotype, cy3 labeled, and the cloning vector, cy5 labeled.

We are evaluating ninety-six potato genotypes polymorphism with polymorphic clones of library 5.

References

Posso Duina, E. Gaitán, M. Ghislain, J. Thome. Diversity arrays Technology (DArT) for potao diversity analysis. In In CIAT (Centro Internacional de Agricultura Tropical). Assessment and utilizing agrobiodiversity through biotechnology. Annual repport 2003. Project SB-2 Cali, Co. pp209- 210.

Edwige Isidore, van Os,b H., Andrzejewskic S., Bakkerd J., Barrenae I., Bryana G. J., Caromelc B., van Eckb H., Ghareeb B., de Jong W., van Koert P., Lefebvrec V., Milbourne D., Rittere E., Rouppe van der Voort J., Rousselle-Bourgeoisc F., van Vlietb J. and Waugha R. Genetics. 2003. Toward a Marker-Dense Meiotic Map of the Potato Genome: Lessons From Linkage Group I. Vol. 165, 2107-2116

Jaccoud, D., K. Peng, D. Feinstein & A. Kilian. 2001. Diversity Arrays: a solide state tecnhology for sequence information. Nucleic Acids Resarch 29.

Lezar Sabine, A.A. Myburg, D.K. Berg, M.J. Wingfield and B. D. Wingfield. 2004. Development and assessment of microarray-based DNA fingerprinting in *Eucalyptus* grandis. Theoretical and Applied Genetics International Journal of Plant Breeding Research.

Wenzl Peter, J. Carling, D. Kudrna , D. Jaccoud, E. Huttner, A. Kleinhofs and A, Kilian. 2004. Diversity Arrays Technology(DArT) for whole-genome profiling of barley. PNAS vol. 101 no. 26: 9915-9920.

Kane Michael D., Jatkoe T. A., Stumpf C. R., Lu J., Thomas J. D. and Madore S. J. 2000. Assessment of the sensitivity and specificity of nucleotide (50mer) microarrays. Nucleic Acids Resarch 28.

Killian Andrzj, P. Wenzel, C. de Vicente, E. Barrera, A. M. Correa, M. Fregene. (2003). In CIAT (Centro Internacional de Agricultura Tropical). Assessment and utilizing agrobiodiversity through biotechnology. Annual repport 2003. Project SB-2 Cali, Co. pp217- 218.

CONTENT

Activity 1.2 Development of molecular techniques for assessing genetic diversity and mapping useful genes

1.2.1 Isolation of Aluminum Resistant Genes: Using Arabidopsis as a Gene Source

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SB-2 Project, CIAT

Introduction

Soils with aluminum toxicity are one of most restrictive factors of agriculture productivity. High aluminum concentrations affect nearly 30 to 40 % of arable land on the planet. To identify key molecular components in crops for Aluminum (Al) resistance we have been taking two different approaches. The first approach is to tackle biological questions for Al resistance in crops. In this case, Brachiaria, a highly Al resistant plant species, is being used for a source for Al resistant genes for plants. Physiological and biochemical aspects of Al resistance of Brachiaria species have been well studied at CIAT (Wenzel et al., 2001) and molecular mechanisms underlying the physiological and biochemical aspects are under investigation through the use of genomics tools. Another approach is the use of Arabidopsis as a gene source for Al resistance. This approach allows us to access the ample genomic tools available in Arabidopsis, such as T-DNA mutant populations. A few research groups have been working on Al tolerance in Arabidopsis, mainly focusing on QTL analysis for Al resistance. However, there are a very few cases reported of large-scale Arabidopsis mutant screening for Al resistance. This is mainly due to the lack of reliable and easy phenotype screening methods. Here we described development of a medium-throughput mutant screening method using a publicly available T-DNA inserted Arabidopsis population and the current status of the mutant screening for Al resistance.

Materials and Methods

Examination of Al resistance in different Arabidopsis ecotypes: Seeds of Arabidopsis Columbia (Col), Landsberg-erecta, RLD and C-24 ecotypes were sterilized and sowed on slides frames (Kodak Ref: 3346) that were immersed in magenta boxes (Magenta, Ref: G-7). About 30 seeds were sowed per slide in the nutrient solution (pH 4.5) reported by Hoekenga & Vision (2003) for QTL analysis. Five different Al concentrations ($AlCl_3$ 0 μM, 5 μM, 10 μM, 20 μM and 50 μM) were evaluated. After 10 days of aluminum treatment, primary root length of the seedlings was measured as a phenotypic response to Al toxicity. The experiments were repeated at least three times to obtain statistical values.
Development of a medium-throughput screening for Arabidopsis mutants involved in Al tolerance: A pooled T-DNA population was obtained from the Arabidopsis Biological Resource Center (ABRC) at the Ohio State University. These T-DNA lines were generated

by vacuum infiltration of Col plants with *Agrobacterium tumefaciens* by the Ecker laboratory at Salk Institute (http://signal.salk.edu/tabout.html). These pools of T2 seeds were grown at ABRC to generate the distributed, T3 seed pools. The sterilized transgenic seeds were germinated and grown for 7 days on MS plates, and then seedlings were transplanted to special glass trays that were designed for phenotypic evaluation for Al tolerance as shown in Figure 1. The trays used for Al treatment contained a double solid/liquid medium, where the solid part was used as support for the aerial part of the seedlings, while in the liquid phase kept the roots immersed in the nutrient solution with a 20 μM aluminum treatment. After 7 days of Al treatment all seedlings with abnormal root length (30% shorter or longer than control plants) were selected as putative mutant and were transferred to MS plates with 50 μg/μl kanamycin for recovery and growth for 2 days. Putative mutants were then transplanted to soil to set seeds to be used for the second screening.

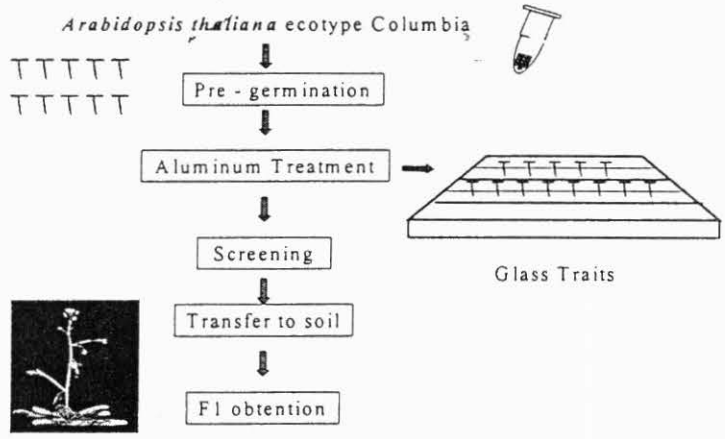

Figure 1. Mutant screening procedure in Arabidopsis for Al tolerance

Results and Discusión

Examination of Al resistance in different Arabidopsis ecotypes: We tested four Arabidopsis ecotypes, Col, Landsberg, C-24 and RLD for Al tolerance by measuring growth inhibition of the primary root at different levels of Al toxicity. Figure 2 shows the dosage dependency of root growth inhibition in Arabidopsis Col and Landsberg ecotypes. Col plants showed less root growth inhibition than the Landsberg up to 20 μM $AlCl_3$. At 50 μM $AlCl_3$ both ecotypes showed severe root growth inhibition (>60%). Therefore, the concentration of 20 μM $AlCl_3$ was used for further analysis.

Figure 3 shows root elongation inhibition of the four ecotypes with 20 μM $AlCl_3$ treatment. The result clearly revealed that the Col ecotype was the most resistant phenotype, as has been reported elsewhere (Hoekenga and Vision, 2003). Ecotypes of Landsberg erecta, C-24 and RLD showed 30-45 % root growth inhibition when compared to growth without Al toxicity. This suggested that at this level of Al toxicity (20 μM $AlCl_3$) Col ecotype plants express resistant mechanism(s) for Al toxicity, while other ecotypes fail to cope with the Al toxicity. Therefore, the distinct Al resistance was revealed among the ecotypes.

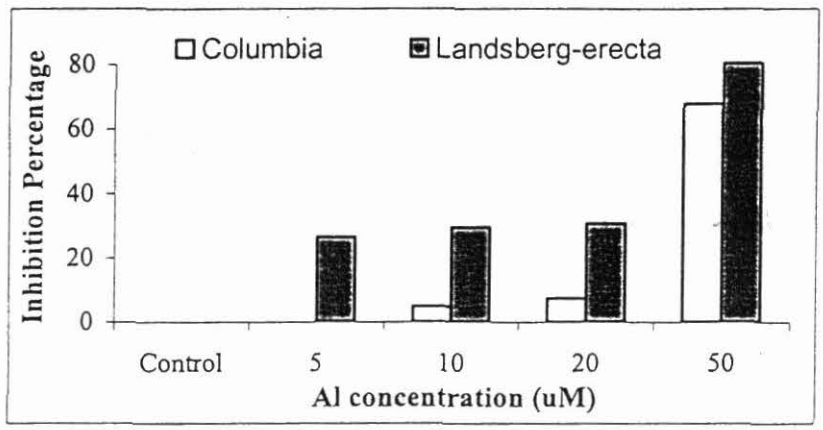

Figure 2. Root elongation inhibition of Colombia and Landsberg ecotypes in different Al toxicity. After 10 days of aluminum treatment using different concentrations, primary root length was measured and compared with that in "control (0 μM)" experiment.

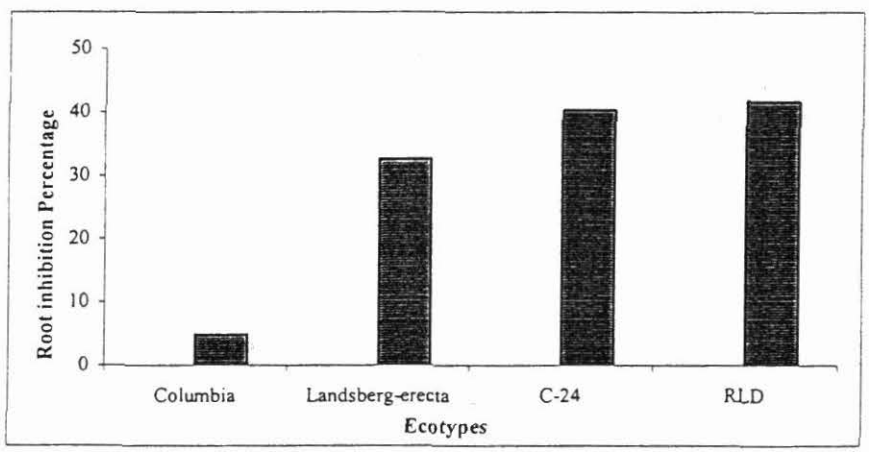

Figure 3. Root elongation inhibition of Arabidopsis Colombia, Landsberg, C-24 and RLD ecotypes under 20 μM $AlCl_3$. The percentage of root elongation inhibition was obtained in comparison with root length under the control condition without Al.

Current status of a medium-throughput screening for Arabidopsis mutants involved in Al tolerance: So far, 29 pools (a total of 2,900 individual lines) of T-DNA have been subjected to the mutant screening and 175 putative Arabidopsis mutants have been isolated. Although it is suggested that multi-genetic components are involved in Al tolerance in Arabidopsis the number of the putative mutants was unexpectedly high. This could be due in part to mutation(s) in molecular components for root growth development itself. The second screening using the offspring of the putative mutants is currently underway.

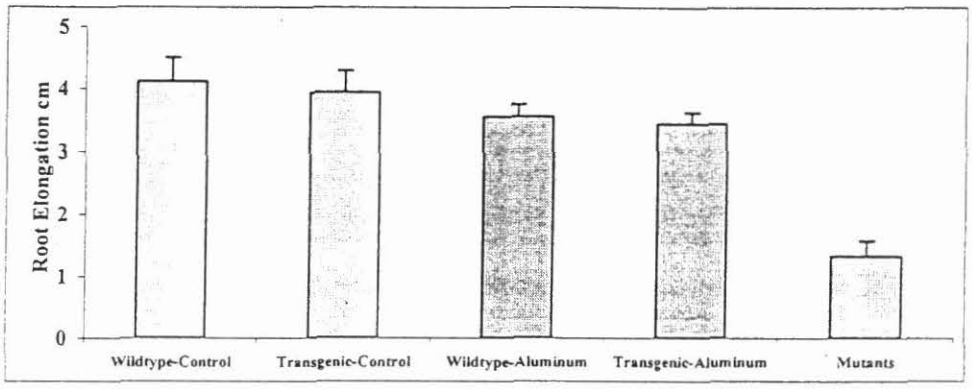

Figure 4. Primary root growth of T-DNA lines with and without Al toxicity using developed screening system. The root growth of 6 putative mutants was shown as "mutants". Wild type-control refers to Col ecotype without Al toxicity. Transgenic-control indicates T-DNA lines without Al toxicity.

Future Plans

- Screening process will be replicated three times to confirm the mutant phenotypes for Al tolerance
- Isolate molecular components that cause the mutant phenotypes
- Apply the identified genes for Al tolerance for crop improvement

References

Feng-Ma, J., Ryan, P., and Delhaize, E. (2001) Aluminum tolerance in plants and the complex role of organic acids. Trends in Plant Science. 6, 273-278.

Hoekenga, O. and Vision, D. (2003) Identification and characterization of aluminum tolerance loci in *Arabidopsis* (*Landsberg erecta* x *Columbia*) by quantitative trait loci mapping. a physiologically simple but genetically complex trait. Plant Physiol. 132, 936-948.

Liu, Y-G., and Whittier, R., (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25, 674-681.

Wenzl, P., Patino, G.M., Chaves, A.L., Mayer, J.E. and Rao, I.M. (2001) The high level of aluminum resistance in signalgrass is not associated with known mechanisms of external aluminum detoxification in root apices. Plant Physiol. 125, 1473-84.

1.2.2 Drought Tolerance and Gene Expression Analysis Using Conserved Water Stress Responsive Gene Sequences Toward Crop Improvement: A Case Study for *DREB* Gene in Common Bean (*Phaseolus vulgaris* L)

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Introduction

This project was initiated last year with the following objectives: 1) isolation of the genes involved in response to water stress, 2) evaluation of gene expression in relation to phenotypic traits (e.g. deeper root) and 3) development of useful molecular markers to select genotypes with better performance under water stress conditions. We selected with the Drought Responsive Element Binding (DREB) gene as an example to understand how stress-inducible genes are involved in drought tolerance in *Phaseolus vulgaris*. The isolated alleles showing correlation to drought-resistance can be used to develop molecular markers.

Materials and Methods

Isolation and characterization of DREB genes: AP2/EREBP domain from *DREB* genes of several dicots was aligned to design degenerate primers from the flanking specific DREB boxes. Resulting isolated sequences from bean were compared to sequences from Genbank to select DREB like AP2/EREBP region. In this region, specific nested primers were designed to isolate the upstream and downstream regions from the AP2/EREBP domain, using a chromosome walk approach. The different analyses for alignment, primer design and putative motif search for the genes were performed using BLAST, VectorNTI 9.0, Pfam, PDBsum, Prosite and jaMBW.

Primer design for gene expression analysis: A total of about 150 genes related to metabolic pathways and response to abiotic stress were selected from databases (Genbank and Soybase) to design primers for Real-Time PCR assays. The genes were divided into 14 functional categories using the MIPS database and primers were designed using the consensus sequences from aligned genes of several dicots and monocots. Primers from the specific isolated *DREB* genes from common bean were also designed for this analysis.

Drought stress treatments: Before examination of gene expression from field-grown plant materials we decided to investigate stress induction of the genes in bean under drought-shock stress, as most of the genes were found from drought-shock treatments. Five bean genotypes (BAT477, DOR364, G21212, BAT881 and G19833) with varying drought-tolerance or susceptibility characteristics were exposed to drought using a drought shock treatment (0, 3, 6 and 12 hours), and to low temperature (4°C for 0, 6, 12, 24 hours). After

the treatment, the aerial and root sections were separated, frozen in liquid nitrogen and placed at -80°C. RNA extraction was performed using a SV Total RNA isolation kit from Promega under manufacturer specifications. Single stranded cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen).

Real-Time PCR assays: Real-Time PCR reactions were performed according to manufacturer specifications with a Brilliant SYBRGreen QRT-PCR Master Mix kit, 1-step from Stratagene in a DNA Engine Opticon® 2 System (MJ Research). PCR efficiencies were calculated using the LinRegPCR software and the relative expression was calculated using the formula: $NE = (E_{target})^{CT\text{-}target} / (E_{ref})^{CT\text{-}ref}$, where NE is the normalized gene expression, E_{target} is the PCR efficiency of the target gene, E_{ref} is the PCR efficiency of the reference gene, $^{CT\text{-}target}$ is the threshold CT value of the target gene, and $^{CT\text{-}ref}$ is the threshold CT value of the reference gene. *PvDREB3* was used as a target gene and ribosomal 18s gene was used as a reference. A standard curve using a cloned ribosomal 18s fragment was used to establish CT values.

Results and Discussion

Isolation of full-length DREB genes from bean and its molecular characterization
Full length *PvDREB2* and *PvDREB3* were isolated with ORFs of 224 and 248 amino acids. Sequence comparisons indicate that these genes have a high sequence similarity to DREB genes of *C. annuum* and *P. avium,* respectively. Both genes have complete AP2/EREBP domains with three beta chains and one alpha helix that are necessary for specific binding to DNA, demonstrating their putative functions as transcription factors (Okamuro et al., 1997; Hao et al., 2002). The previously characterized DREB specific boxes (Jaglo et al., 2001) were found in the flanking regions of the AP2/EREBP domain (Figure 1 – 5'and 3' signals). *PvDREB2* and *PvDREB3* only share 42% identity at the amino acid level further indicating that they are two different genes from the *DREB* gene family. Most of their variability is due to their variable region located upstream and downstream of the AP2/EREBP domain (Figure 1 – 5'var and 3'var). A Nuclear Localization Signal (NLS) was also found upstream from the AP2/EREBP in both genes. In addition, the downstream region of the AP2/EREBP domain has isoelectric points of 3.9 and 3.8, which are typical of acidic domains. The acidic domains in the transcription factors are good candidates for the transcriptional activator function (Stockinger et al., 1999). We also found that several kinase binding motifs were located in the AP2/EREBP domain, which could be important in stabilization, translocation and/or activation of the genes due to the modifications imparted by the transduction signals in response to stress (Tello et al., 1998; Stockinger et al., 1999; Medina et al., 1999). The detailed characteristics of *PvDREB* genes are shown in Figure 1.

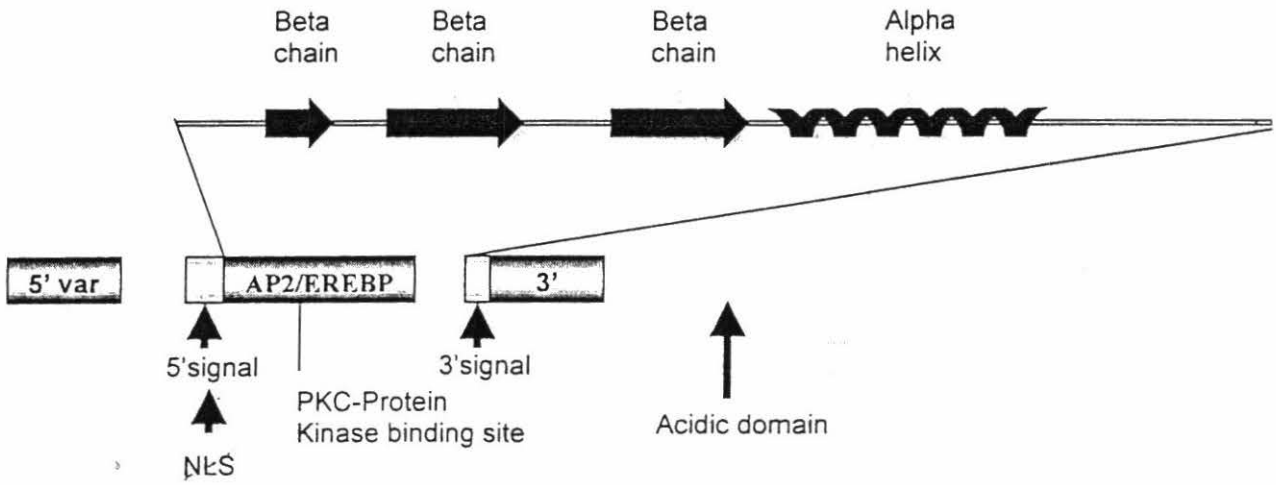

Figure 1. The characteristic motifs and domains of *PvDREB* genes

(2) *Gene expression analysis using Real-Time PCR*

Under drought shock treatment in BAT477, *PvDREB3* gene showed no significant expression at 0 hour and increased expression until 6 hours, followed by a decrease at 12 hours (Figure 2). This indicated *PvDREB3* is not a pseudo gene and is induced by drought stress.

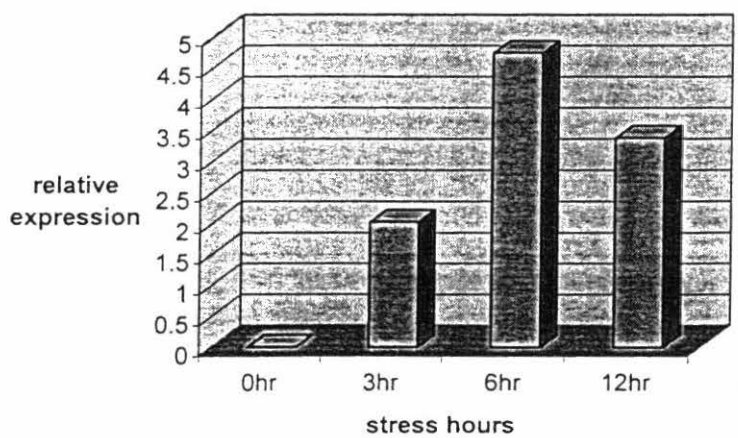

Figure 2. Relative expression of *PvDREB3* under drought shock in BAT477

The expression pattern of the *DREB* gene was similar to the expression demonstrated in *Arabidopsis thaliana, Brassica napus, Secale cereale, Triticum aesvestium* and *Lycopersicon esculentum*, although stress conditions were different in each case (Jaglo et al., 2001). This indicates that *PvDREB3* could act as a master switch to activate downstream genes involved in plant stress responses (Kasuga et al., 1999) as has been suggested in other plants, but further analysis with downstream-related genes is required.

(3) *Functional analysis of the DREB gene from common bean*

It has been demonstrated that the *DREB* gene binds to DRE element in the regulatory region of the target genes in different plants using the one-yeast hybrid system (Kasuga et al., 1999). To examine functionality of the *PvDREB* gene as a transcription factor the we are using the same system to measure its DNA binding activity *in vivo*. This work is currently conducted in the collaboration with Dr. Yamaguchi-Shinozaki at JIRCAS, Japan.

Future Plans

Evaluate *PvDREB* genes, and stress and non-stress responsive genes with Real-Time PCR under controlled (e.g. drought shock) and field-stress conditions (e.g. greenhouse condition that induces phenotypic trait seen in the field and in the field) to examine their stress response.

Investigate correlation of gene expression with stress resistance in the different varieties as a source of molecular markers.

Mapping genes of interest in the bean genome

References

Hao D., Yamasaki K., Sarai A. and Ohme-Takagi M. (2002). Biochemistry. 41, 4202-4208.

Jaglo K., Kleff S., Amundsen K., Zhang X., Haake V., Zhang J., Deits T. and Tomashow M. (2001). Plant Physiology. 127, 910-917.

Kasuga, M., Liu Q., Miura, S., Yamaguchi- Shinozaki, K and Shinozaki, K. (1999). Nature Biotechnology 17: 287-291.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi -Shinozaki, K. and Shinozaki, K. (1998) Plant Cell 10: 1391-1406.

Medina J., Bargues M., Terol J., Pérez M. and Salinas J. (1999) Plant Physiology. 119,463-469.

Okamuro J., Caster B., Villarroel R., Van Montagu M. and Jofuko D. (1997) PNAS. 13, 7076-7081.

Shinozaki, K. and Yamaguchi- Shinozaki, K. (1997) Plant Physiol. 115: 327-334.

Stockinger J., Gilmour S. and Tomashow F. (1997) PNAS. 94, 1035-1040.

Tello A., Oellet F. y Sarhan F. (1998) Molecular and General Genetics. 257, 157-166.

1.2.3 Cassava Gene expression profile in response to *Xanthomonas axonopodis* pv. *manihotis* infection using a cDNA microarray

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The first two authors made equal contributions to this work.

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* pv. *manihotis* (*Xam*) is an important disease in Latin America and Africa and yield losses range between 12 and 100%. Cytochemistry and biochemistry of plant defense responses to CBB have been studied (Kpémoua, K. *et al.*, 1996). However, the response of the plant to pathogen attack at the molecular level remains uncharacterized. The deployment of resistant varieties is still the major means of controlling the disease (Verdier et al. 2001). Identification of genes associated with defense responses is one of most critical steps in the elucidation of disease resistance mechanisms in cassava. Identifying and pyramiding different resistance and defense genes should provide stable resistance against CBB and other diseases. We have developed a large collection of expressed sequence tags (EST) that provides an immediate and productive method of gene discovery. We generated a cassava unigene set with 5,700 sequences (Lopez et al. 2004; Annual Report, 2003). The EST collection is also an unvaluable resource to study global gene expression profiles.
In this study we constructed a cassava cDNA microarray based on the EST information previously generated. We investigated gene expression changes in cassava at different time points during a resistant interaction with *Xanthomonas axonopodis* pv. *manihotis*. Our study provides new insights into the molecular nature of cassava defense mechanisms in response to pathogen attack.

Materials and methods

Plant material and plant inoculation. Cultivar MBra685 was used in the microarray and QRT-PCR experiments. We also used MCol1522 in the QRT-PCR experiments. For this, young plants (4-week old plants) were inoculated by stem puncture with *Xam* strain CIO 151. Stem tissues were collected at 12, 24 and 48 hours post inoculation (pi), and 7 and 15 days pi.
cDNA microarray construction. Cassava unigene set containing 5700 sequences were amplified by PCR and printed on glass slides. Additionally, several control clones from

tomato, potato and cassava housekeeping genes, human genes and spiked controls were included.

Probe preparation and hybridization. Total RNA was extracted from both healthy and inoculated tissues at 12, 24 and 48 hours pi and 7, 15 days pi using the SV total RNA isolation Kit (Promega, Madison, WI). cDNA was synthesized using the SMARTTM PCR cDNA synthesis kit (Clontech, CA). At each time point a comparison was made between cDNA obtained from healthy and inoculated plants (0-12 hpi; 0-24 hpi; 0-48 hpi; 0-7 dpi and 0-15 dpi). Three different biological and technical replicates were used. Fluorescent-labeled probes were prepared using Klenow labelling (for indirect labelling).

cDNA microarray analysis. Spot intensities from scanned slides were quantified using the ArrayPro 4.0. software (BIO-RAD). Output data files (.xls) were used to perform lowess intensity normalization, standard deviation regularization and low intensity filtering using the MIDAS computer program. Normalization between different slides was conducted by centering. Replicate analysis and dye-swap filtering were performed using R language. Using a bootstrap analyses with SAM we determinate the differentially expressed genes (Tusher et al. 2001). K-means clustering analysis was conducted to group ESTs using MeV software.

Quantitative RT-PCR. Fifteen genes that were differentially expressed at different time points over infection were selected for confirmation by QRT-PCR. Additionally, and based on the results obtained, we selected a subset of eight genes that were also tested in a compatible reaction (cultivar MCol1522 infected by *Xam* CIO151).

Results and Discussion

Generation of a cassava cDNA microarray. The microarray was based on a large collection of cassava ESTs, a more complete description of which is given in Lopez et al. (2004). Briefly, the initial data set of 11,954 sequences was reduced to 5,700 unique consensus sequences (unigenes) comprising 1,875 contigs and 3,825 singletons.

Gene expression changes. A total of 199 genes were found to be differentially expressed (126 up-regulated and 73 downregulated). Several up or down-regulated genes were detected at different time points whereas others were regulated at specific times during *Xam* infection. The proportion of differentially expressed genes was relatively low and constant over a period of 48 hours pi (12, 15 and 10 genes at 12, 24 and 48 hours pi, respectively) but increases considerably at 7 days pi (111 genes) before decreasing at 15 days pi (51 genes). This profile is observed for both up and down regulated genes (Fig 1). Of the 199 genes, 155 showed similarity to known proteins while 44 (22%) had no significant similarity to sequences in the public database. Among the 155 genes, 124 showed similarity to proteins of known function and 31 to proteins of unknown function. Some encode proteins previously reported as involved in resistance mechanisms

Cluster analysis of microarray data. A k-means clustering analysis fixing seven clusters was performed in order to gain an overview of the behavior of each gene relative to the others during the course of the infection. Clusters A, B and C are formed by up-regulated genes. In contrast to these clusters, the genes in Clusters D, E and F were down-regulated. Finally, cluster G is formed of genes that showed no significant variation in expression at different time points during infection. Figure 2 show cluster A.

Validation by Quantitative RT-PCR. In all cases except one the differential expression was confirmed. The pattern of gene expression (up or downregulation) was conserved for all genes using the two methods. The direction and degree of differential expression was thus confirmed, although the ratios determined by QRT-PCR were higher than those obtained by microarray analyses.

In this study we have exploited the microarray to evaluate cassava defense responses to *Xam*, although it obviously has much wider applications in the study of cassava gene expression as, for example, changes in gene expression during the post-harvest deterioration or during tuberisation process. The microarray analyses described in this work show that genes involved in the defense mechanisms in cassava are very similar to those governed by R-gene mediated resistance, including oxidative burst, protein degradation, deregulation of photosynthesis and expression of PR genes.

Conclusion

Our study shows that the cassava defense responses to *Xam* infection involve a network that act at different levels and is controlled at different time points during the pathogen infection. Some of the results obtained by microarray analyses confirmed at the molecular level the results observed by a cytochemical approach. We have also identified other mechanisms not described previously in cassava but conserved in other plant-pathogen interactions. This work constitutes an important contribution towards the biology of a neglected plant and a better understanding of the plant-pathogen interaction. This information should help in the development of strategies for managing bacterial blight infection in cassava.

Ongoing Activities

Mapping several ESTs that were identified as differentially expressed in the microarray analysis.

Future Plans

Functional validation of these genes using a reverse genetic approach (VIGS) that has been adapted to cassava (Fofana et al. 2004).

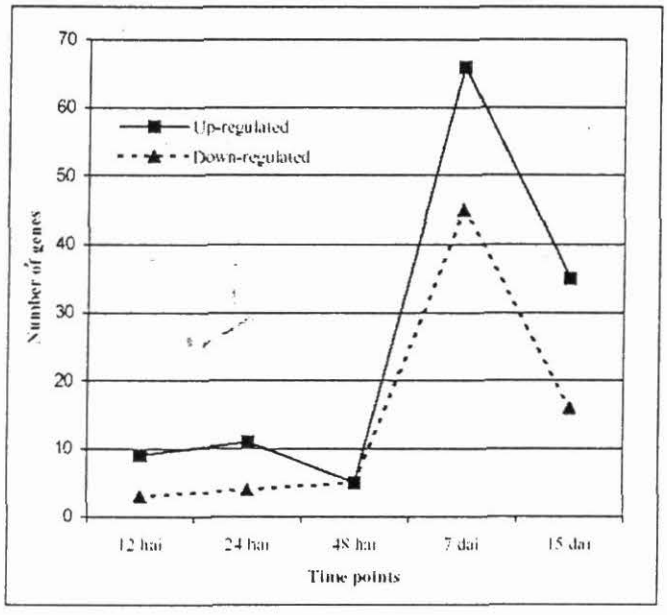

Fig. 1. Number of genes up and down regulated showing differential expression at different time points over *Xam* infection.

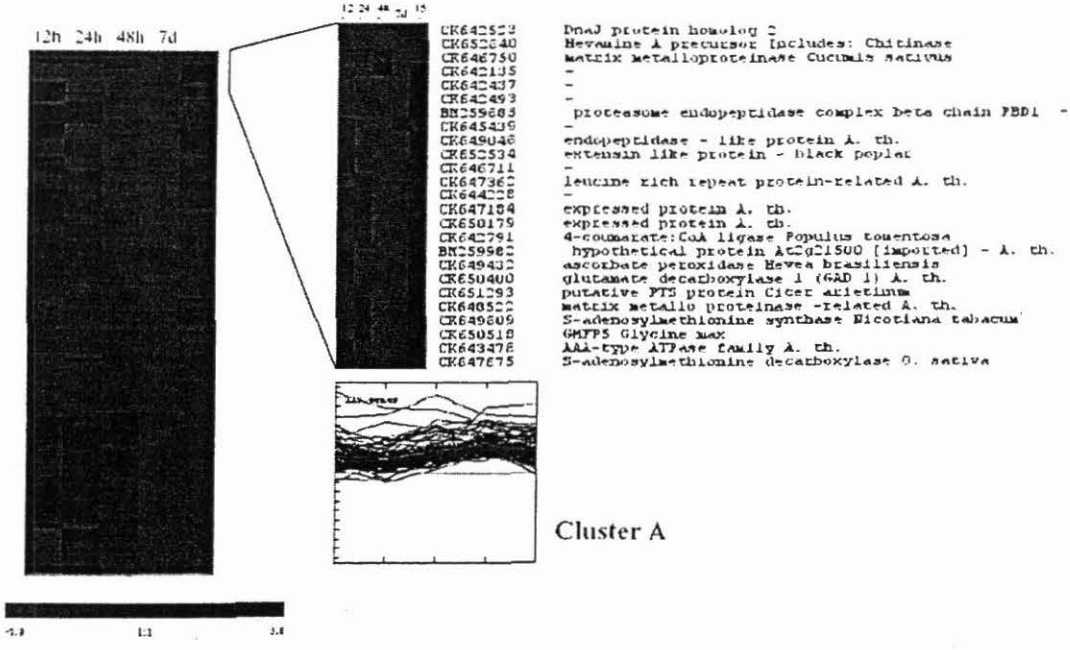

Fig. 2. Clustering analysis showing the behavior of each gene relative to the others during the course of the infection.

References

Verdier, V., Ojeda, S., and Mosquera, G. 2001. Methods for detecting the cassava bacterial blight pathogen: A practical approach for managing the disease. Euphytica 120:103-107.

Kpemoua, K., Boher, B., Nicole, M., Calatayud, P., and Geiger, J. P. 1996. Cytochemistry of defense responses in cassava infected by *Xanthomonas campestris* pv. *manihotis*. Can. J. Microbiol. 42:1131-1143.

Fofana, I. B. F., Sangaré, A., Collier, R., Taylor, Ch., and Fauquet, C. M. 2004. A Geminivirus-Induced Gene Silencing System for Gene Function Validation in Cassava. Plant Mol. Biol. "In press".

Lee, S., Kim, S. Y., Chung, E., Joung, Y. H., Pai, H. S., Hur, C. G., and Choi, D. 2004. EST and microarray analyses of pathogen-responsive genes in hot pepper (*Capsicum annuum* L.) non-host resistance against soybean pustule pathogen (*Xanthomonas axonopodis* pv. *glycines*). Funct. Integr. Genomics "In press".

Mysore, K. S., D' Ascenzo, M. D., He, X., and Martin, G. B. 2003. Overexpression of the disease resistance gene Pto in tomato induces gene expression changes similar to immune responses in human and fruitfly. Plant Physiol. 132:1901-1912.

1.2.4 A unigene catalogue of 5,700 expressed genes in cassava (*Manihot esculenta*): identification of genes implicated in cassava bacterial blight resistance and starch biosynthesis

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Introduction

Cassava (*Manihot esculenta* subsp. *esculenta* Crantz), is the fourth most important starchy staple food crop after rice, wheat and maize with annual production figures of 33 and 52 million tons for South America and Asia respectively, compared with 99 million tons for Africa (FAO, http://www.fao.org/). Cassava Bacterial Blight (CBB), caused by the pathogenic bacterium *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is a major disease for the crop and is endemic in Africa and Latin America. The yield, dry matter content (indirectly starch content) and planting material are severely affected by this disease. It is

necessary to characterize genes that are involved in the downstream signaling cascades in plant defense responses for this crop. This is potentially made easier by considerable progress in the development of genomic and bioinformatics tools that are increasing our knowledge of plant genome structure and organization and gene function.

One tool which permits the unraveling of the complexities of gene expression is the analysis of Expressed Sequence Tags (ESTs). The availability of several EST sequence databases from multiples species makes possible to assign putative functions to the proteins encoded by a large proportion of anonymous cDNA clones. It therefore seemed highly probable that investing in the development of such genomic tools would lead to further elucidation of the cascade of genes involved in the various steps of starch biosynthesis and CBB resistance pathways in cassava. This will ultimately aid the production of new superior cassava varieties targeted for specific market niches.

The objective of this work is to develop a major cassava genomic resource through the generation of a large collection of ESTs that represents a valuable tool for the study of genetic diversity, biological stress resistance and starch production in cassava.

Results

Library construction and sequencing. In all, 15,360 sequences were generated and, after trimming for low quality and vector contamination, 10,280 corresponded to our quality criteria. The overall sequencing success rate, that is useful sequences out of the total sequenced, was approximately 67%. The lengths of good quality sequences varied between 150bp and 700bp with an average of 474 bp. For further analyses we added 848 cassava ESTs available from Genbank and 241 transcript-derived fragments (TDFs) isolated from a cassava cDNA-AFLP analysis and available in our lab (Santaella et al., unpublished data) to the set of 11,954 sequences generated in this study. A total of 13,043 sequences were thus used for subsequent analyses.

Global analyses of the cassava sequences. The initial data set of 13,043 sequences was reduced to 5,700 unique consensus sequences (unigenes) comprising 1,875 contigs (9,218 sequences) and 3,825 singletons. The percentage redundancy for each library varied between 88% (sg_dsc) and 15% (cDNA-AFLP). The contribution of individual libraries to the total unigene set was relatively low (1 to 21%), reflecting the fact that each library contained specific ESTs. This thus demonstrates the great advantage of generating EST from different types of libraries. As for redundancy, the highest level of specificity was obtained from the cDNA-AFLP analysis, making this technique an excellent tool to identify novel genes.

Functional categories. We chose to use the Gene Ontology (GO) scheme (The Gene Ontology Consortium, 2000) to classify our unigene set. For the total unigene set, the GO allowed the placement of 37% of the ESTs into the biological process category and 28% in the molecular function category. The remaining 63% of the unigene ESTs encoded

proteins with insufficient similarity to proteins of know function and were considered as unclassified (Figure 1).

Comparison of the cassava unigene set with other species. To identify cassava-specific genes, the unigene set was screened against the available EST collection from several species (*Arabidopsis*, soybean, tomato, potato, *Medicago*) using TBLASTX to identify ESTs encoding similar proteins. The percentage of cassava sequences that did not shown any similarity to other ESTs varied between 24% and 35%. Overall, predicted protein products of 16% of the cassava unigenes showed no similarity with known proteins of any of these species (Figure 2). This set of sequences was further aligned using BLASTX against the GenBank protein database to identify putative matches, only 6% of this set showed similarity with one or more GenBank entries. The remainder probably represents genes that are unique to cassava or closely-related species.

Cassava multigene families. We studied the gene families identified by our cassava ESTs using a TBLASTX alignment of conceptual amino acid translations of the ESTs against each other. A significant number of sequences (3,866) have no close matches within our data set. However, as the available unigene set only partially represents the cassava transcriptome, the number of single copy sequences is certainly underestimated. As in other plant species, the majority of the cassava gene families identified by sequencing are small, with 2-5 members tagged, although the actual family sizes are probably often greater. However, several very large families are detected, with up to 104 members.

Evaluation of the expression of candidate genes by RT-PCR

To evaluate the transcript abundance of the genes tagged by a selected set of ESTs putatively involved in the defense response, an RT-PCR approach was carried out. We chose genes among the candidates suggested by the *in silico* analysis, seven from families which have previously been implicated in the defense response. Some genes were clearly induced in the plant when challenged with *Xanthomonas*.

Discussion

The cassava EST data presented here is the first effort in large scale sequencing of the expressed genome with the aim of cataloguing cassava genes. Over 13,000 new ESTs, tagging about 5,700 unique genes, have been established, among which 16% show no sequence similarity with known proteins. The unigene contain a redundancy of about 44%. Higher levels of redundancy have been reported, such as 71.7% in soybean (Shoemaker et al, 2002), and 77.5% in tomato (Van der Hoeven et al., 2002), although this may simply reflect the larger numbers of sequences (more than 120,000 EST) from these two species. It is therefore probable that in cassava the redundancy would increase by sequencing more ESTs and alternative strategies would be necessary to tag a large number of new genes.

Using GO terminology we found that the most prevalent categories were metabolism and cell growth and/or maintenance. These categories are also the most frequent in *Arabidopsis* and tomato and may represent a general tendency for all plant species (Van der Hoeven et al., 2002). Though the functional assignment based on sequence similarity requires experimental confirmation, it nevertheless provides a measure of the variety of the genes present in the cassava EST unigene set. Using the biological process class, we obtained at least 20 different categories reflecting the relatively high diversity of sequences. Some of the cassava ESTs showing no similarity with other proteins in the database can be considered as potential cassava-specific genes.

Most of the sequences present in the unigene set were single or low copy genes. Those with the highest copy numbers were the protein kinases, cytochrome P450, a subfamily of transcription factors and ubiquitin. Protein kinases and cytochrome P450s are also the most highly represented in the *Arabidopsis* and tomato genomes. These results seem to confirm the hypothesis that higher-copy gene families are the result of the duplication/diversification events of these families before the divergence of these species (Van der Hoeven et al., 2002).

Temporal expression of a small set of genes was studied by RT-PCR during the infection process in the resistant cultivar. Some genes were clearly induced in the plant when challenged with *Xanthomonas axonopodis* pv. *manihotis* confirming their putative role in plant defense response.

The ESTs generated in this study provide an important tool for understanding the molecular mechanisms involved in two biological processes, resistance to CBB and starch biosynthesis.

Conclusion

In this work we generated 11,954 new cassava ESTs that were assembled into a unigene set of 5,700 sequences and provides new insight in the interaction between cassava and *Xam*. This represents an important contribution to the genomic resource that will permit the beginning of a large scale analysis of expression profiling in cassava, the generation of new molecular markers, the detection of SNPs and other tools that would contribute to a more rapid cassava improvement process as well as ultimately lead to increased cassava production. The information generated will also guide the development of the first cassava microarray for further analysis of gene expression.

Ongoing Activities

Mapping several ESTs that were only present in the cDNA libraries challenged with *Xanthomonas*.

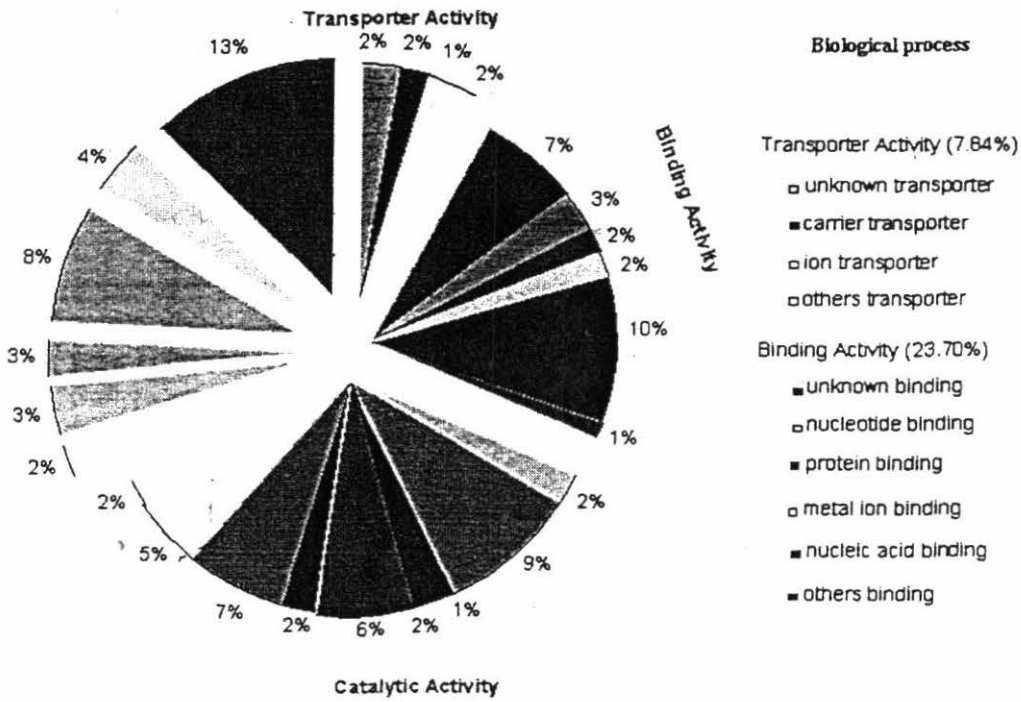

Figure 1. Functional categories were defined using the Gene Ontology (GO) classification scheme

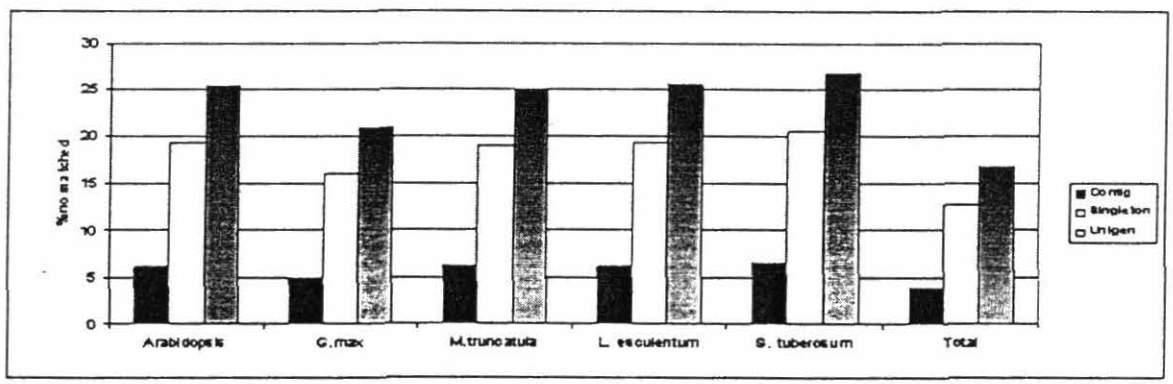

Figure 2. Comparing the cassava unigene set with other plant species

References

Van der Hoeven R, Ronning C, Giovannoni J, Martin G, Tanksley S (2002) Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. Plant Cell 14: 1441-1456.

Shoemaker R, Keim P, Vodkin L, Retzel E, Clifton SW, Waterston R, Smoller D, Coryell V, Khanna A, Erpelding J et al. (2002) A compilation of soybean ESTs: generation and analysis. Genome 45: 329-38.

Ronning CM, Stegalkina SS, Ascenzi RA, Bougri O, Hart AL, Utterbach TR, Vanaken SE, Riedmuller SB, White JA, Cho J, et al. (2003) Comparative analyses of potato expressed sequence tag libraries. Plant Physiol 131: 419-29.

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815

The Gene Ontology Consortium (2000) Gene ontology: tool for the unification of biology. Nat Genet 25: 25-29.

1.2.5 Construction of a TME-3 Bacterial Artificial Chromosome (BAC) Library and Development of a BAC Contig around a CMD Resistance Gene

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1. CIAT, 2. Clemson University Genomic Institute

Funding: CIAT, CUGI

Important output

1) Construction of a bacterial artificial chromosome (BAC) library of cassava from the CMD resistant genotype TME3
2) Construction of 2 BAC contigs around the CMD resistance gene, CMD2, and initiation of chromosome walk

Introduction

High resolution mapping around the cassava genome region bearing a dominant CMD resistance gene, *CMD2*, has lead to the identification of 2 markers, RME1 and NS158 that flank the gene at 1 and 2 cM respectively (Moreno 2004, unpublished data). This year, positional cloning of *CMD2* made progress with the construction of a bacterial artificial chromosome (BAC) library and BAC contigs around *CMD2*. The BAC library construction was carried out at the Clemson University Genome Institute with the participation of CIAT via a graduate student, Ms Isabel Moreno. The BAC library was brought back to CIAT and BAC plate, column and plate pools were created. PCR amplification, using primers of the SSR marker NS158 and the SCAR marker RME1, of the 'BAC Pools' were employed to identify BAC clones containing the markers closest to *CMD2* for BAC contig construction. The BAC contigs will be the start-off point for chromosome walking to the gene of interest.

Methodology

Plant material for BAC library construction was the African local variety TME3 that has resistance to CMD mediated by the dominant gene *CMD2*. Construction of the library was as described earlier by Tomkins et al. (1999a; 1999b; 2004). To estimate the distribution and average size of the clones, a total of 370 clones from the TME3 library were picked at random and grown overnight in 3ml of liquid LB medium + 12.5µg /µl Chlorampenicol. Plasmid DNA was isolated, digested with *Not* I restriction enzymes and inserts separated from the vector by pulsed-field electrophoresis.

Contig construction was by PCR amplification of 'BAC pools', namely 'plate pools' (PP), 'column pools' (CP) and 'row pools' (RP). Briefly, all 192 384-well plates were duplicated using a 384-pin replicator and allowed to grow in LB/ Cloramfenicol (12.5 ug/ml) medium at 37° C over night. For BAC plate pools, all the bacteria culture in a 384-well plate was combined into an omnitray and 200ul of this transferred into a single well in a 96-well plate to yield 2 'BAC pool' plates. Simultaneously, every 10 plates of the library were inoculated into a single 384-well plate using a 384-pin replicator to give 20 384-well plates. Each row of each of the 20 plates was inoculated, using a sterile tooth pick, into a single well containing LB/ Cloramfenicol (12.5 ug/ml) medium to form 'Row plates (RP)' 5 RPs of 96-well plates in all (Fig 1). The same was done for each column of the 20 384-well plates combined into a single well to form 4 96-well 'Column plates (CP)' (Fig 2)

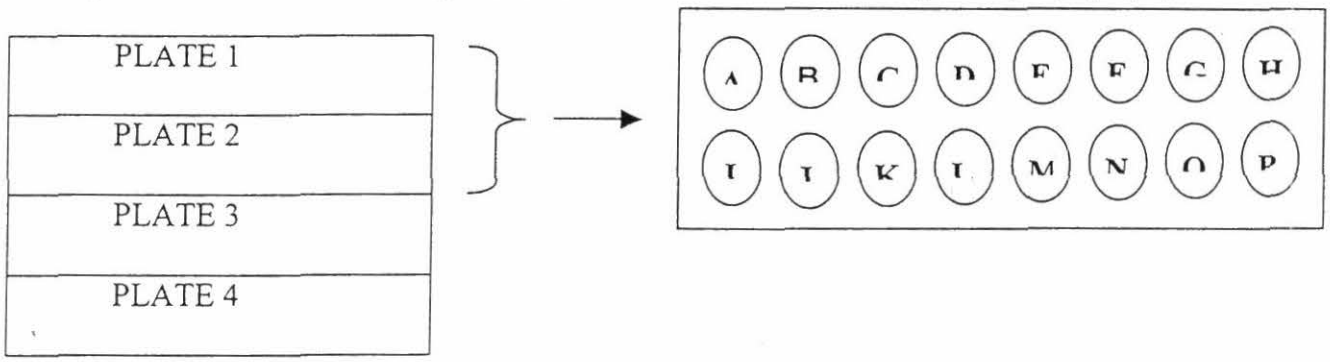

Fig 1. Schematic diagram of how the 'Row plates' were formed, sixteen rows from each of the plates (on the right) were combined into a single well (on the right).

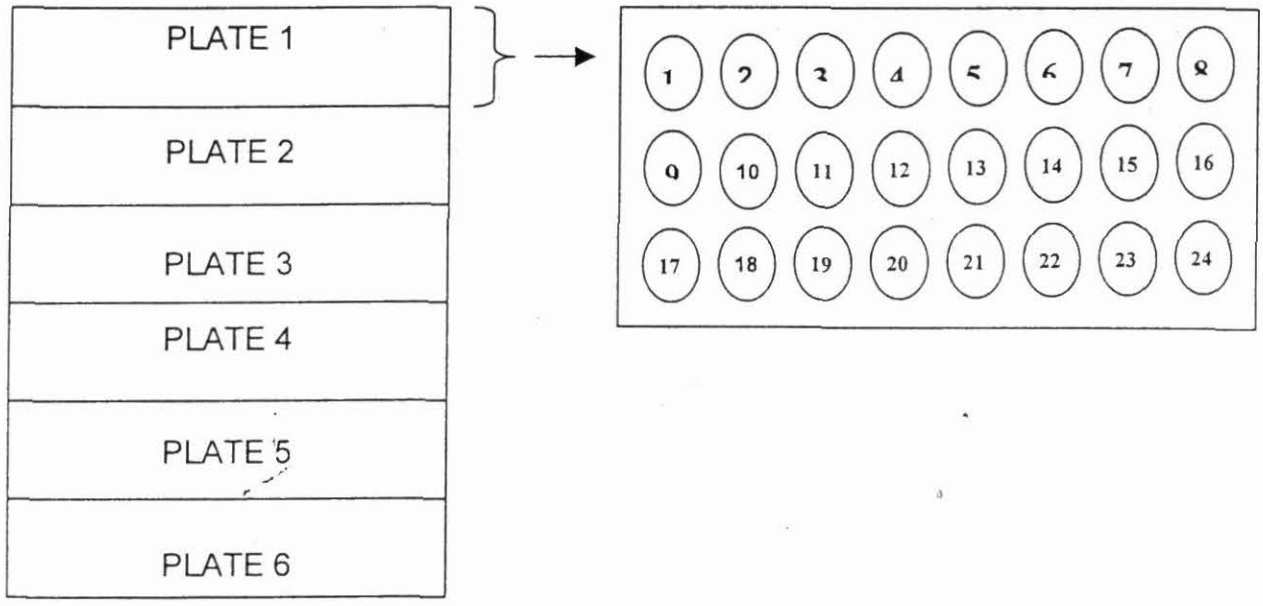

Fig 2. Schematic diagram of how the 'column plates' were formed, 24 column from each of the plates (on the right) were combined into a single well (on the right).

The 4 column and 5 row plates were incubated at 37°C overnight. A total of 11 plates PP, RP and CP were obtained. For PCR amplification, 5ul of the bacteria culture was transferred using a multi-pipette to a clean 96 well plate and the bacteria pelleted at 4500rpm for 10 minutes in a Sorvall centrifuge. The supernatant was discarded and the pellet re-suspended in 5ul of sterile water and used as template for PCR amplification. PCR amplification condition for the SSR marker was 2 mM of $MgCl_2$, 0.2 mM of DNTPs, 0.2 uM of each primer, 1 U of taq-polimerasa, in a final volume of 50ul. Thermal cycle profile was an initial *denaturation step* at 95°C 2 min, 30 cycles 94° 30s, 55°C 1 min, 72°C 1 min, and a final extension step of 72°C for 5 min. For the SCAR marker, $MgCl_2$ and DNTP concentrations were increased to 2.5 mM and 0.4 mM respectively. PCR cycling conditions were: 95°C 2 min, 30 cycles: 94°C 30 seg, 52°C 1 min, 72°C 1 min; and a final extension of 72°C for 5 min. PCR products were visualized in a 1.5% agarose gel stained with ethidium bromide.

Results

The TME3 BAC library is made up of 73 728 clones in 192 384-well plates. Insert size ranged from 20kb to 130kb with an average insert size of 100kb (Fig 3). The BAC library has a 10X coverage of the cassava genome.

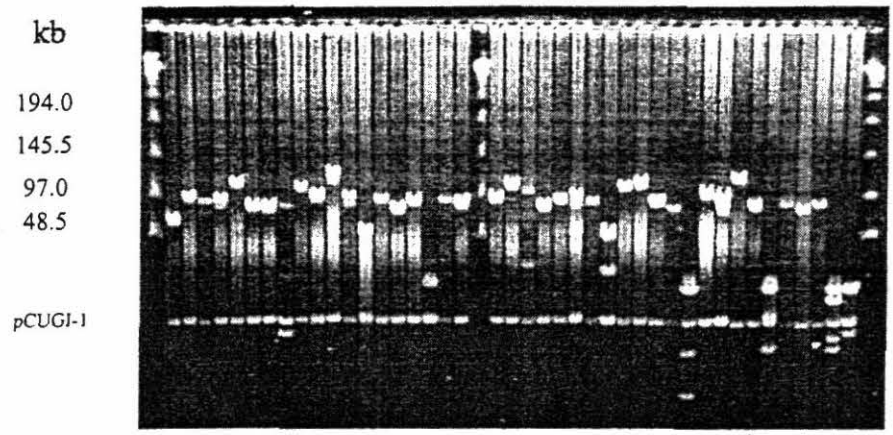

Fig 3. Random BAC clones from the TME3 library digested with *Not 1* and run in a 1% agarose gel in 0.5X TBE at 14°C at 6V/cm with a switch time of 5s-15s pulse time for 14 hours.

Results of screening the 'BAC pools' with NS158 yielded 2 positive clones while screening with RME1 yielded 34 positive clones. NS158 is a single copy SSR marker while RME1 was developed from a multiple copy RAPD marker. The clones were digested with 20U of HindIII overnight and run for 24 hours on 1.2% agarose gel to obtain a BAC clone fingerprint (Fig 4.)

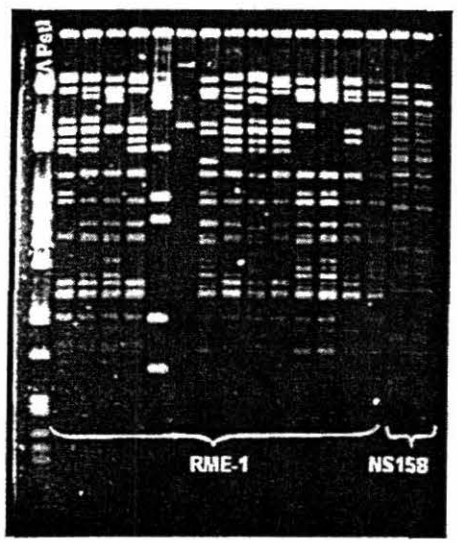

Fig 4. Fingerprinting of positive BAC clones.

The positive clones are listed below (Fig.5)

"PP" ("Pool Plate")	"RP"("Row Plate")	"CP"(Column Plate")
90	N	18
189	M	19

"PP" ("Pool Plate")	"RP"("Row Plate")	"CP"(Column Plate")
12	N	14
17	M	21
34	L	16
47	O	3
51	I	1
52	A	23
54		
85	J	23
130	D	18
136	J	8
139	G	22
155	K	24
173	F	1

Fig 5: List of positive clones from the 'BAC pool' screening using the SSR marker NS158 and the SCAR marker RME1.

Conclusion and Perspectives

A BAC library has been constructed from the CMD resistant genotype TME3. The BAC library was screened with markers closest to the resistance gene CMD 2 and a total of 36 BAC clones obtained. A Hind III digest of the clones yielded a fingerprint which is being used in BAC contig construction. Primers will be designed from the end of the BAC clones that make up the extreme of the contigs and mapped in a set of recombinants from the fine mapping population. The new markers will be used in another round of screening of the BAC library and a 'walk' to the CMD2 inititiated.

References

CIAT. (2003). Annual report Proyect IP3, Improved cassava for the developing world, CIAT, Cali, Colombia, 8.68-8.70

Tanksley, S.D; Ganal, M.W; Martin, G.B. 1995. Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends in Genetics. 11 (2): 63-68.

1.2.6 Isolation of Full-Length cDNA Clones of Transcripts Differentially Expressed Between Full-Sib Genotyped Resistant and Susceptible to the Cassava Mosaic Disease (CMD)

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1. SB-2 Project, CIAT ; 2. IBRC, Iwate, Japan

Funding: CIAT

Important Outputs

1) Dot blot screening of cDNA library constructed from a group of CMD resistant full-sib genotypes for identification of full-length cDNA clones representing SAGE tags
2) Identification of full-length cDNA for 7 SAGE tags

Introduction

Serial analysis of gene expression (SAGE) revealed many genes differentially expressed between CMD resistant and susceptible genotypes from a full-sib family (Fregene et al. 2004). To elucidate the role above genes in host-plant resistance to CMD mediated by the dominant resistance gene CMD2, genetic complementation experiments have been planned in cooperation with the Iwate Biotech Research Center (IBRC), Kitakami, Japan. Full-length cDNA clones are required for these experiments. We describe here screening of a cassava cDNA library of cassava was obtained using as template mRNA from a group of 40 CMD resistant genotypes and short PCR amplification product of the SAGE tags using as probe.

Methodology

cDNA clones from a cDNA library developed from CMD resistant clones were hand picked and arrayed on small sized nylon membrane (Hybond-N Amersham Biosciences) filters placed on solid LB agar media having ampicillin (100mg/ml). The filters were incubated at 37 °C for 16 hours and the lysis of the colonies were done following the protocol described by Sambrook *et al.* (1989). The filters were stored at 4°C until use.

SAGE tags had earlier been amplified from the cDNA library (described above) using the tag sequence as forward primer and a primer from the plasmid vector in which the cDNA was cloned (pYES2, invitrogen) as reverse (CIAT 2001). The PCR amplification product are small in size, 200 and 400bp, because the SAGE tag was obtained from the 5'end of cDNA transcripts. These PCR amplification products were cloned into the pGEMT-easy (Promega) vector. To generate probes for dot blot screening of the cDNA library, plasmid containing the SAGE tags was PCR amplified using SP6 and T7 primers. PCR amplification conditions included 3mM $MgCl_2$, 0.16mM de DNTPs, 0.10 uM of each primer and 1U of taq in final volume of 25ul, using 5ul of a 50X dilution of the plasmid DNA template. The thermal profile program was an initial denaturation step of 94°C for

1minute, 30 cycles of: denaturation 94°C for 30seconds, annealing 50°C for 30 seg, extension 72°C for 2min, follow by a final extension step of 72°C for 5 min. Five microliter of the PCR reaction was used as probe for dot blot hybridization as described by Sambrook *et al.* (1989).

Because only a single set of filters was prepared, the SAGE tags were combined after PCR amplification and only 8 probes were prepared from 25SAGE tags available

Probes for screening dot blots	Combination of SAGE Tags
C1	Tags 2,3,8,9
C2	Tags 12,18,19,21
C3	Tags 25,7,15,16
C4	Tags 17,26,28,1
C5	Tags 4,5,7,13
C6	Tags 14,11,13,20
C7	Tags 4,11,13,14
C8	Tags 15,16,25

The probes were labelled radioactively with P^{32} using the kit Mega prime DNA Labelling System RPN 1605 (Amersham Biosciences) following the instructions of the supplier. Post hybridization washes were 2XSSC, 0.1% SDS for 10 minutes at 65°C and 0.5XSSC, 0.1% SDS for 5 minutes at 65°C. In some cases an additional high strigency wash, 0.1XSSC 0.1% SDS, for 5 minutes at 65°C was added. Filters were exposed to X-ray films for 2-4 days at –80°C with an intensifying screens film. Positive cDNA clones were identified and verified by PCR using T7 and pYES primers and PCR condictions as described above. Sequencing of the cDNA clones was using the big dye cycle sequencing kit (APPLIED BIOSYSTEMS reference # 4303152) following the manufacturer's instructions on a 377 sequencing machine (PERKIN ELMER/APPLIED BIOSYSTEMS). Sequence homology was obtained using BLAST (www.ncbi.nlm.nih.gov).

Results

More than 19,960 cDNA clones were hand picked from a cDNA library developed from CMD resistant clones and arrayed on 52 small sized filters, 96 clones per filter. Dot blot screening yielded over 100 clones (Fig1) of which 66 positive clones were selected for sequencing after PCR amplification to check size of the cDNA clone (Fig 2). Sequence homology revealed that several of the clones had homology to the following genes related to the SAGE tags from which they were obtained homology for the next proteins:

β- Tubulina
Elongación factor Alpha1α

1. Ribosomal protein 40S
2. Histone
3. Hypothetical proteína (A. Thaliana)
4. Rubredoxine

5. Importin Protein

Analysis of the sequence data revealed that close to all the cDNA clones sequenced were full-length or almost full-length clones.

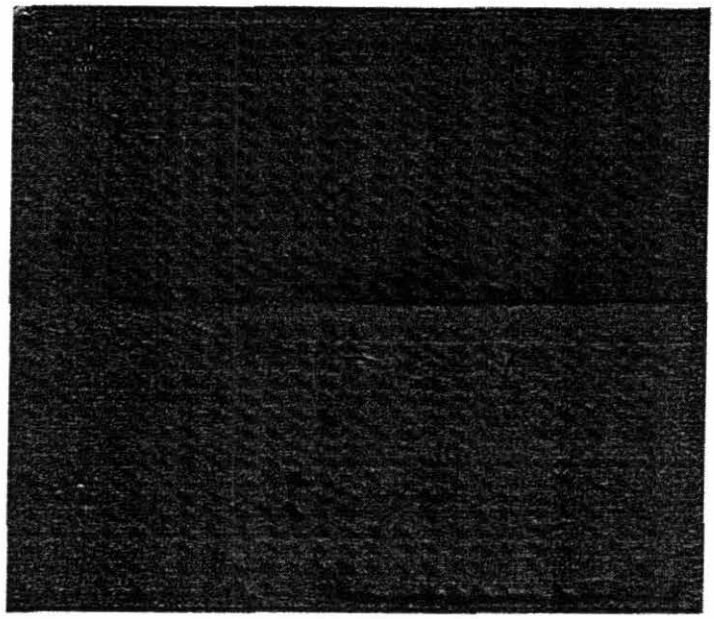

Fig1. Dot blot hybridization of a cDNA library using SAGE Tags as probes, dark spots are putative cDNA clones with homology to the SAGE tag used as probe.

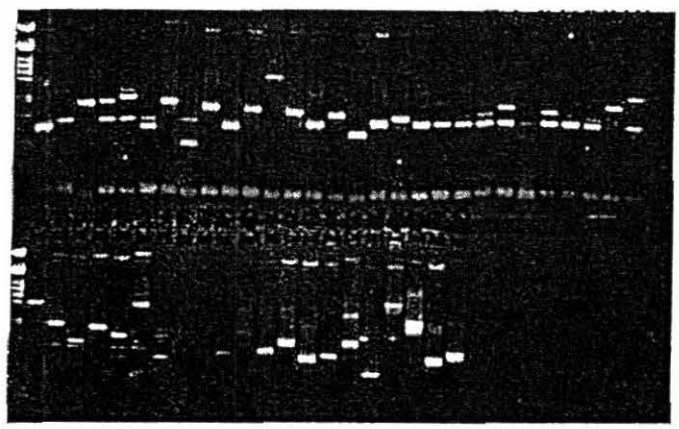

Fig 2. Positive clones from dot blot screening of the TME3 cDNA library amplified with the primers T7 and pYES

The full-length cDNA clones are going to be used in genetic complementation experiment to assess the function of these differentially expressed genes in relation to host-plant resistance to CMD.

Conclusions and Perspective

Dot blot screening of cDNA library constructed from a group of CMD resistant full-sib genotypes has lead to the identification of full-length cDNA clones for 7 SAGE tags. These genes will be used in genetic complementation experiments to determine the relationship of these genes of host-plant resistance to CMD mediated by *CMD2*.

References

CIAT 2001.Annual report SB-2 project. Annotation of SAGE tags (Transcripts) differentially expressed in CMD resistant and susceptible genotypes. CIAT, Cali, Colombia.

Sambrook, J; Frish,E; Maniatus, T. 1989. Lysis of Colonies and Binding of DNA to Nitrocellulose Filters : a Laboratory manual , 2nd edn. Cold Spring Harbor laboratory, Cold Spring Harbor. New York.

1.2.7 Edaphic adaptation of *Brachiaria*

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CIAT, 2. CAMBIA, Australia; 3. Virginia Tech University, USA 4. Hokkaido University, Japan

Previous research on mechanisms of adaptation of *Brachiaria* species to acid soil stress factors indicated that *Brachiaria decumbens* cv. Basilisk is highly resistant to toxic levels of Al and low supply of P. Based on this knowledge, rapid and reliable screening procedure to evaluate Al resistance was developed to improve the efficiency of the on-going *Brachiaria* breeding program. The use of improved screening methods and identification of QTLs and candidate genes responsible for Al resistance and adaptation to low P supply will contribute toward development of superior genotypes that combine several desirable traits to improve pasture productivity on acid, infertile soils and to combat pasture degradation.

Investigating physiological and genetic aspects of aluminum 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*.

1.2.8 Aluminum Resistance Coincides with Differential Resistance to Trivalent Lanthanide Cations in *Brachiaria*

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1. CAMBIA, Australia; 2.CIAT, Cali - Colombia

Introduction

Signalgrass (*Brachiaria decumbens*) has evolved a highly effective Al-resistance mechanism that does not appear to rely on chelation of Al ions with organic-acid anions. Electrical charges at the external surface of root cells generate an electrostatic potential that modulates cell-surface ion activities and hence ion uptake and intoxication. We hypothesized that the superior Al resistance of signalgrass compared to closely related ruzigrass (*B. ruziziensis*) could be due to a less negative surface potential at root cells that are critical to root growth and elongation. We tested this hypothesis by investigating whether Al resistance of signalgrass was associated with superior resistance to other cations toxicants and greater susceptibility to anionic toxicants.

Materials and Methods

Seeds were germinated in continuously aerated 200 μM $CaCl_2$ (pH 4.20) for 3-4 days. Homogeneous seedlings were selected and their root lengths were recorded. The seedlings were then transferred to continuously aerated treatments solutions in the greenhouse (toxicant + 200 μM $CaCl_2$, pH 4.20; see left panel below). After three days root length were measured again. Each experiment comprised six toxicant levels plus the toxicant-free basal solution. Three independent experiments were performed for each toxicant. The concentration of a toxicant that inhibited relative root elongation (RRE) by 50 % (C_{50}) was determined for each of the two species after fitting a Weibull function to the pooled data from the three replicate experiments by using the Marquardt-Levenberg algorithm (right panel above). The SE of C_{50} was computed based on error propagation rules. The C_{50} values of the two species for a particular toxicant were considered to be different if their 95 % confidence intervals did not overlap.

Results and Discussion

The superior Al resistance of signalgrass compared to ruzigrass was associated with greater resistance to all the trivalent lanthanide cations tested (Figure 1a). If a lower root cell surface negativity was the cause for the greater lanthanide resistance of signalgrass, signalgrass should be more resistant to other cationic toxicants and more sensitive to anionic toxicants. The two species, however, were equally sensitive to the majority of divalent and monovalent cations (Figure 1b) and most anions (Figure 1c). Apart from lanthanides and Cd^{2+}, signalgrass was more resistant than ruzigrass only for those inorganic toxicants that are in redox equilibrium with a trivalent cationic form: $Fe^{2+} \leftrightarrow$

Fe^{3+}, $Co^{2+} \leftrightarrow Co^{3+}$, $Cr_2O_7^{2-} \leftrightarrow Cr^{3+}$. An organic trivalent cation (spermidine^{3+}), by contrast, was equally toxic to both species.

These results suggest that Al resistance in signalgrass is part of a more generic resistance mechanism that is effective against trivalent cations in general, a finding that confirms the unique physiological basis of Al resistance in signalgrass.

The pattern of resistance to cationic and anionic toxicants, however, is not consistent with the idea that a less negative root cell surface potential confers resistance to cationic toxicants as a result of electrostatic interactions, that is, solely based on the charge but not the structural properties of a toxicant. The cross-resistance of signalgrass to Al and other, mostly trivalent inorganic cations may instead be based on interspecific differences in critical cellular sites to which trivalent cations such as Al^{3+} and lanthanides bind.

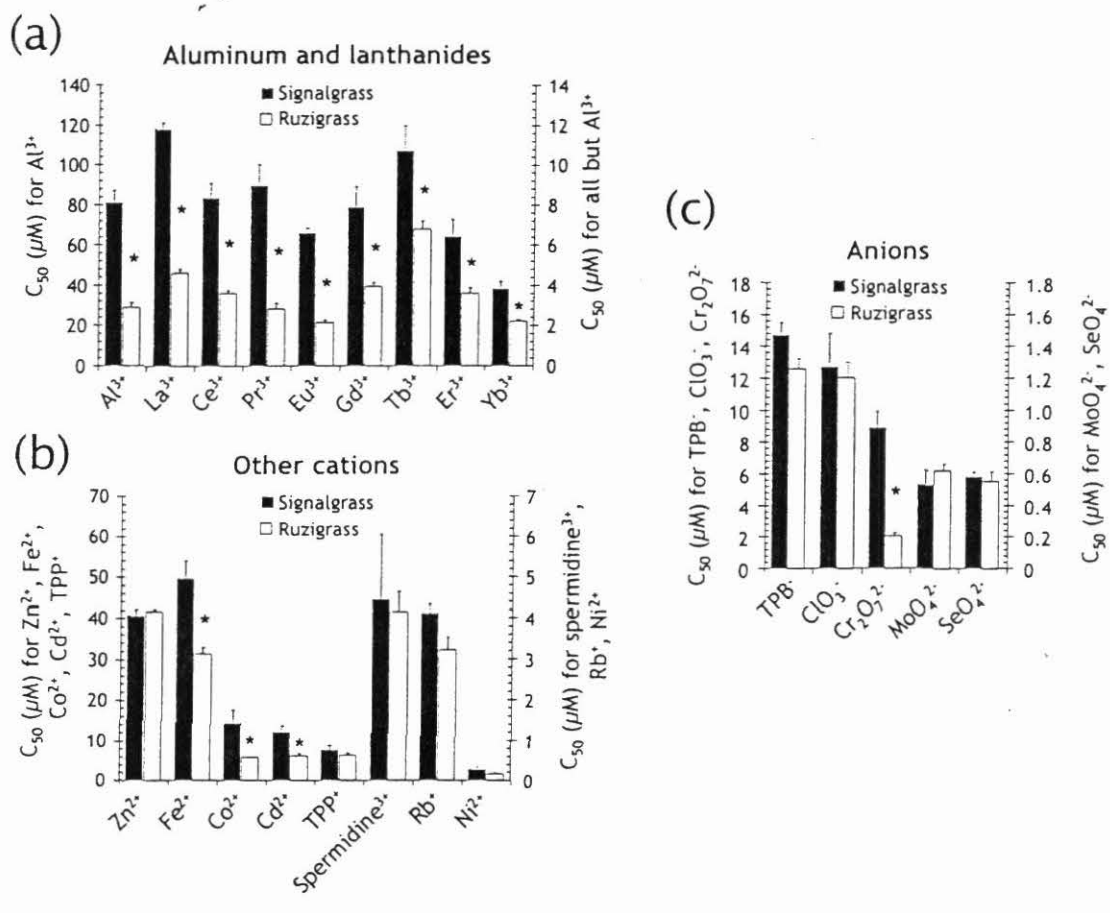

Figure 1. Concentrations of cationic and anionic toxicants required to inhibit root elongation of signalgrass and ruzigrass by 50 % (C_{50}).

More work is required to elucidate the nature of these sites and to develop biochemically-based strategies to isolate the underlying genes. It may be possible to use lanthanide

cations as proxies for Al to circumvent some of the problems and ambiguities caused by the difficulties to predict Al speciation.

1.2.9 Accumulation of callose and aluminum in root tips of *Brachiaria* spp

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Introduction

The effects of aluminum (Al) toxicity on callose accumulation were evaluated in root tips of *Brachiaria* populations previously evaluated for physiological and genetics response (IP-5 Annual report 2002, 2003). *Brachiaria decumbens* (Al resistant), *B. ruziziensis* (Al sensitive) and two contrasting *B. ruziziensis* x *B. decumbens* hybrids were evaluated after 3, 12 and 21 days of Al treatment (200 μM $AlCl_3$).

Materials and Methods

Rooted stem cuttings of *B. decumbens*, *B. ruziziensis* and two contrasting hybrids were cultivated as described previously (IP-5 Annual Report, 2003). Root apices (5 mm), collected after different times of exposure to Al, were fixed during 48 hours in 2.5 % glutaraldehyde to detect callose with aniline blue, or in a 1:1 mixture of 3.7 % phormol (pH 7.4) and 2.5 % glutaraldehyde to detect Al accumulation with hematoxylin. Samples were cut (7 μm) and processed and aluminum was visualized by staining with 0.1% (w/v) hematoxylin and 0.01% (w/v) KIO_3 for 20 min. Callose was visualized by staining with 0.1% (w/v) aniline blue and 1M glycine NaOH (pH 9.5).

Results and Discussion

Differential hematoxylin staining was observed between sensitive and tolerant genotypes. Root apices of *B. decumbens* (Fig. 1A) and an Al-resistant hybrid (Fig. 1C) did not accumulate much Al. By contrast, root apices of *B. ruziziensis* (Fig. 1B) and an Al-sensitive hybrid (Fig. 1D) accumulated Al in the outer layer of root meristems.

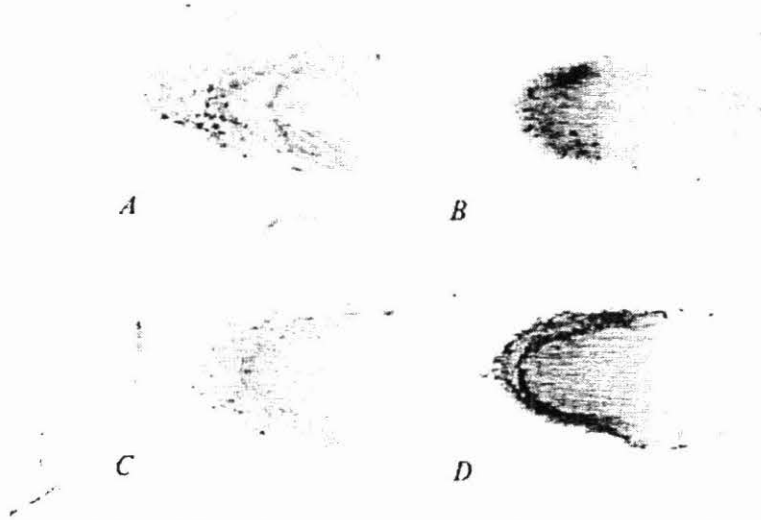

Figure 1.Hematoxylin staining of *Brachiaria*.root apices. A. Al-tolerant parent (*B. decumbens*); B. Al-sensitive parent (*B. ruziziensis*); C. Al-tolerant hybrid; D. Al-sensitive hybrid (12 days of Al treatment).

Aniline-blue staining of histological sections revealed a higher content of callose for Al-sensitive genotypes. The pattern of callose accumulation was only partially correlated with that of Al (visualized by hematoxylin). Al-tolerant *B. decumbens* accumulated callose exclusively in the root cap and at the surface of the root meristem. Al-sensitive *B. ruziziensis* accumulated a large amount of callose in cortical and vascular tissues, an area where little Al was detected in the hematoxylin stain (Fig. 2).

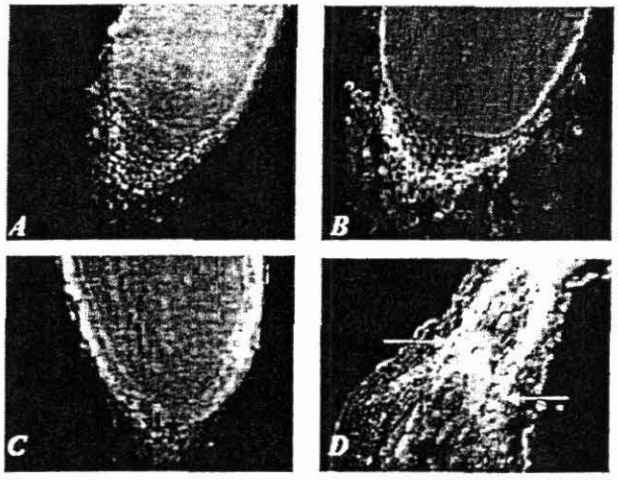

Figure 2. Callose detection by aniline-blue staining in histological sections of *Brachiaria* root apices. A, B. *B. decumbens* (without and with Al). C, D. *B. ruziziensis* (without and with Al). Arrows designate callose deposition (3 days of Al treatment).

Hematoxylin staining could be employed as a quick selection criterion to discard Al-sensitive geotypes in the *Brachiaria* breeding program, because most of the Al

accumulates in the external layer of root meristems and should be readily stainable with hematoxylin in intact apices.

1.2.10 Identification of candidate genes associated with aluminum resistance in *Brachiaria decumbens*

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Introduction

The 3'-UTR sequences of candidate genes associated with aluminum (Al) resistance were identified by comparing expression levels between genotypes and treatments. Subtractive libraries were prepared from root apices of contrasting genotypes grown in the presence and absence of Al: *Brachiaria decumbens* (Al-resistant parent), three Al-resistant *B. ruziziensis* x *B. decumbens* hybrids, *B. ruziziensis* (Al-sensitive parent), and seven Al-sensitive hybrids (Annual Report 2003). Microarray technology was then used to catalogue clones derived from genes that were differentially expressed between samples.

Materials and Methods

Subtractive libraries of 3'UTR fragments were prepared with the differential subtraction chain (DSC) method (IP-5 Annual Report, 2003). Inserts were amplified and arrayed in duplicate on glass slides. Pairs of contrasting RNA populations (control, target) were hybridized to microarrays (Table 1). Two pairs of dye-swap hybridizations were performed per combination of control and target.

Table1. Populations evaluated in conditions of Al toxicity by Microarrays

Combination	Control	Treatment		Target	Treatment
1	Al-sensitive parent + hybrids	$AlCl_3$ (200 µM)	*vs*	Al-resistant parent + hybrids	$AlCl_3$ (200 µM)
2	Al-resistant parent + hybrids	Al (0 µM)	*vs*	Al-resistant parent + hybrids	$AlCl_3$ (200 µM)
3	*B. ruziziensis*	$AlCl_3$ (200 µM)	*vs*	*B. decumbens*	$AlCl_3$ (200 µM)
4	*B. decumbens*	Al (0 µM)	*vs*	*B. decumbens*	$AlCl_3$ (200 µM)

Microarray sample pools (MSPs) were synthesized from 2.4 to 240 ng of cDNA to cover a 100-fold range of signal intensities. They were arrayed together with other controls, such as negative controls (spotting buffer, polylinker of the vector used for library preparation, unrelated genes such as insulin, Sp1 β-cell, HPH) and positive controls (GADPH of *Brachiaria*, α-tubulin, Spy genes). A total of 768 controls were spotted onto the array. The logarithms of the crude ratios between the two channels were first normalized by using the

lowess algorithm and then analyzed with the Significance Analysis of Microarrays (SAM) software.

Differentially expressed clones were amplified, purified (Qiagen kit) and sequenced (ABI BigDye terminator kit). The sequences obtained were compared against those in the UTR database at http://bighost.area.ba.cn.it/BIG/UTRHome.

Results and Discussion

The microarray hybridizations identified a total of 35 3'-UTR fragments of candidate genes that were expressed differentially in the four comparisons between target and control pools (Fig. 1, Table 1).

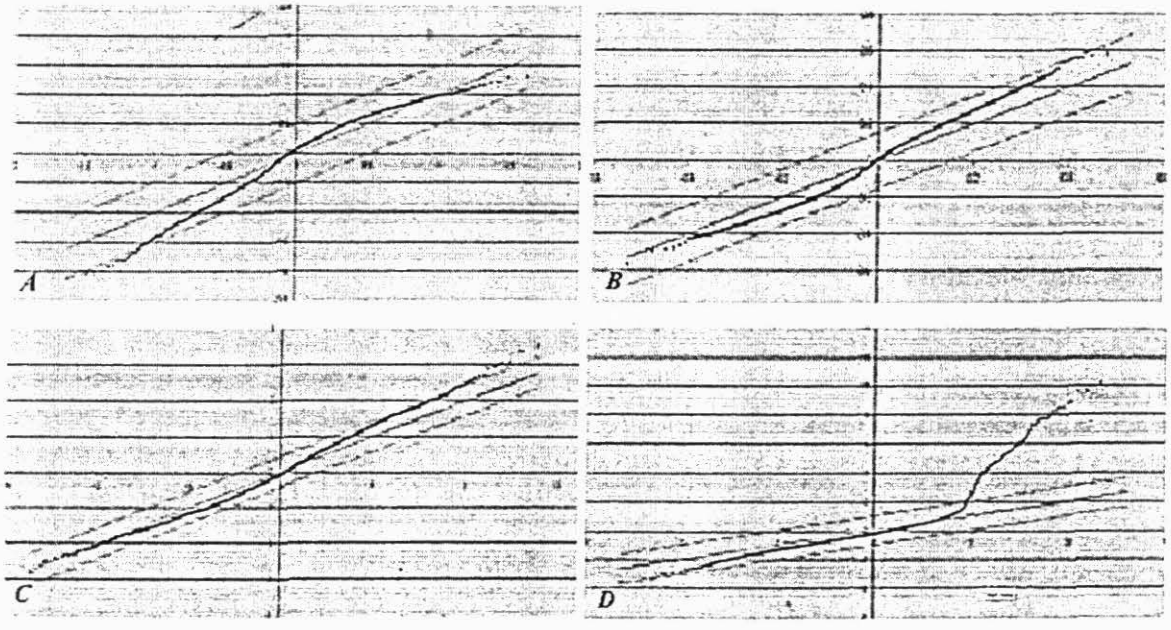

Figure 2. Differentially expressed genes associated with Al resistance (red points). A. Al-sensitive parent + hybrids in Al treatment *vs* Al-resistant parent + hybrids in Al treatment; B. Al-resistant parent + hybrids in control treatment *vs* Al-resistant parent + hybrids in Al treatment; C. Al-sensitive parent (*B. ruziziensis*) in Al treatment *vs.* Al-resistant parent (*B. decumbens*) in Al treatment; D. Al-resistant parent (*B. decumbens*) in control treatment *vs.* Al-resistant parent (*B. decumbens*) in Al treatment.

Seven clones contained the post-transcriptional control sequence 15-LOX-Dice (15-Lypoxigenase Differentiation Control Element), seven clones contained the ribosomal regulatory element IRES (Internal Ribosome Entry Site), and one clone was homologous to the 3'-UTR of a *Arabidopsis thaliana* gene coding for the germination protein GLP2 (EMBL: BT002170). Five clones had no match in the data base.

The 3'-UTR clones of differentially expressed genes identified in these experiments will be used as probes to isolate the corresponding full-length genes in a cDNA library previously prepared from root apices of the Al-resistant parent (*B. decumbens*).

1.2.11 Identification of genomic regions responsible for conferring resistance to white fly cassava

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Introduction

White fly is one of the most serious pest and disease vectors that affect the agricultural production around the world. In cassava (*Manihot esculenta* Crantz), white fly causes between 70 to 80 percent of economical losses. The most important source of resistance genes was a genotype MEcu-72 (Arias, 1995). Due to the white fly importance as a pest, it is necessary to know about the nature of genes that confer resistance to white fly in genotypes like MEcu-72. For this purpose we are using F1 segregation and the genetic expression of cross MEcu-72 (resistant genotype) x any very susceptible genotype (MCol-2246) and molecular markers. This would help to accelerate selection of resistant materials to white fly and also the isolate resistant genes. It I hypothesized that these resistant genes may also be effective against other whitefly species, especially *Bemisia tabaci*, the species that is a vector of ACMD, a virus that causes severe crop losses in Africa and Asia. Whitefly resistant genotypes (such as MEcu 72) from the neotropics are displaying resistance to *B. tabaci* in greenhouse trials being carried out by NRI in the UK (CIAT Progress report 2003).

The application of molecular genetic analysis for cassava breeding has been limited compared to others crops. Recently progress has been made in the development of genomic and bioinformatics tools to increase our knowledge of cassava genome structure and cassava gene function. Expressed Séquense Tag (EST) provides an immediate and productive method of gene discovery. In cassava a total of 14168 ESTs were obtained in CIAT and Perpignan Universite (Lopez, et al, submitted), of these 105 have SSRs, for which we designed primers.

Materials and Methods

For the present work we have used the F1 cross (family CM 8996, 276 individuals) between MEcu-72 (as the resistance parent) and MCol-2246 (as the susceptible parent) elite cassava cultivars from Ecuador and Colombia, respectively. The parents and its offspring were evaluated in the field in two places: Nataima (Tolima) and Santander de Quilichao (Cauca). With this evaluation we pretend to identify the 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 developed (Mba et al, submitted). We are using AFLPs (Vos, et al, 1995) and to find markers associated to resistance for mapping and ultimately cloning the resistant genes. We are using silver staining to visualize the allelic segregation of the markers.Cassava RGAs primers were done in the parentals and the polymophics were mapping in the F1.
We designed primers SSRs from ESTs sequences using the software Primer 3 and these SSR were amplified in the parentals and the polymorphics were mapping in the F1.

Results

Field evaluation
The field evaluation showed high pressure being exerted by the pest in Nataima (2003 and 2004), and Santander de Quilichao (2004) where test materials had high damage ratings; however, some materials had lower levels of damage in the evaluations. We can conclude that these genotypes show a resistance level similar to parental MEcu 72. These evaluations were analyzed with the molecular markers to found putative associations.

SSRs from ESTs

We designed 51 pairs of SSRs primers (Table 1) which 29 were polymorphics for cross (Figure 1)

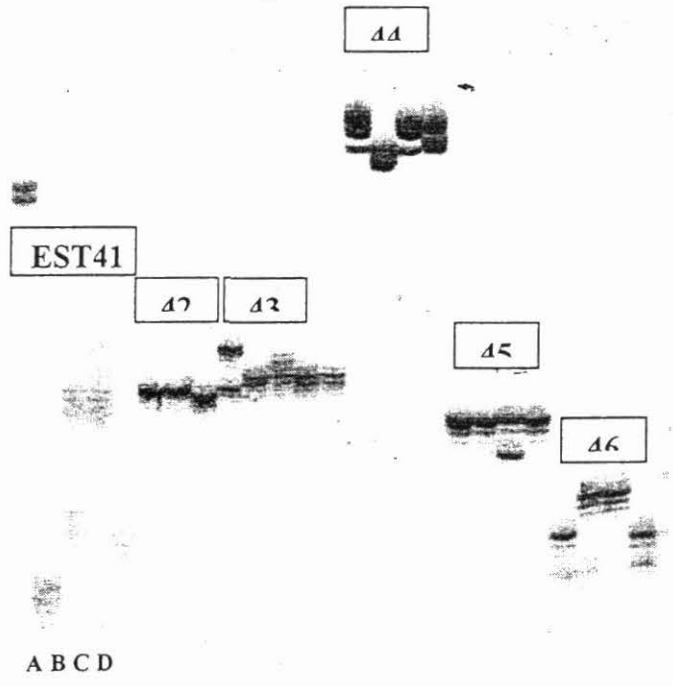


Figure 1. SSRs from ESTs in parentals, A is MNig2, B is CM21772 (both parentals used in ESTs), C is MEcu 72 and D is MCol 2246.

Table 1. SSRs from ESTs primers designed

No.	EST Name	Motif	No. Repeat	No.	EST Name	Motif	No. Repeat
1	cn1375-1	atgg	5	27	cn1304-1	atg	9
2	cn1004-1	tatt	6	28	cn1351-1	aga	10
3	cn1098-1	aga	6	29	si.03.G1.5-1	cca	10
4	cn1388-1	tct	6	30	gi17923193gbBM260153.	taa	11
5	cn1457-1	agc	6	31	mi.06.I21.5-1	tct	11
6	cn255-1	tcc	6	32	si.02.O10.5-1	aat	11
7	cn416-2	gat	6	33	cn1635-1	aag	12
8	cn44-1	tta	6	34	m.01.H14.5-1	tc	12
9	cn700-3	ttc	6	35	si.01.E12.5-1	tc	12
10	c.04.C18.5-3	atg	7	36	cn1460-1	ag	13
11	c.05.I1.5-1	tct	7	37	cn1498-1	at	13
12	cn1186-1	ttc	7	38	cn1587-1	ata	13
13	cn2269-1	tgg	7	39	cn2418-1	ag	13
14	cn393-1	cat	7	40	m.04.K18.5-1	ct	13
15	cn732-1	aag	7	41	aflp_28-2	ga	15
16	cn764-2	tca	7	42	m.06.H4.5-1	ct	15
17	gi17922797gbBM259765.1BM259765-2	aag	7	43	mi.06.N9.5-1	at	15
18	m.04.K21.5-1	ctt	7	44	cn1009-1	ct	16
19	m.05.I2.5-1	ttc	7	45	cn1722-1	tct	17
20	mi.06.N10.5-1	tta	7	46	m.05.L3.5-1	ag	17
21	mi.09.D10.5-1	ctg	7	47	cn47-1	ct	18
22	si.03.B22.5-1	tct	7	48	m.09.N13.5-1	ct	18
23	cn1131-1	tcc	8	49	gi17922797gbBM259765.1BM259765-1	agc	19
24	m.10.J19.5-1	gat	8	50	m.08.G23.5-1	at	20
25	m.11.K5.5-1	gat	8	51	cn1880-1	at	29
26	mi.05.L17.5-1	tga	8				

Mapping

For the construction of linkage map were analyzed 246 markers, 103 SSRs, 2 RGAs, 121 AFLPs and 20 SSRs from ESTs. A genetic linkage map of cassava was constructed with 111 markers segregating from the heterozygous female parent (MEcu-72) of an intraspecific cross (see Figure 2A and 2B). The map consists of 20 linkage groups. These linkage groups span is 879.8 cM and the average marker density is 1 per 7,9 cM. The position of 111 markers, shown in the figure 2, on the framework (LOD = 25 and tetha (θ) = 25) molecular genetic map of cassava. Map distances are shown in Kosambi map units and analyzed by Q gene. So far, 41 SSRs markers were mapped on the cassava framework map (Fregene et al, 1997), the other 70 markers are new. The molecular data are being analyzed using QTL packages (Q gene) to determine linkages between the SSR, RGAs and AFLPs markers and the phenotypic characterization.

Association between Molecular Markers and Resistance

The molecular data are being analyzed using QTL packages (Qgene) and Simple Linear Regression at the 5% level was done using SAS. Putative associations s were found between molecular markers and the field phenotypic characterization (65 markers SSRs, RGA and AFLPs, shown by ***bold*** in the Figure 2A-2B). We observed that SSRY39 marker anchored in the linkage group K are associated with the resistance.

Figure 2A. Location of putatives QTL's (identified by colors) for partial resistance to whitefly in three localities on the female (MEcu-72) derived framework map. These results are based on Qgene analysis. Distances in centimorgans (cM) and significance levels are indicated on the right.The most significants markers are indicated in bold and blue square show the Linkage Group K which is localized a putative QTL in the marker SSRY39

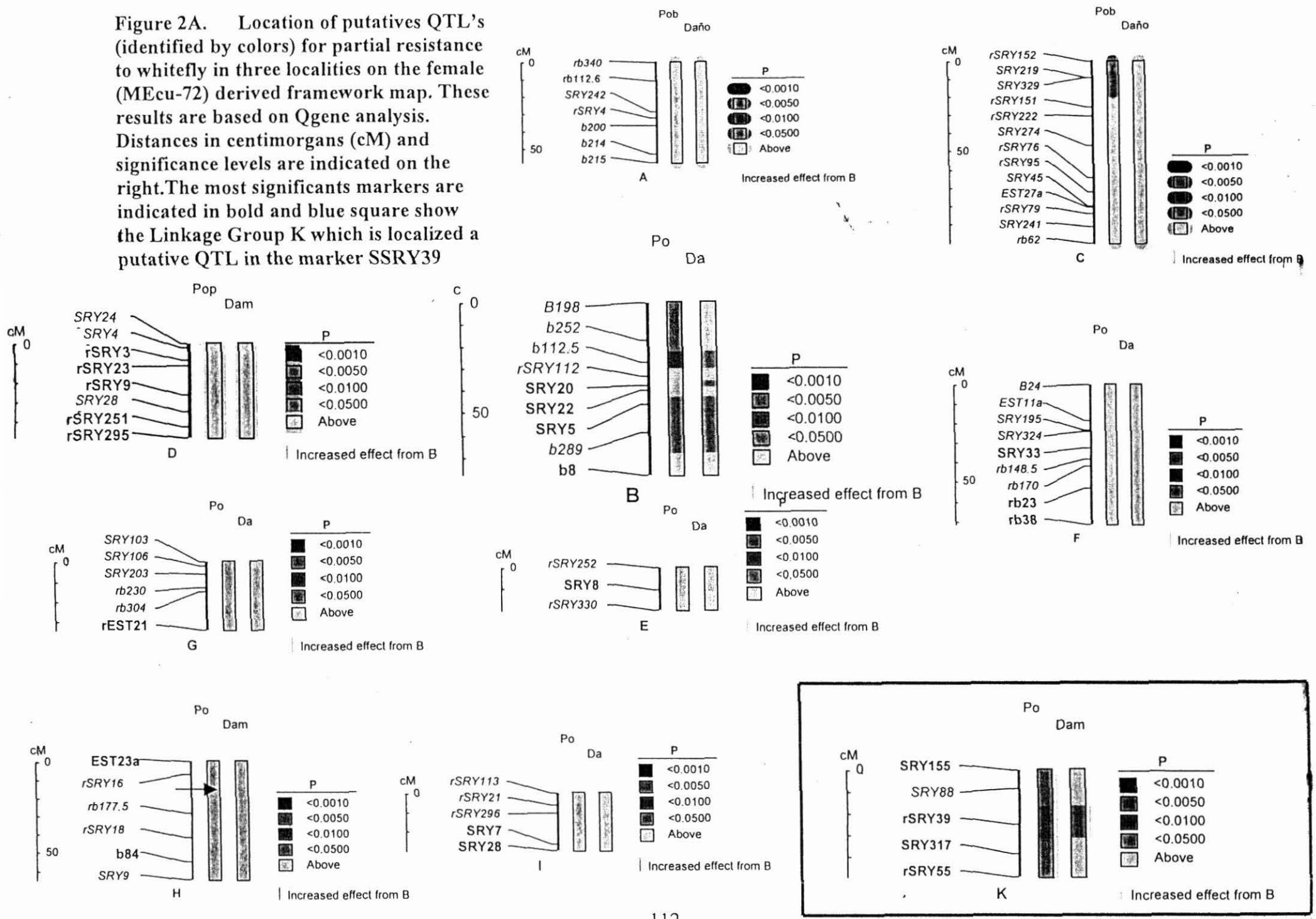
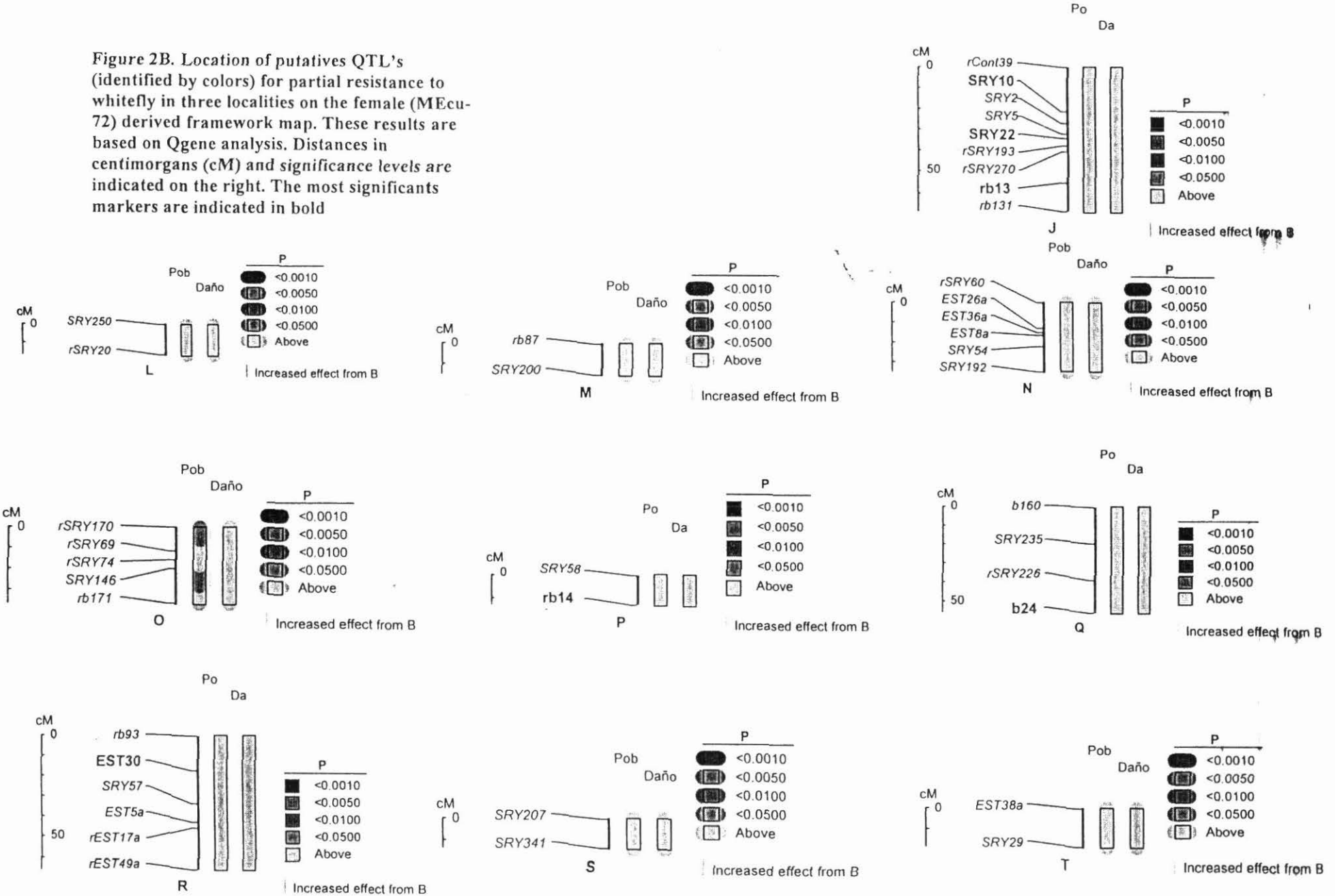


Figure 2B. Location of putatives QTL's (identified by colors) for partial resistance to whitefly in three localities on the female (MEcu-72) derived framework map. These results are based on Qgene analysis. Distances in centimorgans (cM) and significance levels are indicated on the right. The most significants markers are indicated in bold



Ongoing activities

- Saturation of linkage map of MEcu 72, using SNPs.
- Design of SCARs for marker-assisted selection.
- QTL analysis for whitefly resistance.
- Subtractive hybridization of the amplicon MEcu 72 (tester) and MCol 2246 (driver), during which amplified portions of differentially expressed genes are enriched and common sequences are depleted.
- Cloning and screening of the resulting products of expressed sequences during the defense response of MEcu 72 to whitefly attack.
- Microarray of clones in order to identify differentially expressed sequences.

References

Arias B. 1995. Estudio sobre el comportamiento de la "Mosca Blanca" *Aleurotrachellus socialis* Bondar (homoptera: Aleyrodidae) en diferentes genotipos de Yuca, *Manihot esculenta* Crant'z. Tesis.

CIAT Progress Report to NZAID 2003-2004: Sustainable Integrated Management of Whiteflies Through Host Plant Resistance. Pp. 2-3.

Fregene, M., Angel, F., Gomez, R., Rodriguez, F., Chavarriaga, P., Roca, W., Tohme, J. and M. Bonierbale. 1997. A molecular genetic map of cassava (*Manihot esculenta* Crantz). Theor. Appl. Genet. 95: 431-441.

Mba, R.E.C., Stephenson, P., Edwards, K., Melzer, S., Mkumbira, J., Gullberg, U., Apel, K., Gale, M., Tohme, J. and Fregene, M. 2001. Simple Sequence Repeat (SSR) Markers Survey of the Cassava (Manihot esculenta Crantz) Genome: Towards an SSR-Based Molecular Genetic Map of Cassava. Theoretical and Applied Genetics Journal. 102:21-31.

Vos, P., Hogers, R., Bleeker, M., Reijans., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research. Vol. 23 No. 21.

1.2.12 Identification of molecular markers and QTLs associated with gene(s) conferring aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* F1.

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SB-02 Project, CIAT

Introduction

Acid soils have been estimated to occur on about 40% of the arable land (3.95 billions of ha). Plant growth on these soils is constrained mainly by aluminum (Al) toxicity and

deficiencies of nutrients such as phosphorus (P), nitrogen (N), and calcium (Ca). There is considerable variation within and between plant species in their ability to resist Al, and this variation within some species has allowed breeders to develop genotypes that are able to grow on acid soils. Within the *Brachiaria* genus, Al resistance of signal grass (*B. decumbens* Stapf cv Basilisk), a widely sown tropical forage grass, is outstanding compared with the closely related ruzigrass (*B. ruziziensis* Germain and Evrard cv Common) (Wenzl et al., 2001).
The main objective of this work is to identify microsatellites, AFLPs and QTLs associated with the gene(s) conferring aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* cross.

Materials and Methods

An F_1 hybrid population of 263 individuals (*B. ruziziensis* x *B. decumbens*) was used for this study.

Young leaves were cut and placed in paper bags in an incubator previously set at 45-50°C. Samples were allowed to dry for at least 20h, or until the leaves were dry enough that they break easily. Samples were stored at –80°C until grinding.

Dried leaf tissue was grinded with stainless steel spheres with vigorous shaking. Genomic DNA was extracted using a CTAB-Chloroform protocol with some modifications for small amounts of tissue. DNA was quantified on a DyNA Quant 200™ Flourometer (Hoefer Scientific Instruments) and diluted at 4ng/ul for SSRs amplification and 25ng/ul for AFLPs amplification.

Microsatellites: The isolation of microsatellites, and the methodology for PCR amplification and evaluation of polymorphism, have been described previously (Gaitan et al., 2000; Giraldo et al., 2001), with some modifications.

AFLPS: The AFLP Analysis System I kit, and AFLP Analysis System II Small Genome, from Invitrogen® are being used for AFLP amplification, following the instructions with some modifications.

Silver staining (Promega Inc., USA), is used to visualize allele segregation of the markers on 6% denaturing polyacrylamide gels with 5M Urea and 0.5X TBE.

Results and discussion

Microsatellites: 73 SSRs were evaluates in the parental genotypes of which 40 were found to be polymorphic. When run in the progeny, three sets of primers did not amplify in 30% of the progeny, so they were discarded together with three more monomorphic microsatellites.

Ninety-seven polymorphic alleles were scored in the population, out of which 63 were found with the *B. decumbens*, Al-tolerant, 606 genotype, while 34 carried the *B. ruziziensis*, Al-sensitive, 44-2 genotype (Figure 1).

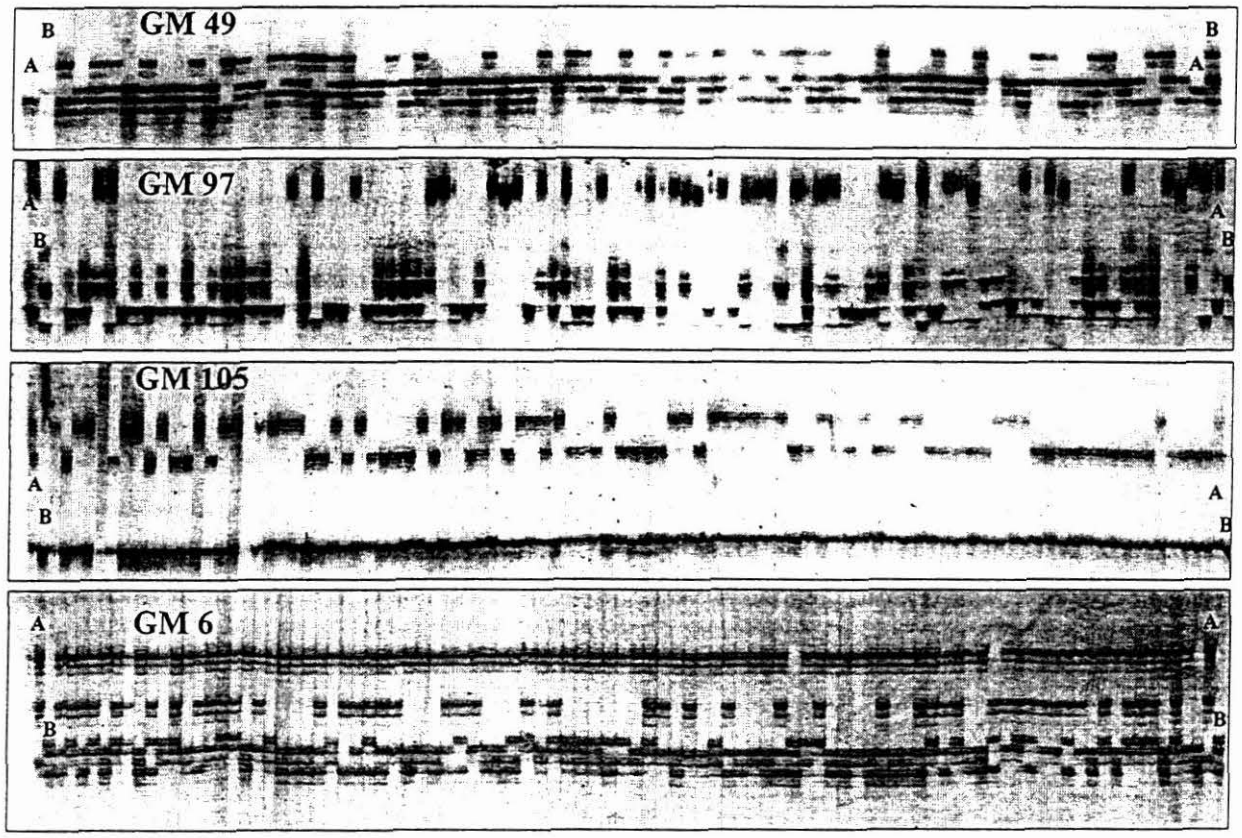

Figure 1. Segregation of four SSR alleles in some individuals of the F_1 *B. ruziziensis* x *B. decumbens* progeny. A, Parental genotype 606 (Al-resistant) and B, parental genotype 44-2 (Al-sensitive)

AFLPs: 64 combinations of primers were assayed with the two parental genotypes. Among them, 12 having high number of polymorphic bands, were chosen. To date 3 combinations (E-ACC/M-CAC; E-ACT/M-CTA and E-ACG/M-CAG) were run in the progeny and yielded 63 polymorphic bands distributed as follows: 50 were found in the Al-tolerant parental and 13 in the Al-sensitive genotype. (Figure 2)

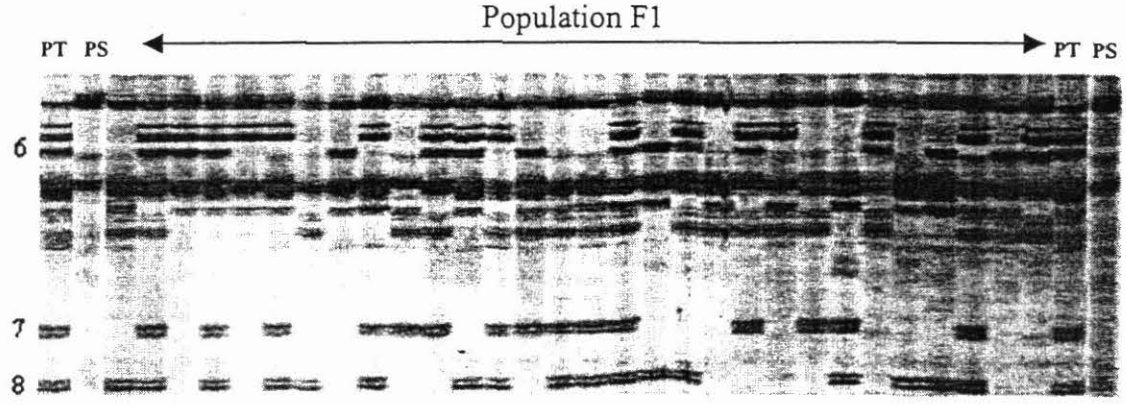

Figure 2. Segregation of AFLPs bands in individuals of the F_1 *B. ruziziensis* x *B. decumbens* progeny. PT, Parental genotype 606 (Al-resistant) and PS, parental genotype 44-2 (Al-sensitive).

Linkage analysis

Segregation of markers in a 1:1 ratio, as single dose restriction fragments (SDRF), will be determined by a Chi-square test. The data matrixes for presence or absence of bands were analyzed with MAPMAKER v3.0b for PC (Lander et al. 1987), and MAPMAKER v2 for Macintosh.

Using 56 SSR and 50 AFLPs molecular markers, we constructed a putative linkage map with 18 linkage groups. These linkage groups span 445.3 cM, and the average marker density is one per every 6.1 cM. The position of 78 markers is shown in Figure 2, on the framework molecular genetic map of *Brachiaria* (LOD = 25 and tetha (θ) = 25). Map distances are shown in Kosambi map units.

Association between Molecular Markers and Al resistance

To find association with molecular markers, a preliminary analysis of 106 markers at the 10% level was done using SAS. Putative associations were found between 78 SSRs and AFLPs markers and the phenotypic characterization under greenhouse conditions.

The three phenotypic variables for Al resistance (root length, abundance of root tips, mean root diameter) were analyzed for association with molecular markers. We found 13 molecular markers with R^2 between 0.0124-0.0267 that explain the variance for these traits, molecular markers associated with phenotypic characterization are in blue (Table 1 and Figure 3).

Table 1. Association analysis between molecular markers and phenotipic variable for Al resistance in a *Brachiaria decumbens* x *Brachiaria ruziziens*es hybrid population.

Marker	R^2	Linkage Group	Phenotipic Variable
GM 44d2	0.0267	B	Rl[a]
GM 44d2	0.0218	B	Tips[b]
C1b4	0.0181	R	Tips
GM 109c	0.0177	N	Rd[c]
C1b17	0.166	D	Rd
C2b17	0.0161	R	Tips
C2b15	0.016	I	Rd
GM 109d	0.0156	N	Rd
GM 79c	0.0156	A	Rl
GM 58a	0.0151	D	Rd
GM 44d1	0.0149	B	Tips
C2b12	0.0145	B	Rd
C1b12	0.014	R	Tips
GM 79d	0.013	D	Rd
GM 44d2	0.0124	B	Rd

[a] Root Length [b] Abundance of root Tips [c] Root Diameter

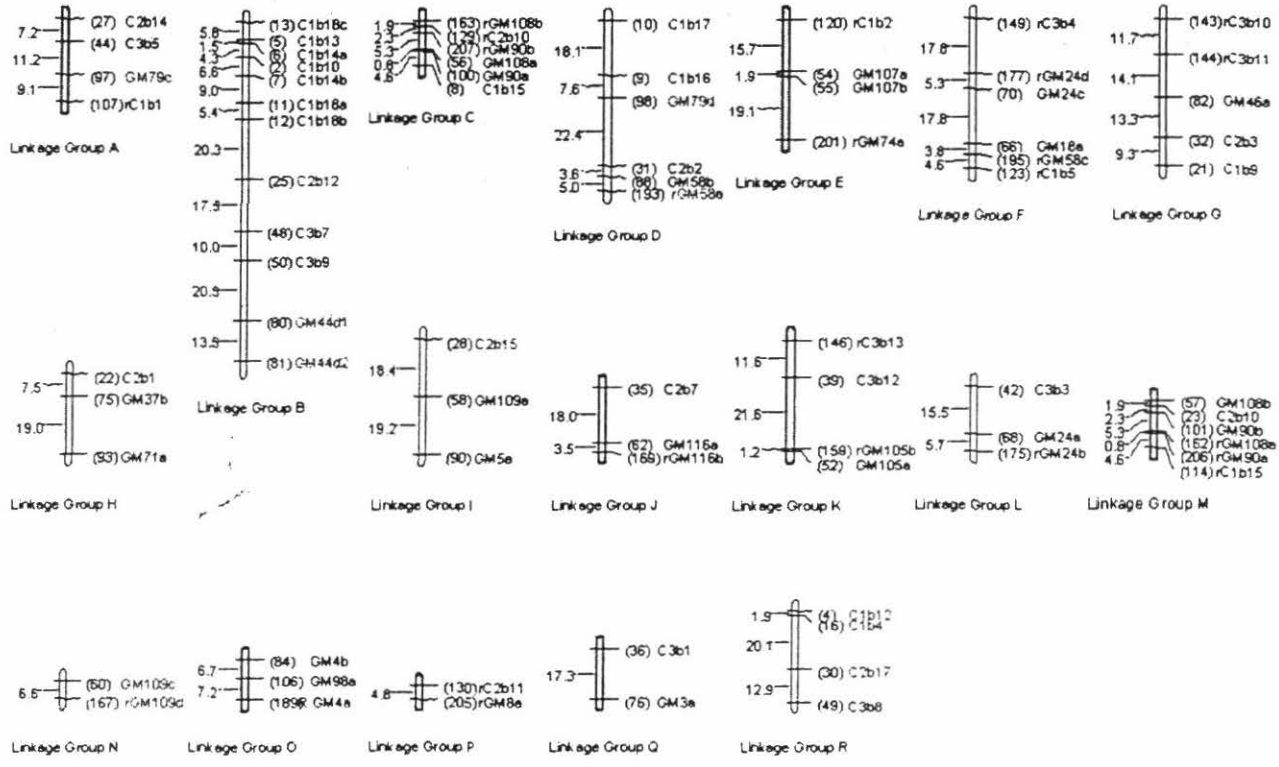

Figure 3. Preliminary *Brachiaria* linkage map of a *B, ruziziensis* × *B. decumbens* F1. Markers in blue indicate putative association with Al resistance.

On-going activities

- Saturation of linkage map of 606 parent with SSR and AFLPS.
- Analysis of the data to mapping for Al resistance
- QTLs analysis for Al resistance.
- Design of SCARs for marker assisted selection

Main achievements

Evaluation of 50 % of the polymorphic SSRs and AFLPs in the F1.

Preliminary associations between markers SSRs and AFLPs and phenotypic evaluation.

References

Gaitán, E., OX Giraldo, JW Miles and J. Tohme. (2000). Development and use of SSR and AFLP markers for constructing a molecular genetic map for *Brachiaria*. *In*: CIAT. Annual report 2000 pp 123-127.

Giraldo, O.X., Vargas, J., Gaitán, E., Duque M.C., Miles, J., Cardona, C., and Tohme, J. (2001). Construction of a molecular genetic map of Brachiaria and QTL analysis of spittlebug resistance. *In*: CIAT Annual report 2001 pp186-190

Wenzl P., G.M. Patiño, A.L. Chaves, J.E. Mayer, and I.M. Rao. 2001. The high level of aluminum resistance in signalgrass is not associated with known mechanisms of external aluminum detoxification in root apices. Plant Physiol, 125:1473-1484.

1.2.13 Candidate genes involved in the defense response of *Brachiaria* to the Spittlebug (II)

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SB-2 Project

Introduction

Regardless the numerous losses caused by arthropods in agriculture, the study of plant molecular defense responses has been focused for decades in plant-pathogen interactions. The rapid increase in the biochemical and genetic characterization of signal cascades underlying responses to pathogens had provided the required framework to integrate numerous parallel and interdependent processes in a genome-wide scale (Glazebrook *et al.*, 2003). The study of the salicylic acid (SA) signaling pathway, which plays a major role in defense against pathogens, has mainly driven the research in this field. Although jasmonates (JA) and ethylene (ET) pathways have also been found in plant-pathogen interactions, their relationship with SA is antagonistic (Spoel *et al.*, 2003). The JA and ET activities have a more central role in defense against necrothrophic fungi and wounding, which are stresses that involve mechanical damage in plant tissue.

In contrast to the knowledge achieved in plant-pathogen systems, the genetic network involved in defense responses to insects is largely unknown. First, the initial perception of insect attack is not well determined. Second, the consequent transcriptional changes had not been explored in a genome-wide scale. The initial differential expression analysis, performed through the construction of substractive hybridization libraries in tobacco (Hui *et al.*, 2003) sorghum (Shu-Salzman *et al.*, 2004) and *Brachiaria* (SB-2 Project Annual Report, 2003), suggests that the changes elicited by herbivore attack are comparable in scope and magnitude to those elicited by pathogens. However, due to the partial nature of

these studies (which consider only few hundreds or tens of genes), and to the lack of understanding on the signaling events that lead to the transcriptional reprogramming, is difficult to articulate the results found in each specific plant-herbivore system. Without the integration provided by signaling pathways, the extrapolation and generalization of the defense genetic network in different plants responding to different enemies is even more demanding.

Although the JA signaling was recognized as part of the abiotic and biotic wounding responses fourteen years ago (Farmer & Ryan, 1990), the delineation of the proteins and genes regulated by these phytohormones is incipient (Liechti & Farmer, 2002). Nevertheless, notable advances in the understanding of JA biosynthesis had been achieved in recent years (Blée, 2002). Particularly, the realization that several members of the oxylipin family have differential biological activities as signals, broadens unexpectedly this field of research (Howe & Schilmiller, 2002). Currently, the onset of JA biosynthesis under insect damage is only understood in tomato, where the peptide systemin is the trigger after wounding (Ryan, 2000). However, no analogous initial trigger in other plant species has been found. In general, under insect damage, rapid jasmonate biosynthesis occurs, with differential control over the concentration of each oxylipin compound (Stintzi *et al.*, 2001). These molecules, in turn, generate a massive transcriptional reprogramming, which include the upregulation of i) genes of their own biosynthetic pathway (Ryan, 2000), generating a positive feedback and amplification of the signal, and ii) effector genes, involved in specific insect resistance mechanisms. The proteinase inhibitors and polyphenol oxidase in solanaceas (Ryan, 2000) and the defensins and thionins in Arabidopsis (Thomma *et al.*, 2002) are some of the characteristic antidigestive mechanisms induced by JA.

Besides SA, JA and ET, the three major accepted hormones in defense responses, a recent study implicates the brassinosteroids (BR) as an alternative overlooked systemic signaling pathway. Nakashita *et al.*, 2003, showed an enhancement of resistance capacity against virus, bacteria and fungi, in both monocotyledonous and dicotyledonous plants after exogenous application of BR. This induction of resistance is completely independent of the SA accumulation, and therefore, independent of Systemic Acquired Resistance. Furthermore, the recent characterization of the brassinosteroid receptor from tomato revealed that this protein is identical to the previously isolated systemin receptor (Montoya *et al.*, 2002). The fact that both hormones bind to the same cell receptor suggests that BR could be related to the activation or modulation of JA biosynthesis. Additionally, BR induces the expression of three genes involved in JA production (Bishop & Yokota, 2001) as well as genes of their own biosynthetic pathway (Müssig *et al.*, 2002). Regardless this evidences, the action of BR as an insect defense-signaling pathway has still not been tested.

Through the construction of a substractive hybridization library performed in *Brachiaria* resistant plants (SB-2 Project Annual Report, 2003), we previously depicted part of the genetic network involved in the defense response against *Aeneolamia varia*. The

sequencing of 295 EST (Expressed Sequence Tags) and the bioinformatic analysis of 74 unique sequences, show the activation of several biological processes in different levels, among them the biosynthesis of JA and BR. The induction of four genes involved in the production of JA during the defense response of *Brachiaria* to spittlebug, strongly suggests the participation of JA signaling in the regulation of the defense response to this herbivore. Likewise, the presence of a CYP90B1 steroid 22-alpha-hydroxylase, one of the earlier enzymes in the BR biosynthetic pathway, raises interesting questions about the possible role of this hormone in this defense response (SB-2 Project Annual Report, 2003).

To determine the role of these two hormones as part of the defense response of *Brachiaria* at molecular and phenotypic level we pursuit two different goals. Initially, we are looking for the precise quantification of expression levels of JA and BR related sequences in infested vs non-infested plants throughout qRT-PCR (quantitative Real Time-PCR). An additional sequence of interest, a transcription factor known as SCARECROW, was also included in this quantification. Secondly, in order to manipulate those particular signaling pathways during the defense response, we carried out two bioassays on *Brachiaria* susceptible plants under exogenous applications of jasmonic acid and brassinolide. Our objective is to evaluate the phenotypic consequences of the JA and BR priming in the plant-insect interaction.

Methodology

qRT-PCR

The molecular quantification of gene expression levels was performed on DNA Engine OPTICON 2^{TM} System (MJ Research) with SYBR green dye (DyNAmo kit, Finnenzymes). Since this fluorescent dye binds to all dsDNA molecules present in each round of amplification, the specificity of PCR product is essential for the reliability of the quantification. For this reason the optimum melting temperature and $MgCl_2$ concentration was stringently standardized on genomic DNA for each PCR product. Additionally, in order to obtain template of the exact coding sequence length to build the quantitation curve in each run (10^6-10^2 molecules as template), PCR products from cDNA were amplified, cleaned and quantify in a GENios espectrophotometer (TECAN) with PicoGreen^R dsDNA quantitation kit. The sscDNA template was synthesized with Superscript Reverse Transcriptase (CLONTECH) from a bulk of total RNA obtained from superficial roots of resistant infested (RI) and non-infested (R) plants, extracted during different stages of the infestation process (1-30 days). The comparison between these two samples was first attempted by relative quantification, method based on differences in cycle threshold values; however, due to dramatic differences in PCR efficiencies between sequences, we decided to make the comparison by the absolute number method. The exact number of molecules found in each sample was normalized against two different housekeeping sequences (reference genes) and compared through mean normalization $(\text{target}_{RI}/\text{reference}_{RI})/(\text{target}_{R}/\text{reference}_{R})$ (QIAGEN, 2004) with three replicates per sequence.

Exogenous application of JA and BR on *Brachiaria* susceptible plants Bioassay1

We executed a preliminary assay in order to evaluate two hormone application methods: foliage dipping and spray on roots. Initially, we began with low dosages: 1, 10, 100 and 1000 µM of jasmonic acid (SIGMA) and 0.02, 0.2, 20 y 200 µM of brassinolide (CIDTech Research Inc.), ranges previously reported for transcriptional change experiments in other species (Casaretto *et al.* 2004, Farmer *et al.* 1992, Nakashita *et al.*, 2003, Müssig *et al.*, 2002). All solutions were diluted in 0.05% (v/v) water/surfactant (INEX-A). Infestation with *Aeneolamia varia* eggs was done on roots 24 hours after hormone application. We used ten replications for each of the treatments, each replication (one plant) infested with ten mature eggs. Upon hatching the number of eggs hatched was recorded. The infestation was allowed to proceed until all the nymphs matured or became adults. Plant damage scores and percentage of nymph survival were recorded. Percentages of insect mortality were transformed to arcsine of square root of mortality proportion plus one and analyzed using STATISTIX software.

Bioassay2

Higher concentration and periodic applications of hormones were performed in the second bioassay. Dosages of 2.5 mM and 25mM of jasmonic acid and 0.5 mM and 5mM of brassinolide (Thaler *et al.*, 2001, McConn *et al.*, 1997, Khripach *et al.*, 2000) were applied every five days through dipping of the foliage on the respective solution. Since hormone effect on insect performance could vary over the infestation period, we decided to evaluate mortality at 10, 20 and 32 days post infestation (2, 4 and 6 applications respectively). We used 17 replications for each of the treatments, each replication infested with six mature eggs. Upon hatching the number of eggs hatched was recorded and the unhatched eggs were replaced. Percentage of nymph survival was recorded and analyzed independently for each evaluation time. Plant damage scores were only assessed at 32 days. Percentages of insect mortality were corrected for natural mortality using the Abott formula (= 100 x (M_{obs}– $M_{control}$/100-$M_{control}$)) and transformed to arcsine of square root of mortality proportion plus one. Statistical analysis was performed on STATISTIX software.

Results and Discussion

qRT-PCR

The upregulation of three out of five attempted sequences was corroborated through independent normalization events against two different reference genes: glycerol-3-phosphate dehydrogenase (GAPDH) and tubuline (Table 1). From the four JA related sequences (Lypoxigenase 1 and 2, Phospholipase and Fatty Acid Desaturase) only lipoxygenases 1 and 2 were found upregulated (RI/R>2). Additionally, the SCARECROW transcription factor, a protein activated by chitin in other monocotyledonous plants, was also found induced in the defense response of *Brachiaria* to spittlebug. Unfortunately, the

primers designed to amplify the steroid 22-alpha-hydroxylase, sequence involved in the production of BR, did not work in either genomic DNA or sscDNA. The future quantification of this specific sequence depends on the isolation of a longer sequence with higher quality.

Table 1. Mean ratios RI/R for each gene of interest, normalized against two different reference genes.

Target \ Reference	*GAPDH*	*Tubuline*
Lypoxygenase 1	3.66	3.59
Lypoxygenase 2	2.42	2.37
SCARECROW	2.34	2.29
Phospholipase	1.21	1.18
Fatty Acid Desaturase	0.60	0.59

In plants, the differences on expression pattern, subcellular location and substrate utilization between different lipoxygenases isoforms generates part of the oxylipins structural and functional diversity (Howe & Schilmiller, 2002). In our hands, the participation of at least two different isoforms of lipoxygenases in *Brachiaria* defense response to spittlebug was supported by structural and expression data. 1) The divergence between the two lipoxygenases sequences, which exhibit similarity to the same proteic region (770 to 840 amino acids) of different reported lipoxygenases and 2) their differing expression levels, suggest that two separate isoforms are involved in this defense response.

Is important to notice here that the quantification of expression levels was carried out on bulked samples, which represents the overall population of genes expressed in different stages of infestation. Additional quantifications with special focus in earlier stages of defense response, when the signaling events are expected to be more relevant, have to be done in order to confirm the expression levels herein found.

Exogenous application of BR and JA on *Brachiaria* susceptible plants

Bioassay 1

None of the hormone concentration tested had an effect on insect mortality. Moreover, we observed high levels of mortality (39%) in control plants (susceptible plants treated with water / INEX-A 0.05%) at the end of the infestation. This increased mortality could be attributed to competition among nymphs, effect that became stronger in later infestation stages, when susceptible plants were completely dry and nymphs are subjected to food depletion. Based in this observation six nymphs per pot were chosen as the infestation level for the second bioassay.

Bioassay 2

The insect mortality did not show any significant difference at 10 days post infestation. This evaluation was hinder by the small size of nymphs (2-3 mm), factor that made difficult their detection on roots. At 20 days after infestation the difference in insect mortality found in low BR was statistically significant (P value= 0,0027). The mean mortality of nymphs in control plants was 8.83% SE 2.89 compared with the corrected mortality in low BR plants, that was 24.73% SE 6.28. At 32 days after infestation the difference in mean corrected mortalities between treatments was also statistically significant (P value= 0,0042). However, in this later stage of infestation, we also observed high insect mortality levels in control plants, probably due to food depletion. The mean mortality of nymphs in control plants was 16.66% SE 4.3 whereas the mean mortality of low BR descend from 22.93% SE 5.45 (crude mortality) to 12.5% SE 5.43 (corrected mortality) due to the high value of the correction factor. After 30 days of infestation the treated plants exhibited the same damage extent than the control.

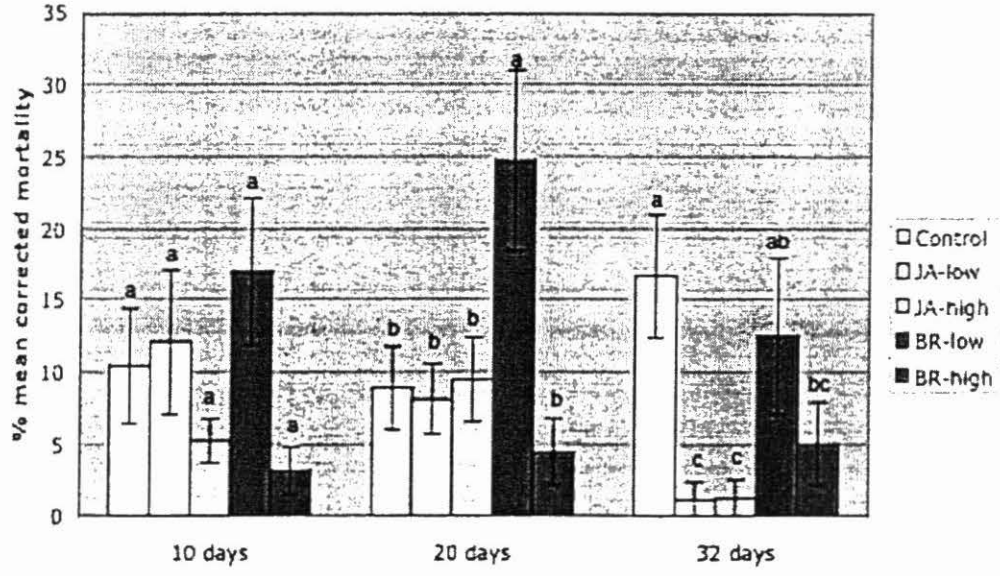

Figure 1. Response of *A. varia* populations to low and high concentrations of JA and BR. Each stage of infestation was recorded and analyzed separately. Bars with the same letter did not differ at the 5% level by LSD.

Although data shows statistically significant differences between control and low BR treatment at 20 and 32 days after infestation, the observed mortality increment was very low and does not have true biological value. Treatment mortalities did not surpass the 30% meanwhile natural mortality oscillates between 8.8 and 16.66%. Additionally, the increment of mortality observed in low BR treatment appeared to be inconsistent with the absence of effect on high BR treatment.

The high standard error values make evident the need of a larger number of replications in future bioassays. Furthermore, higher hormone concentrations, frequency of application and suitable times of evaluation (to avoid additional effects of food depletion) are some of the additional variables that must be adjusted for our experimental system. Besides those experimental variables, is important to take into consideration the branched pattern of JA and BR biosynthetic pathways. The present study is focused in the use of jasmonic acid and brassinolide, the signal molecules with higher biological activities within each family of compounds (Bishop & Koncz, 2002, Liechti & Farmer, 2002). However, we could not discard the possibility that other different biochemically modified products of JA and BR pathways happen to be active in the signaling of the defense response of *Brachiaria* to the spittlebug. For this reason, we could think on testing other active members of JA and BR (as methyl-jasmonate and OPDA for JA or catesterone and 24-epibrassinolide for BR) in the future experiments.

Conclusion and Perspective

Molecular evidence for the differencial expression of 2 out of four genes implied in JA biosynthesis was achieved. However, at the phenotypic level, the application of JA and BR did not show any enhancement in the defense capacities of susceptible plants of *Brachiaria*. In this context our next goals are:

To obtain the quantification of expression levels of phospholipase and fatty acid desaturase in earlier and specific times of infestation.

Further characterization of steroid 22-alpha-hydroxylase sequence, in order to achieve the quantification of BR related gene expression level.

Compare early gene expression levels of JA and BR related sequences in resistant infested vs susceptible infested plants, in order to clarify if the susceptible plants are delayed or show any difference in the production of these signaling molecules under the herbivore attack.

Execute a bioassay with higher hormone concentrations.

References

Bishop, G. J. & Yokota, T. (2001). Plants steroid hormones, brassinosteroids: current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response. *Plant Cell Physiol* 42 (2):114-120.

Bishop, G.J. & Koncz, C. (2002). Brassinosteroids and plant steroid hormone signaling. *Plant Cell*, S97–S110, Supplement.

Blée, E. (2002). Impact of phyto-oxylipins in plant defense. *Trends Plant Sci* 7 (7):315- 322.

Casaretto, J.A., Zuniga, G.E., Corcuera, L.J. (2004) Abscisic acid and jasmonic acid affect proteinase inhibitor activities in barley leaves. *J Plant Physiol.* 161(4):389-9

Farmer, E. E. & Ryan, C. A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves, *PNAS* 87: 7713-7716.

Farmer, E. E. & Ryan, C. A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4 (2):129-134.

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J.* 34(2):217-28.

Howe, G. A. & Schilmiller, A. L. (2002). Oxylipin metabolism in response to stress. *Curr Opin Plant Biol* 5(3):230-236.

Hui, D., Iqbal, J., Lehmann, K., Gase, K., Saluz, H. P., Baldwin, I. T. (2003). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*: V. Microarray analysis and further characterization of large-scale changes in herbivore-induced mRNAs. *Plant Physiol* 131 (4):1877-1893.

Khripach, V., Zhabinskii, V., De Groot, A. (2000). Twenty Years of Brassinosteroids: Steroidal Plant Hormones Warrant Better Crops for the XXI Century. *Annals of Botany* 86: 441-447

Liechti, R. & Farmer, E. E. (2002). The jasmonate pathway. *Science* 296 (5573):1649- 1650.

Mcconn, M., Creelman, R. A., Bell, E., Mullet, J. E. & Browse, J. (1997). Jasmonate is essential for insect defense in *Arabidopsis*. *Proc Natl Acad Sci U S A* 94 (10):5473-5477.

Montoya, T., Nomura, T., Farrar, K., Kaneta, T., Yokota, T., Bishop, G. J. (2002). Cloning the tomato *curl3* gene highlights the putative dual role of the leucine-rich repeat receptor kinase tBRI1/sr160 in plant steroid hormone and peptide hormone signaling. *Plant Cell* 14 (12):3163-3176.

Müssig, C., Fischer, S. & Altmann, T. (2002). Brassinosteroid-regulated gene expression. *Plant Physiol* 129(3):1241-1251.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I. & Yoshida, S. (2003). Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *Plant J* 33 (5):887-98.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 2001 May 1;29(9):e45

QIAGEN (2004) Critical Factors for Successful Real-Time PCR. Romero, C., Acosta, I.F, Tohme, J. (2003) Identification of genes induced during the defense response of *Brachiaria* to the spittlebug. SB-2 Project Annual Report

Ryan, C. A. (2000). The systemin signaling pathway: Differential activation of plant defensive genes. *Biochim Biophys Acta* 1477 (1-2):112-121.

Spoel, S.H., Koornneef, A., Claessens. S,M., Korzelius. J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., Pieterse, C.M. (2003). NPR1

modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15(3):760-70.

Stintzi, A., Weber, H., Reymond, P., Browse, J. & Farmer, E. E. (2001). Plant defense in the absence of jasmonic acid: The role of cyclopentenones. *Proc Natl Acad Sci U S A* 98 (22):12837-12842.

Thaler, J., Stout, M., Karbanan, R., Duffey, S. (2001). Jasmonate-mediated induced plant resistance affects a community of herbivores. *Ecological Entomology* 26, 312-324

Thomma, B. P., Cammue, B. P. & Thevissen, K. (2002). Plant defensins. *Planta* 216 (2):193-202.

Zhu-Salzman, K., Salzman, R.A., Ahn, J.E., Koiwa, H. (2004). Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. *Plant Physiol* 134(1):420-3

CONTENT

Activity 1.3 Identification and mapping of useful genes and gene combinations

1.3.1 Analysis of iron reductase as a mechanism for enhanced iron uptake in common beans: QTL analysis and candidate gene cloning

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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*. Iron reductase is a member of the protein superfamily 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. The gene for iron reductase was first isolated from Arabidopsis and Pisum (Pea). Both species are fairly efficient at extracting iron from the soil and serve as models for enzyme activity. Other candidate genes include a family of zinc transporters.

Methodology

Library screening: the Grusak lab selected conserved primers for RT-PCR based on the Pisum iron reductase gene (PsFRO1) and used them to amplify Common bean reductase candidates which were then used to screen filters from a leaf cDNA library made in the CUGI-CIAT collaboration.

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.

Plant Material: The reductase assay has been carried out on two populations of recombinant inbred lines that are being used to genetically map seed micronutrient content QTL: DOR364 x G19833 and G21242 x G21078. The first population is from an Andean x Mesoamerican inter-gene pool cross and the second is from and Andean x Andean intra-gene pool cross.

Results and Discussion

Library Screening:

The Grusak lab has been screening a common bean leaf cDNA library which we made at CIAT and has found partial sequences with homology to iron reductase using the Medicago reductase clone and sequences with similarity to Aquaporins using the Zinc transporter family. They will begin next year to screen a set of two root leaf cDNA libraries, where the mRNAs for iron reductase and zinc transporters are more likely to be expressed.

Reductase assays:

The iron reductase assay is producing interesting differences between the parents of both mapping populations for their ability to reduce iron at two different iron regimes. 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.

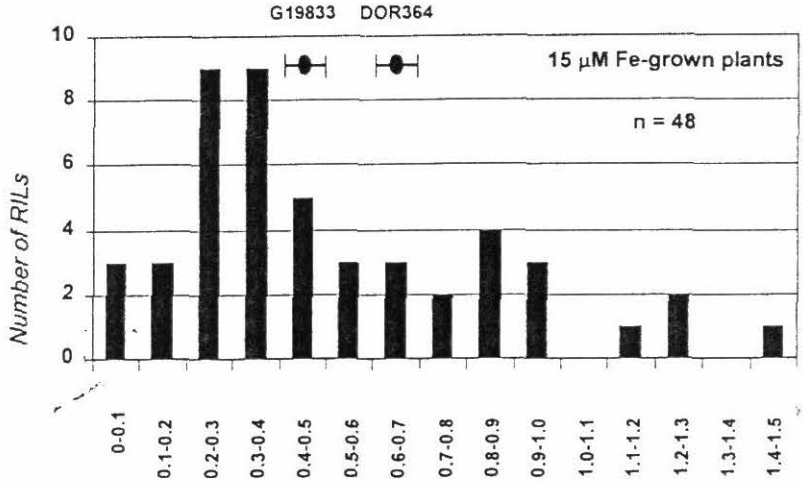
The bimodal nature of the population distribution for DOR364 x G19833 recombinant inbred lines (Figure 1) shows that inheritance of iron reductase activity looks like one or a few genes control it. QTL analysis confirms that there is one major QTL under iron sufficiency and this QTL is different from the one under iron deficiency. Therefore it can be postulated that iron reductase is represented by several differentially expressed genes, whereby: one iron reductase gene is expressed in iron deficiency (1 uM) and is found on Chromosome b02; another iron reductase gene is expressed in iron sufficiency (15 uM) and is found on Chromosome b11.

To understand the relationship of iron reductase to seed iron accumulation we analyzed ICP results that we had obtained on seed of the recombinant inbred lines and identified two QTLs controlling seed iron accumulation in this population – one very important QTL on Chromosome b11 and another important QTL on Chromosome b08.

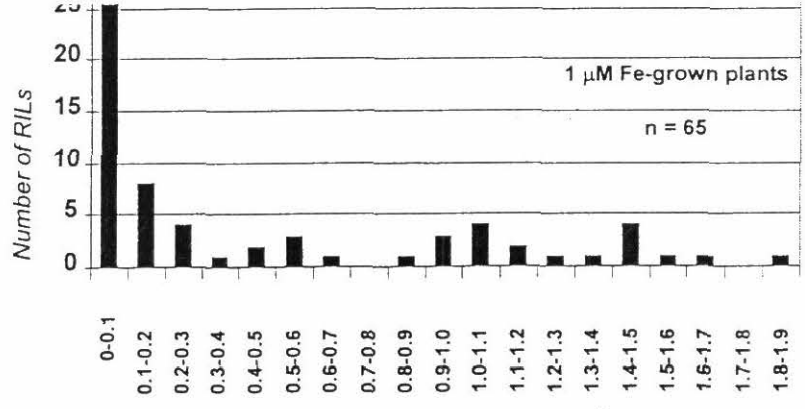
Although the correlation of iron reductase activity with seed iron accumulation was not significant in this populations at either 1 uM (r=0.044) or at 15 uM (-0.163), it was interesting to see that the iron reductase activity QTL on chromosome 11 was located in the same place as the seed iron content QTL. This did not hold true for the other seed iron QTL on chromosome 8.

Future Steps:

- Evaluate Fe reductase activity in a greater number of parents of other populations and a range of Fe concentrations for each parent.
- Perform QTL analysis with the G21078 x G21242 population and an improved genetic map that we have constructed.
- Identify any other potential QTL for iron accumulation and for Fe reductase activity at other genomic locations based on other populations.
- Develop a DNA marker for Fe reductase activity either based on QTL mapping or cloning and mapping of orthologs of the Fe reductase gene.



Range of root reductase values (µmol Fe reduced g FW^{-1} h^{-1})



Range of root reductase value (µmol Fe reduced g FW^{-1} h^{-1})

1.3.2 Evaluation of five common bean mapping populations for tannin content

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SB-2 Project, 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 the seed coats of common beans (*P. vulgaris*) and their relatives. 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. Some of the tannin fractions and flavinoid precursors have been suggested to have a positive effect on health through anti-oxidant activity. Therefore it is important to have a greater understanding about the inheritance of total tannin content and its component fractions. Given this we began a program to identify genetic variability for seed coat tannin and soluble vs. insoluble tannin components in mapping populations. In the previous annual we report the results in the DOR364 x G19833 population. In this study we applied the extraction techniques developed over the last two years to the analysis of four additional populations of recombinant inbred lines. Our ultimate objective is to identify the QTLs associated with tannin accumulation in common bean.

Materials and Methods

Plant Material: A total of 500 lines from five populations (averaging 100 Rills each) were analyzed in this study. The populations represented both Inter.-gene pool crosses (DOR364 x G19833, G19839 x G2333, BAT93 x JALOEEP558) and intra-gene pool crosses (Andean - G21242 x G21078 and Mesoamerican G14519 x G4825). Each population was grown at a single location and seed was freshly harvested for analysis.

Seed Coat preparation: Seed coats were peeled from common bean seed and dried at 60 C for one hour and ground into a fine powder to use in the analysis. An n-heptane treatment was used facilitate seed peeling which consisted in 12 hours immersion in n-heptane after which the seed was dried and peeled by hand. 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.

Tannin Extraction: Total tannin extraction and analysis were as reported last year using 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.

Tannin Purification and Establishment of the Calibration Curve: Seed coat of the parents of each population was extracted for tannins and purified with Sephadex LH-20. A dilution series of different tannin concentrations was evaluated in the colorimetric assay described above. The calibration curve was then established by plotting average absorbance against tannin concentration for the two parents of each population.

Results and Discussion

Soluble and insoluble tannins were successfully purified from the parents of each population to use for the determination of the calibration curves for absorbance vs. concentration and to use in estimating the amount of tannins in the progeny. When the progeny and parents were evaluated, a range of seed coat tannin concentrations (expressed in percent) were observed for the four populations (Figure 1). Tannin measurements were consistent between repetitions showing that the Butanol-HCl method used for analysis is technically sound.

Average soluble, insoluble and total tannins were found to vary significantly between populations (Table 1). The DOR364 x G19833 population had the highest soluble tannins and since soluble tannins make up the bulk of total tannins this population was also highest in this category. G2333 x G19839 and G21242 x G21078 has lower amongst of soluble tannin but had the highest amounts of insoluble tannins. The other two populations, especially BAT93 x Jalo EEP558 were low in both soluble and insoluble tannins. The normal distribution found in total tannin concentration (and both soluble and insoluble tannins in most of the populations suggests that the traits are inherited as quantitatively (Figure 1). However given that the BAT93 x Jalo population was distributed binomially, there is the possibility that inheritance of tannin content is more qualitative. Correlations between the amount of soluble and insoluble tannins (Table 2) was low in the intra-gene pool populations but was significant for the inter-gene pool populations (ranging from $r=0.20$ to $r=0.55$). This may indicate that the inheritance of soluble and insoluble tannin content is independent in the first set of populations but linked in the other populations.

The search for QTLs in the DOR364 x G19833 population identified two QTLs for total tannin content on linkage groups b03 and b10 (Figure 2). The QTL on linkage group b10 was also associated with QTLs for both traits independently while the QTL on linkage

group b03 was only significant for total tannin content. These QTLs map to the same locations as the seed coat color genes "Z" (b03) and "J" (b10) on the classical genetic map of common beans showing an association between seed color and tannin content which will be investigated further.

Conclusions and future plans

We plan to complete the QTL analysis with the additional populations 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. We also plan to evaluate the flavinoid components of the tannin fraction using HPLC analysis and determine if there are correlations between tannin content and iron/zinc accumulation in the seed..

Table 1. Average tannin content in five populations of common bean recombinant inbred lines

Population[1]	Soluble	Insoluble	Total
DOR364 x G19833	26.45 A[(2)]	2.88 B	29.38 A
G2333 x G19839	19.31 B	3.41 A	22.74 B
G21242 x G21078	18.34 B	3.52 A	21.88 B
G14519 x G4825	15.88 C	2.98 B	18.88 C
BAT93 x JALO	9.49 D	2.36 C	11.87 D

1/ Population differences significant at P<1%
2/ Means separation by Ryan Einot Gabriel Welch test – at P

Table 2. Correlation between soluble and insoluble tannin content and total tannin content for each of five recombinant inbred line populations.

Population	Correlation		
	Ins vs. Sol	Ins. vs Tot	Sol vs. Tot
DOR364 x G19833	0.20	0.39	0.98
G21242 x G21078	0.05	0.21	0.99
G14519 x G4825	-0.04	0.18	0.97
G2333 x G19839	0.44	0.58	0.99
BAT93 x JALO	0.55	0.69	0.99

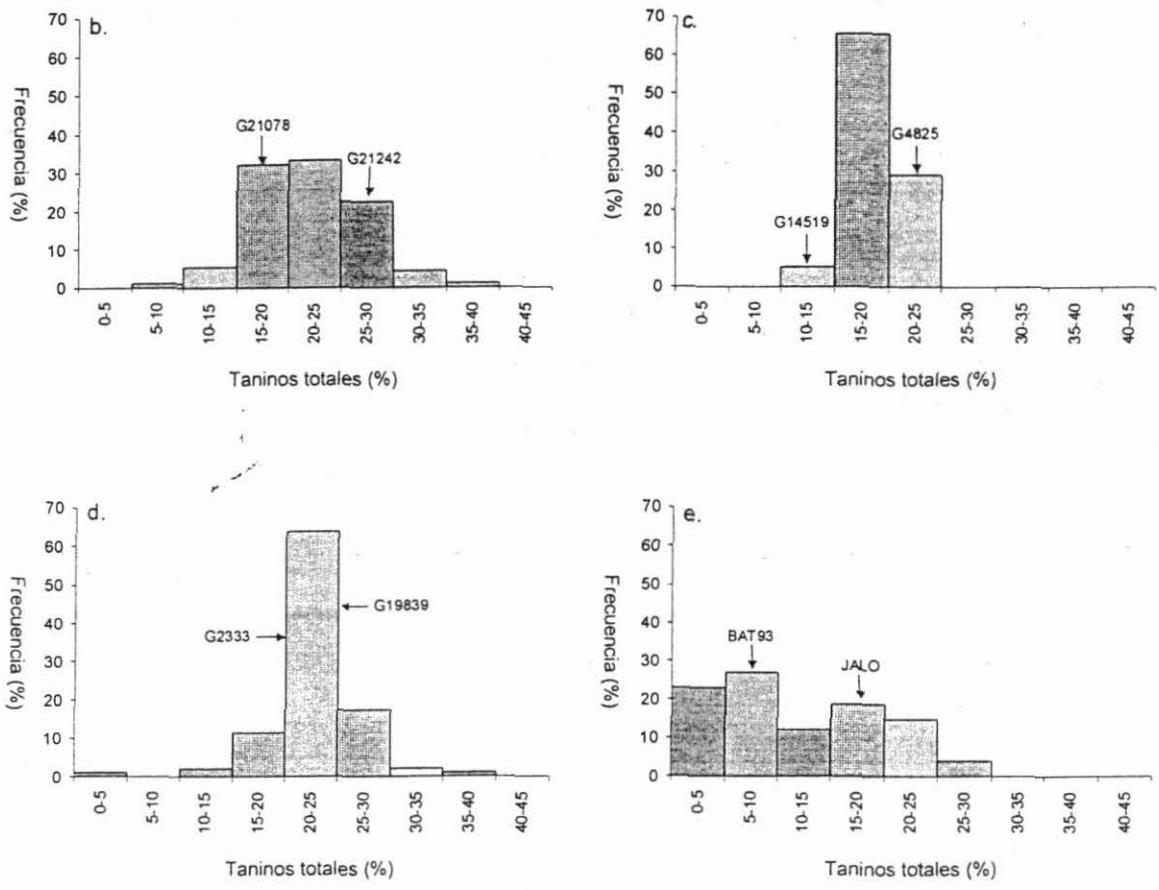

Figure 1. Population distribution for total condensed tannins in four populations of common bean: a. G21242 x G21078, b. G14519 x G4825, c. G2333 x G19839, d. BAT93 x JALO EEP558.

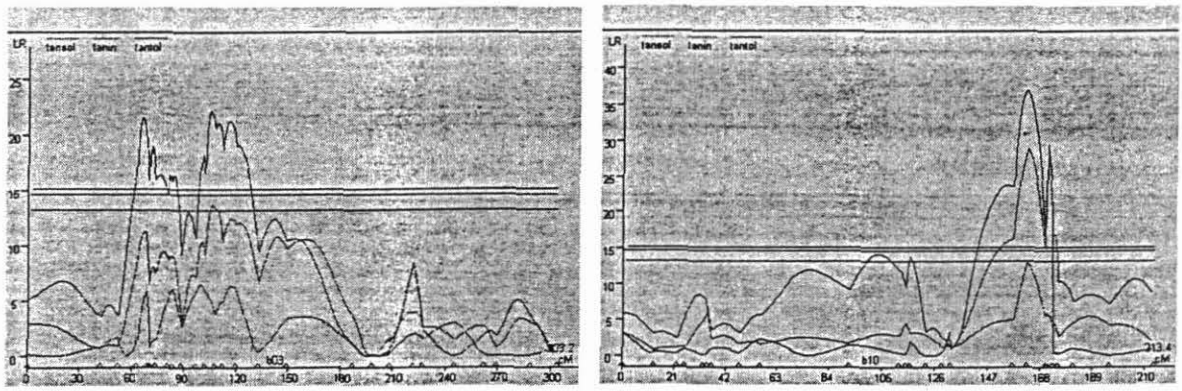

Figure 2. Quantitative trait loci (QTLs) for tannin content detected with composite interval mapping analysis in the DOR364 x G19833 mapping population of common bean.

1.3.3 Adaptation and use of SCAR markers for marker assisted selection of two anthracnose resistance genes in Andean bean breeding

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Introduction

Anthracnose, caused by the fungal pathogen *Colletotrichum lindemuthianum*, is a serious biotic constraint on common bean (*Phaseolus vulgaris*) in many areas of East Africa, Central America and South America. A set of differentials and the mapping population BAT93 x Jaló EEP558 have been used to identify over a dozen genes and QTLs affecting resistance/ susceptibility reactions of different races of the pathogen to different common bean genotypes. Co –evolution is known to have occurred between fungal pathotypes and the two gene pools found in common bean – so that Andean races attack Andean genotypes and Mesoamerican races attack Mesoamerican genotypes, while Andean genotypes resist Mesoamerican races and Mesoamerican genotypes resist Andean races. Therefore the best sources of resistance for breeding programs is often found in the complementary gene pool and genes for resistance must be introgressed through wide crosses and recurrent selection or backcrossing between Andean and Mesoamerican genotypes. Our objective in this study was to introgress two important resistance genes against Andean races of Anthracnose into Andean climbing bean breeding lines from their Mesoamerican sources. The principal source used for this work was G2333, a climbing bean landrace from Mexico that is known to have several target genes, namely *Co-4*^{2} and *Co-5* (Young et al., 1998).

Materials and Methods

Plant Material: Two extraction techniques were used: 1) Alkaline Extraction (a high-throughput "microprep", 96-well format method based on alkaline lysis of fresh leaf tissue disks) and 2) Miniprep (ammonium acetate based method using liquid N2 ground tissue from newly-emerging trifoliates). A total of 574 genotypes were extracted with the first method and 140 genotypes were extracted with the second method. The first group consisted in advanced lines from the Andean bean breeding program, while the second group consisted in advanced lines, breeding lines, backcross derived families and anthracnose differentials as controls. Alkaline extraction DNA was diluted 1:1, 1:5, 1:10, 1:20, 1:30 and 1:50 to determine the optimum concentration for amplification, while miniprep DNA was diluted 1:10.

SCAR markers: A set of four SCAR markers were used in this study to target the two resistance genes: SAS13 (Young et al., 1998), SH18 and SBB14 (Awale and Kelly, 2000)

for *Co-4*^{2} and SAB3 (Vallejo and Kelly, 2000) for *Co-5*. PCR amplification protocols were modified to provide a touchdown annealing temperature that worked best with the diluted alkaline extraction DNA.

Results

All four SCAR markers worked well with miniprep DNA diluted 1:10 and presented the expected size band (Table 1) in the correct source genotype (G2333) and other sources of the same gene or alternate allele of the same gene. In the case of SAS13 a band was amplified in the *Co-4* containing genotypes: G-2333, TO, PI, Widusa. This agrees with the results of Young et al. (1998) who found that the marker was not specific to G2333 and other *Co-4*^{2} containing genotypes but also amplified a band in several genotypes that have the *Co-4* allele. Meanwhile in the case of the co-dominant marker SBB14 which was reported to only produce the expected band in G2333 and derived genotypes according to Awale and Kelly (2001) there was also amplification of the resistance-associated allele in the genotypes AB136, Cornell 47-292 and TO, in addition to G2333. The other specific marker from Awale and Kelly (2001), SH18, behaved as expected and amplified a band exclusively in G2333. The *Co-5* marker, SAB3 amplified in both G2333 and TU, as expected from the report of Vallejo and Kelly, (2000).

Alkaline extraction DNA at 1:1 or 1:5 dilutions as template for PCR reactions, was not as reliable as miniprep DNA for SAS13, SBB14 or SH18 amplification but was for SAB3 amplification, which produced the expected bands for the genotypes G2333 and TU at this concentration (Table 1). Increasing the dilution to 1:20 was effective for the amplification of the correct band in G2333 by the markers SAS13 and SBB14 although band intensity was still low (Figure 1). The SAS13 marker which was reported by Young et al. (1998) to amplify a number of Co-4 containing genotypes and which did so when miniprep DNA was used only produced a band for G2333. The SBB14 marker which was reported by Awale and Kelly (2001) to be co-dominant became a dominant marker when alkaline extraction DNA was used but still amplified the correct resistance-associated band in G2333 and AB136. Other dilutions were also tried, including 1:30 and 1:50, but only SAB-3 was observed to amplify the correct genotypes (G-2333 and TU) under all dilutions (data not shown). With alkaline extraction DNA, the most effective annealing temperature for SAS13 was 68°C rather than the 72°C as previously reported (Young et al., 1998).

Table 1. Results of dilution series tests on SCAR marker amplification in the 12 Anthracnose differential genotypes.

Marker	Size bp	Gene	MP	Alkaline		
			Dil. 1:10	Dil. 1:5	Dil. 1:10	Dil. 1:20
SAB-3 (5.9cM)[2]	400	Co-5 (dominant)	+	+ (Tu only)	+ (Tu, G2333)	+ (Tu, G2333)
SAS-13 (0cM)[3]	950	Co-4 Co-4 (dominant)	+	-	-	+ (G2333)
SBB-14 (5.89cM)[1]	1150-1050	Co-4^2 (co-dominant)	+	-	-	+/- (G2333,AB136)
SH-18 (4.2cM)[1]	1100	Co-4^2 (dominant)	+	-	-	-

1. Awale, H.E., and J.D. Kelly. 2001. Development of SCAR markers linked to Co-4^2 gene in common bean. Annu Rept. Bean Improv. Coop. 44:119-120.
2. Vallejo, V., and J.D. Kelly. 2001. Development of a SCAR marker linked to Co-5 gene in common bean. Annu Rept. Bean Improv. Coop. 44:121-122.
3. Young, R.A., M. Melotto, R.O., Nodari and J.D. Kelly. 1998. Marker assisted disseaction of the oligogenic anthracnose resistance in the common bean cultivar G2333. Theor. Appli. Genet. 96:87-94

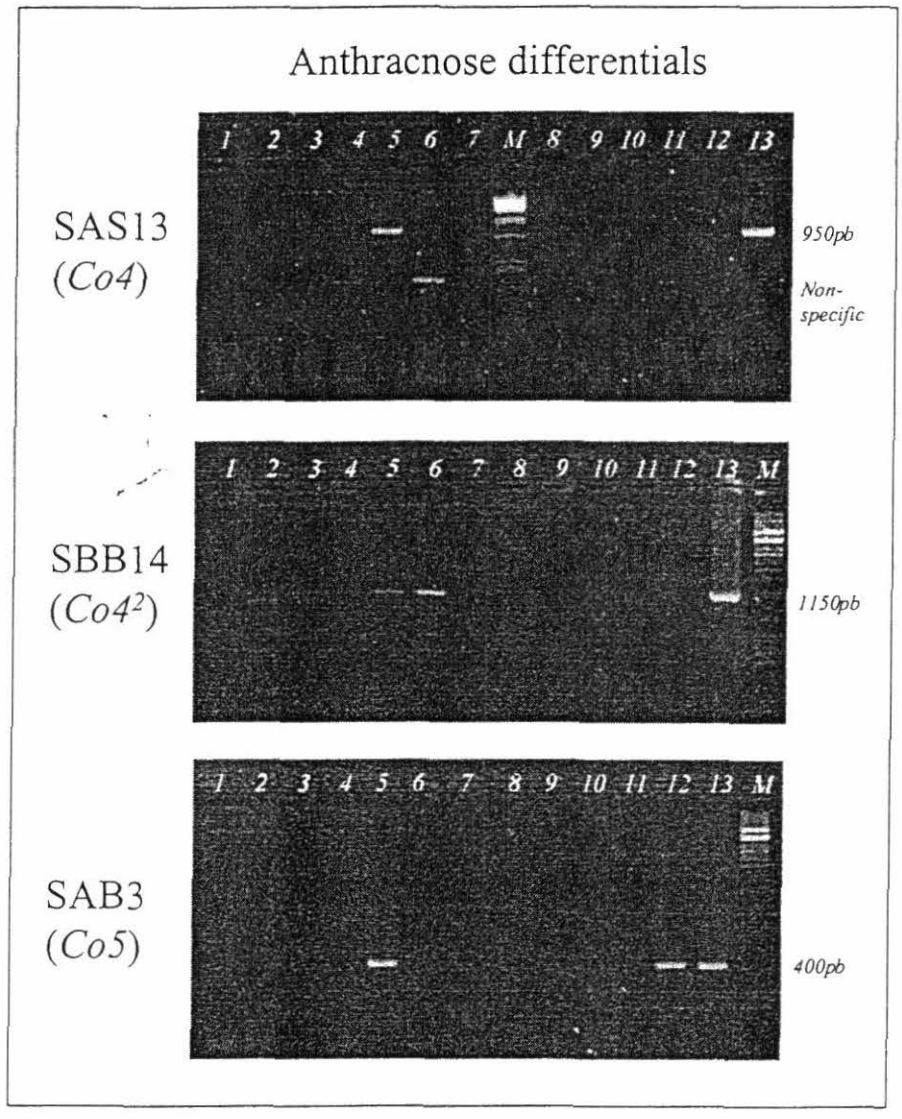

Figure 1.Amplification of Anthracnose SCAR markers using alkaline extraction DNA for twelve differentials (lane 1. MDRK, 2. Perry Marrow; 3. Kaboon; 4. Michelite; 5. G-2333; 6. AB136; 7. Cornell 47-292; 8. Mexico 222; 9. PI207262; 10. Widusa; 11. TO; 12. TU) and one control (lane 13. 1:10 dilution of miniprep DNA for the source genotype, G2333).

1.3.4 Microsatellite-based marker assisted selection of Arcelin-derived bruchid resistance in Andean genotypes of common bean

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Introduction

The Arcelin resistance gene is the most effective resistance factor for the most common storage pests of common bean, namely the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman). We have tested a series of microsatellite markers that are linked to the Arcelin resistance gene and found two to be very effective at distinguishing resistant (of which there are seven variants) and susceptible alleles. Last year we tested these two markers using both miniprep and alkaline extraction derived DNA and found that miniprep DNA worked best and that Pv-ATCT001 (M68913) was the most closely linked marker. This year we improved the amplification conditions for alkaline extraction derived DNA so as to test for marker assisted selection in the field with a total of 261 genotypes segregating for Arc 1 resistance allele. 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 microsatellite over the time-consuming protein based selection was that it was amenable to high-throughput, rapid analysis.

Methodology

Genetic materials and DNA extraction: A total of 261 advanced lines from two families of red mottled bush beans from the Andean bean breeding program were planted in Darien in the 2004A season. DNA was extracted by alkaline lysis from leaf disks harvested into 96 well plates that were packed on ice and processed at the CIAT marker lab. The alkaline extraction techniques is a rapid, high-throughput "microprep" method based on alkaline lysis. The resulting DNA was diluted by 1:10 in sterile water before being used in PCR reactions.

Microsatellite markers: The microsatellite marker Pv-ATCT001 (M68913) was used for the marker assisted selection scheme. Microsatellite amplifications were conducted according to standard PCR protocols. Microsatellite amplifications were run at 1800 volts, 120 Wats and temperature of 45C for one to two hours on 4% polyacrylamide gels and silver-stained with a re-circulating tank system. Alleles were identified as reported in the 2002 annual report for the parental materials used in multiple crosses to generate the advanced lines tested this year.

Results and Discussion

The microsatellite Pv-ATCT001 (M68913) presented only two alleles. This was an advantage for multiplexing. In addition, the marker presented a unique allele for the parent that provided Arcelin 1 (RAZ44) so was used as a diagnostic test for this widely used allele of the Arcelin gene. This marker produced single amplification products where the resistant allele of Arcelin 1 was associated with the 190 bp band while all susceptible alleles were associated with the 195 and/or 200 bp bands. The amplification of Pv-ATCT001 with alkaline extracted DNA (Figure 1) was comparable to the previous amplifications with miniprep DNA. As marker assisted screening proceeded, improvements were made in the experimental technique resulting in successful amplification of over 96% of the genotypes using Pv-ATCT001 (only 10 null alleles were registered among the 261 tested genotypes and these were not scored).

Marker assisted selection proved useful for screening the advanced lines derived from single plant selection in the F5 generation. Of the overall total of 261 advanced lines screened from the two cross combinations, a total of 58 positives were selected with 161 negatives and 32 heterozygotes (Table 1). In the case of the first cross A36 x (A36 x ((RAZ44 x ROYAL RED) x (CATRACHITA x WILK2))), the segregation ratio fit the expected 3:1 negative:positive ratio for the test crosses between the negative recurrent parent A36 and the positive Arcelin1 containing heterozygotes that had been selected by the Bean Entomology project in the F1 generation using the protein assay. These results show that the two assays can be combined effectively in a breeding program and the strength of the molecular assay in screening a large number of advanced lines.

While it appeared that all the lines derived from the cross A483 x ((MAR1 x RAZ50) x (PVA9576-34-1 x G 17340)) were fixed, a substantial number of the lines derived from the cross A36 x (A36 x ((RAZ44 x ROYAL RED) x (CATRACHITA x WILK2))) were scored as heterozygous. The larger than expected number of heterozygotes may be a misinterpretation of a faint amplification product for the upper band (Figure 1). We will confirm whether these genotypes continue to segregate or whether the marker assay is sensitive to DNA quality or mixtures.

Future work

- Improve the efficiency of the screening technique, adapting additional arcelin linked microsatellites to the alkaline extraction technique.
- 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 microsatellite screening for the Arcelin resistance gene in two families of Andean red mottled bush bean lines.

CROSSES	Positive	Negative	n. a.	Het	Overall
A36 x (A36 x ((RAZ44 x ROYAL RED) x (CATRACHITA x WILK2)))	42	161	10	32	245
A483 x ((MAR1 x RAZ50) x (PVA9576-34-1 x G 17340))	16	0	0	0	16
Grand Total	58	161	10	32	261

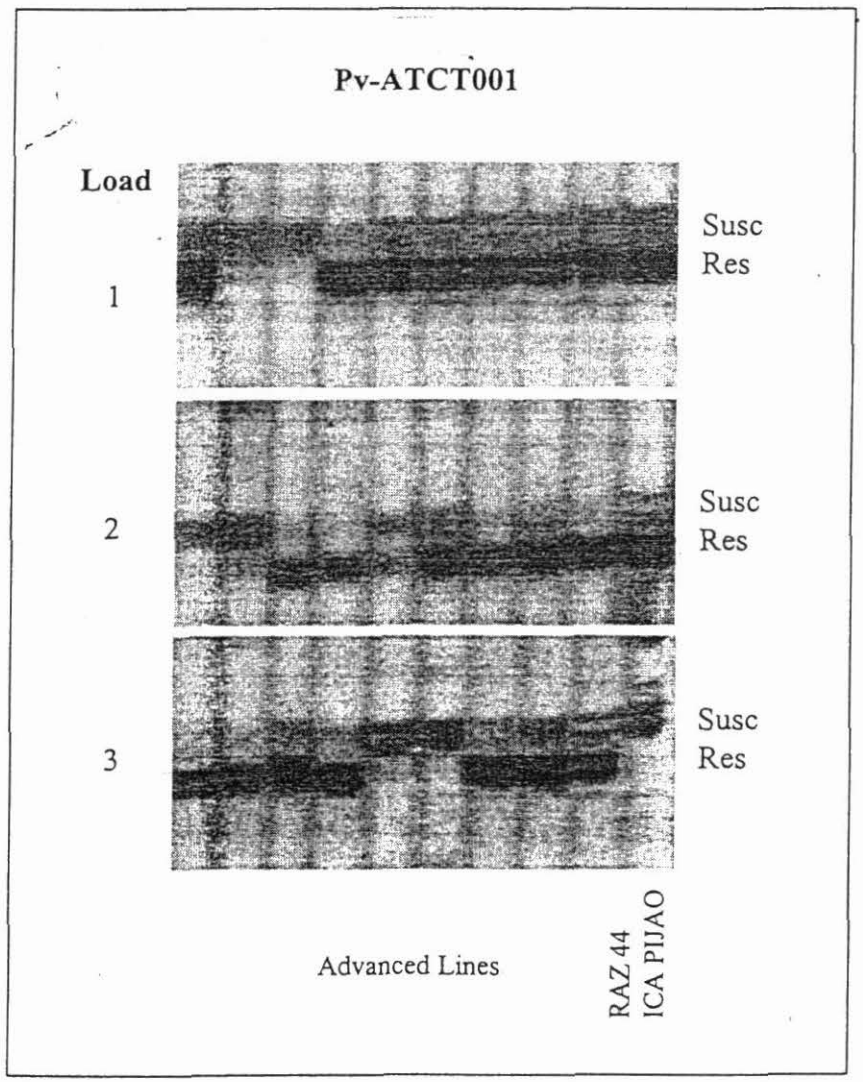

Figure 1. Examples of the marker assisted selection for the Arcelin gene in Andean red mottled bush bean lines. Control genotypes were RAZ44 (Arc1 positive) and ICA Pijao (Arc1 negative).

1.3.5 Development of SCAR and microsatellite markers for Apion resistance

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Introduction

The bean pod weevil (*Apion goodmani* Wagner) is a destructive insect pest 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 objective of this research was to create additional SCAR markers linked to the genes controlling resistance in the recombinant inbred line (RIL) population derived from Jamapa x J117.

Methodology

Plant material: Plant material consisted in susceptible and resistant bulks (4 lines each) and the Jamapa x J117 with 104 F5 derived recombinant inbred lines (RILs). Jamapa is a susceptible cultivar released in Mexico and J117 is a resistant breeding line. The population has been evaluated in Mexico for Apion resistance over five seasons.

RAPD cloning: RAPD bands were purified from 1% low melting point agarose gels using Wizard PCR prep purification system (Promega). The purified insert DNA was cloned into the PGEM-T easy vector system for further analysis. Several recombinant clones were picked per ligation reaction, checked with *Eco*RI digestions, and their inserts sequenced using standard techniques, T7 and Sp6 primers, Big Dye sequencing kits and an ABI377 DNA sequencer.

SCAR primer design and CAPs assays: Specific primers were designed for each unique cloned RAPD 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, *Cfo*I, *Hae*III, *Hsp*92II, *Mbo*I, *Rsa*I and *Sau*3AI) to convert the markers into CAPS (Cleaved Amplified Polymorphisms).

Results and Discussion

A total of seven RAPD bands, U11400R, F10500S (on linkage group b01), M12800S (b07), C1800S (b08), B11400R, R2012CS (b11) and W6800R (unlinked), that were polymorphic from the parental survey and which were significantly associated with the resistance phenotype were selected for cloning. This brings to a total of nine the bands that have been targeted including W91300S and Z4800R which were converted to SCARs last year. BLAST searches identified homologies for several of the nine cloned RAPD bands (Table1). Several clones had homologies to retrotransposons from a range of dicotolydenous species. This is a common feature of cloned RAPD bands. Two of the clones were similar to gene sequences from soybean: 1) Z4-800R with similarity to an unknown gene and 2) F10500S with similarity to a seed coat peroxidase.

SCAR and CAPS development is also summarized in table 1. A total of 15 primer sets were designed for the nine RAPD band sequences and these were tested on the population parents and on the bulks. Most of the PCR products of these primer sets, except those derived from W6800R, were monomorphic as SCARs. A single SCAR (W6800R) showed a polymorphic fragment with clear positive and negative signals in PCR amplification (Figure 1). All monomorphic SCARs were tested with frequently-cutting restriction enzymes (all with 4 bp recognition sites). CAPs polymorphisms were revealed for four of the PCR fragments (W91300S, C1800S, B11400R and R2012CS) when digested with different restriction enzymes, two of the fragments being polymorphic with *Alu*I digestion while one each was polymorphic with *Rsa*I or *Taq*I digestion. All the polymorphic SCAR and CAPS markers genetically mapped to the same locations as the original RAPD bands from which they were derived.

Unfortunately we were not able to make a polymorphic marker for the peroxidase gene that we found in the cloning process (F10500S). The peroxidase represents an interesting candidate gene for providing insect resistance because peroxidases have been involved in hypersensitive response and have been shown to be up-regulated by wounding. Given that *Apion goodmani* is a pod borer whose main site of action is at developing seed within the pod placenta and that beans resist the pod borer through a modified hypersensitive response, the peroxidase may be one of the mechanisms common bean uses for resistance to this pest. Therefore, we will continue to pursue this mechanism of resistance and try to genetically map the peroxidase gene. Among other results worth highlighting is the potential of the new markers to dissect the inheritance of resistance and to help with marker assisted selection.

Future Plans

- The peroxidase gene markers will be converted into polymorphic SNP markers so as to genetically map this gene in the bean genome.
- QTL analysis will be carried out when phenotypic data is available for the entire set of recombinant inbred lines which is expected for 2005.
- We will test the ability of the markers to be used in marker assisted selection.

Table 1. Development of SCAR and CAPS markers from RAPD bands that were significantly associated with *Apion goodmani* resistance in common bean.

RAPD band	Linkage group	Aprox. Size	Blastx results	No. of primer sets	SCAR polym	CAPS polym.
W91300S	b01	1300	Retrotransposon	1	-	+ AluI
Z4800	b01	800	Unknown gene *Glycine max*	1	-	-
U11400R	b01	1400	NS	1	-	-
F10500S	b01	500	Peroxidase *Glycine max*	4	-	-
M12800S	b07	800	NS	1	-	-
C1800S	b08	800	NS	1	-	+ RsaI
B11400R	b11	1400	Retrotransposon *Cicer*	2	-	+ TaqI
R2012CS	b11	1200	Retrotransposon *Arabidopsis*	2	-	+ AluI,MboI
W6800R	unlinked	800	NS	2	+	na

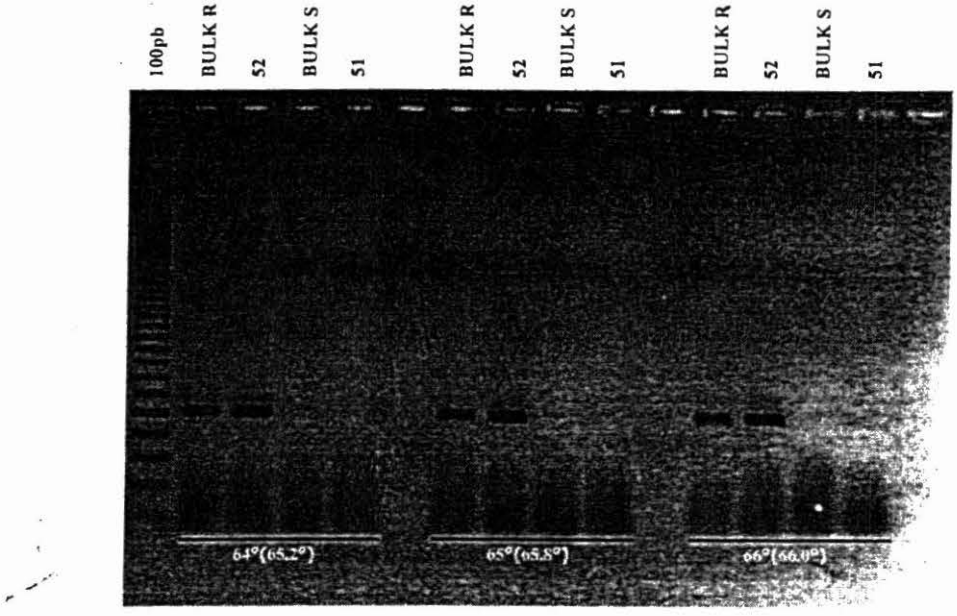

Figure1. Dominant SCAR for resistance based on the W68C RAPD showing amplification in the resistant parent no. 52 and in the resistant bulk at a range of Annealing temp. gradient =55° - 66°C.

1.3.6 Evaluation of geminivirus resistance sources for SCAR and microsatellite polymorphisms

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Introduction

Bean golden yellow mosaic virus (BGYMV) is an important disease of tropical lowland bean production areas caused by a member of the Geminiviridae family. Symptoms include intense yellowing, pod deformation, stunting and flower abortion that cause important and often devastating yield loses. BGYMV is transmitted by the sweet-potato white fly (*Bemisia tabaci*) a widespread and cosmopolitan pest that is often found on a wide range of horticultural crops as well as tobacco, soybean and common beans. The disease is endemic in Brazil, Central America and the Caribbean. Breeding for resistance to the virus has been the most effective strategy for controlling the disease since pest control is expensive and impractical given the rapid lifecycle of the insect and the repeated development of insecticide resistance. A few resistance genes have been identified in common beans (*P. vulgaris*) and these function by attenuating symptoms and yield losses

when the plant is infected. In the case of a few relative species (*P. acutifolius* and *P. coccineus*) there appears to be immunity. Most of these resistance genes remain under-utilized or have only been bred into a limited pool of advanced breeding materials or cultivars. For example several BGYMV resistance genes originated in the Mesoamerican genepool and these are only now beginning to be transferred to the Andean gene pool. Given this, there is an important role for marker assisted selection to play in encouraging the use of and effecting the transfer of BGYMV resistance genes. Pyramiding of resistance genes is important for the greatest protection from the disease's symptoms. Our objectives for this study was to screen all the sources of geminivirus resistance for polymorphic microsatellites from the linkage groups which are thought to be involved in resistance and to evaluate all the SCARs developed for geminivirus resistance against this same panel of genotypes.

Methodology

Plant material and DNA extraction: A panel of genotypes was constructed with all the known sources of resistance to BGYMV (Table 1). A total of 16 genotypes were included and represent the parents of six populations that have been used to determine the genetic control of resistance (two at CIAT and four elsewhere). A miniprep DNA extraction technique was used.

Marker genotyping: Microsatellites were selected from linkage groups b04, b06, b07, b08, and b10 based on central CIAT map for the DOR364 x G19833 population and previous reports that suggested that these regions of the genome were important in virus resistance in common beans. The second set of markers were SCARs related with virus resistance developed by other authors, including SR2 (CIAT, unpublished, Singh et al., 2001), SW12 (Miklas et al. 2000) and SAS 8 (Larsen et al., 2004). Microsatellites were run on 4% PAGE gels while SCARs were run on 1.5% agarose gels.

Results and Discussion

Among the SCAR markers, SR2, which is linked to the *bgm-1* gene, was co-dominant as expected, showing a resistant associated band (530 bp) and a susceptible associated band (570 bp) (Figure 1). The genotypes Morales and DOR476 had the resistant associated band, as did the BGYMV resistant bulk R2. All the other genotypes and bulks had the susceptible associated band for this marker. Since the *bgm-1* gene is known to have originated in a specific Durango landrace it is only found in genotypes derived from this original source through many of the CIAT breeding lines that were bred to contain this resistance gene which provided the best levels of control for severe yellowing symptom caused by BGYMV.

The SW12 marker, linked to a QTL for BGYMV resistance on linkage group b04 was dominant and present in the genotypes Arroyo Loro, DOR303, DOR364, DOR476 and SEL1309 and Raven most of which are genotypes that have been bred for BGYMV resistance (Figure 1). The same band was also present in both the resistant and susceptible bulks R1, S1, R2 and S2 (data not shown). Light bands were amplified for G122 and

Montcalm, two Andean genotypes that have not been improved for BGYMV and would not be expected to have the band. A new banding pattern (allele) with two bands, one higher than the expected band and one lower than the expected band, was observed in A55.

The SAS8 marker developed for the *Ctv*-1 gene for resistance to beet curly top virus was dominant and was present in Morales, Arroyo Loro, A55, Montcalm, DOR476, DOR 364, XAN176, DOR303 and Raven. This marker was reported by Larsen et al. (2003) to amplify a band in all Mesoamerican genotypes and to not amplify a band for all Andeans. This seems to be the case in our survey where most of the positive genotypes are typically Mesoamerican and where the two positive Andean genotypes, Montcalm (S Phaseolin) and DOR303 (Pedigree A25 x Redkloud), are the result of Andean x Mesoamerican crosses.

In the microsatellite survey, the parental combinations varied in their level of polymorphism (Table 2). As expected the Andean x Mesoamerican cross DOR364 x G19833 was the most polymorphic. The cross DOR303 x IJR was almost as polymorphic as DOR476 x SEL1309, while the population Arroyo Loro x X0157-4 (G35172) had the lowest polymorphism rate. The number of microsatellite per linkage group varied from 5 to 12 and the relative polymorphism in different parts of the genome varied as well.

Future work

- Based on parental polymorphism and bulked segregant analysis, we will select microsatellite markers for mapping on the populations represented by this survey.
- QTL analysis on the selected populations.

Table 1. Evaluation of SCAR markers on the geminivirus parental survey.

No.	Genotype	Gene pool[1]	BGYMV reaction	SCAR[4] SAS8	SCAR[4] SR 2		SW 12
					530 (R)	570 (S)	
1	Morales	Meso	S	+	+	-	-
2	G35172	P.coccineus	R	-	-	+	-
3	Arroyo loro	Meso	S	+	-	+	+
4	X 015741	Meso	S	-	-	+	-
5	Bulk R1	--	R	+	-	+	+
6	Bulk S1	--	S	+	-	+	+
7	A55	Meso	S	+	-	+	NA[3]
8	G122[2]	Andean	--	-	-	+	+
9	Montcalm[2]	Andean	--	+	-	+	+
10	DOR476	Meso	R	+	+	-	+
11	SEL 1309	Meso	S	-	-	+	+
12	Bulk R2	--	R	-	+	-	+
13	Bulk S2	--	S	-	-	+	+
14	G19833	Andean	S	-	-	+	-
15	DOR364	Meso	R	+	-	+	+

16	XAN 176	Meso	S	+	-	+	-
17	DOR 303	Andean	S	+	-	+	+
18	IJR	Andean	S	-	-	+	-
19	Raven[4]	Meso	S	+	-	+	+
20	I 9365-31	Meso	--	-	-	+	-

Abbrv: 1/ MA: Mesoamerican, A: Andean, R: Resistant, S: susceptible 2/ BCTV-resistant (G122) and partially resistant (Montcalm) checks 3/ new allele observed 4/ BCMV resistant check

Table 2. Number of polymorphic microsatellite markers in each linkage group for each population and overall percentage population polymorphism.

Population	Number of polymorphic markers					% polymorphism on each population
Linkage Group	b04	b06	b07	b08	b10	
No. of markers tested	n = 11	n =12	n=9	n = 5	n = 5	
Arroyo Loro x X0157-4 (G35172)	2	2	2	2	1	23
DOR 476 x SEL 1309	2	7	7	2	2	49
DOR 364 x G19833	8	9	8	5	5	83
DOR 303 x IJR	4	4	5	2	2	41

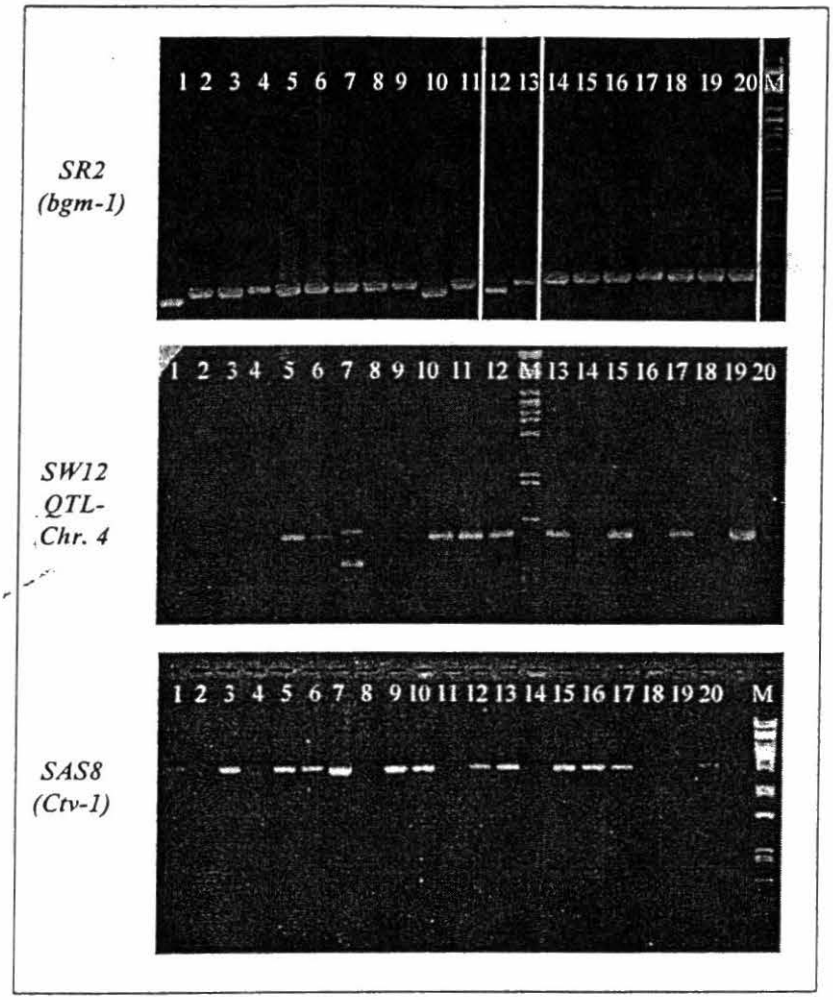

Figure 1. Parental survey of geminivirus resistance sources.

1.3.7 Marker assisted selection for BGYMV resistance in small-seeded common bean

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Introduction

Common bean (*Phaseolus vulgaris* L) is, among four other *Phaseolus* species (*P. coccineus, P. acutifolius* and *P. polyanthus*), the most important and widely cultivated. It originated in Latin America were more than four million tons are produced per year with a consumption of 10-20kg.per capita. Among several bean production problems, diseases caused by whitefly-transmitted viruses are some of the most important factors reducing crop production in regions between 0-1000 m.a.s.l (Morales, 2000). As in previous years a marker assisted selection breeding scheme has been applied using two SCAR markers linked to the resistance to bean golden yellow mosaic virus (BGYMV).

Materials and Methods

Samples from individual F_1 plants were collected directly from the field into microtiter plates. Alkaline DNA extraction was performed for M.A.S. purposes as described previously (Quintero *et al.*, 2002)

Usually W12 and *bgm-1* SCARs were amplified independently. This year, multiplex PCR (amplification of both markers in the same PCR reaction) was standardized using different primer pairs for W12 designed last year (Quintero *et al.*, 2003).

Once annealing temperatures and oligonucleotide concentrations were standardized, multiplex PCR was carried out in a set of 4498 F_1 plants of multiple crosses and amplified products were resolved in a 0.5X TBE agarose gels stained with ethidium bromide at a final concentration of 0.02µg/ml. Presence or absence of both SCAR markers was scored.

Results and discusion

M.A.S. for introducing BGYMV resistance in small-seeded beans continued as reported last year and then F_1 plants belonging to forty-two segregant populations (BGMV code 608 to 649) were screened for the presence of *bgm-1* and W12 markers.

Last year a multiplex PCR of these markers was proposed to speed up MAS process. Some assays were conducted using 27 varieties and pure DNA so that a new primer set for W12 was chosen for being more specific than the original sequence (Quintero *et al.*, 2003). When working with alkaline DNA extractions, some modifications of PCR profiles and/or reagent concentrations have to be done. So when trying multiplex PCR, original primer

pairs of W12 markers yield more confident results since the new primers pairs proposed last year did not segregated as expected.

Then, after standardization of oligonucleotide concentration and annealing temperatures, multiplex of *bgm-1* and W12 SCAR markers was applied to a set of 4498 plants permitting some savings in time and reagents (Figure 1).

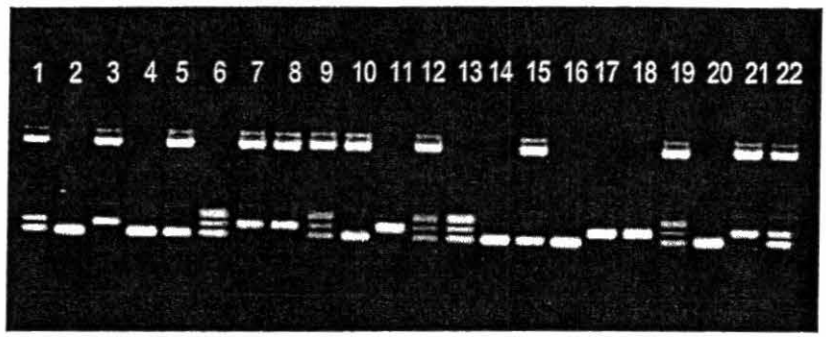

Figure 1. **Multiplexing W12 and DOR21 markers for BGYMV resistance. Lane 1 and 22, Tio Canela 75 showing W12 (730 bp) and *bgm-1* bands, susceptibility allele (upper) and resistance allele (lower); Lanes 2-21, segregation of the markers in one of the F_1 crosses evaluated**

This year, selection was stricter than in previous ones. Only 1182 plants (27%) having both markers were selected (Table 1), contrasting with previous years when 45 to 50% of the plants were chosen. This group (1182) was then checked for seed quality and another F_1 plants were discarded. The resulting 580 selections belonging to 32 multiple crosses were planted under drought stress where 157 were chosen for their best performance and will be evaluated for disease resistance.

Table 1. Number of F_1 families expressing or lacking two markers for resistance to BGYMV.

		State of *bgm-1*		
		Present	Heterocygote[§]	Absent
State of W12	Present	1086	96	797
	Absent	1080	73	1366

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

Conclusion and On-going activities

The *bgm-1* gene and QTL linked to BGYMV resistance are being introduced through marker-assisted selection using multiplex PCR on small seeded families also tolerant to main biotic and abiotic constraints to bean production.

Looking for SNPs in DNA fragments linked to biotic or abiotic stresses could be a strategy to improve molecular breeding in beans and other crops.

References

Quintero, C; H. Terán, S. Beebe and J. Tohme. 2003. Marker assisted selection for BGYMV resistance in small-seeded beans. SB-2 Project, Annual Report 2003, CIAT. 105-106p.

Quintero, C; H. Terán, E. Tovar; S. Beebe and J. Tohme. 2002. Marker assisted selection for BGM resistance in common bean. SB-2 Project, Annual Report 2002, CIAT. 103-104p.

Morales, Francisco José. 2000. El mosaico dorado y otras enfermedades del frijol común causadas por geminivirus transmitidos por mosca blanca en la América Latina. Centro Internacional de Agricultura Tropical. 169p.

1.3.8 Construction of a BAC Library for Phaseolus vulgaris G19833

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Introduction

Large insert libraries are a fundamental resource for map-based cloning, physical mapping and genome characterization. Several large insert libraries exist for common bean but none for Andean dry beans (the first BAC library having been made for the cultivar 'Sprite' which is a snap bean variety). There is a need, therefore, to develop a library for this gene pool. With this in mind we began a project to develop a BAC library for the Andean parent of the CIAT mapping population, G19833, a Peruvian landrace that has proven to have many unique alleles in genotypic screening and many unique characteristics in phenotypic screening. G19833 has good levels of disease resistance (against anthracnose and angular leaf spot) and abiotic stress tolerance (especially for low phosphorus tolerance) as well as being contrasting in nutritional characteristics (both in types of tannins and high mineral content).

Materials and Methods

High molecular weight DNA was prepared for G19833 from fresh, greenhouse-grown leaf tissue. This DNA was embedded in agarose plugs and digested with *Not*I restriction

enzyme before ligation into pCUGI BAC vector. The ligation mix was transformed into electro-competent cells which were plated on Q-plates and grown overnight. A total of 384 clones were picked randomly and analyzed for insert size by *NotI* digestion and visualization on EtBr stained agarose gels after pulsed field gel electrophoresis on a CHEF-DR mapper. Clones were then picked by a Q-bot robot and arrayed into library master plates and then replicated onto 6 x 6 high-density filters. The filters were probed for chloroplast sequences and a random set of clones were sequenced on an ABI3730 to determine BAC end integrity and to characterize their sequences.

Results

The BAC library for G19833 consists of 55,296 clones arrayed onto 384-well plates providing 12 X coverage of the common bean genome based on a genome size of 650 Mb. Average insert size is 142 kb and ranges from 28 to 420 kb for individual BACs based on the sampling of 384 randomly picked clones from the library (Figure 1). Chloroplast contamination is below 4% based on hybridization of chloroplast sequences. The percent of empty vectors is 0.8%.

BAC end sequencing of 384 clones, sequenced from both the 5' and 3' ends uncovered a number of microsatellites and retrotransposon sequences that will serve to physically anchor BACs to the genetic map.

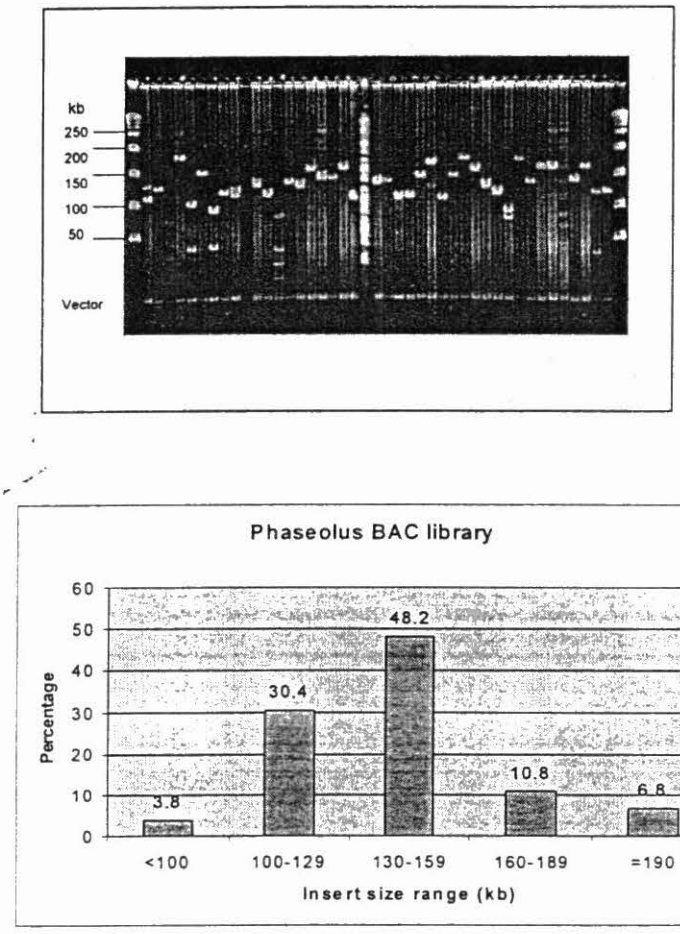

Figure 1. Characterization of the G19833 BAC library.

Future Work

- Our objective for next year will be to utilize the library in characterization of gene families involved in these traits for which physical mapping will link QTL analysis to candidate genes in regions of interest. We plan to focus first on genes of interes for nutritional quality.
- It will be of interest to anchor selected BAC to the CIAT (DOR364 x G19833) and UC-Davis (BAT93 x JaloEEP558) integrated genetic maps.
- We also plan to compare sequences between comparable sites in the genomes of Mesoamerican and Andean representative using this library and others that have been developed for Mesoamerican genotypes.

1.3.9 Single Nucleotide Polymorphisms in CIAT's crops: Genotyping implementation on Luminex-100

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Introduction

SNPs have been shown to be the most abundant type of molecular genetic markers in the genome (Cho et al. 1999) and are quickly becoming the marker of choice in agricultural research, especially for use in high-throughput marker-assisted breeding (Rafalski 2002). In plants, studies on the occurrence and nature of SNPs are beginning to receive considerable attention, particularly in Arabidopsis. In this plant more than 37000 SNPs have been identified through the comparison of two accessions (Jander et al. 2002). In soybean, the presence of 280 SNPs in 143 amplicons totalling about 76.3 kpb of DNA sequence has been reported (Zhu et al. 2003). Because of the high importance given to this kind of molecular markers at the present, the implementation of flexible and reliable high-throughput technologies for analyzing SNPs is key for their wide use in mapping and biodiversity studies. Here we present the implementation for CIAT of Single Base Extension Primer (SBEP) protocols used on a Luminex-100 for common bean and rice.

Methodology

Plant Material: Ten accessions from *Phaseolus vulgaris* used initially for SNP discovery (Gaitán and Tohme, 2002) were used to standardize and validate SNP genotyping on Luminex-100 for 32 Single Base Extension Primers (SBEP) developed from *Phaseolus vulgaris* and 75 SBEP developed from soybean (Quigley, personal communication). Additionally, 16 individuals from rice were used to standardize 16 SBEP published by Nasu et al., 2002, for genotyping on Luminex-100.

SNP detection: The SNP detection was carried out using Single Base Extension Primer (SBEP) methodology as described by Chen et al (2000). This protocol was standardized with some modifications by Cregan at BARC-USDA. Between 5-9 PCR reactions containing SNPs were pooled and cleaned up for primers and dNTPs using shrimp alkaline phosphatase and exonuclease I enzymes. Then, the clean PCR reactions were hybridized with a specific SBEP, which have attached to their 5 end a 25 oligonucleotide (ZIP code or tag) for future detection with microspheres. The single base extension was carried out using ddNTPs (one labeled with biotin) and Thermosequenase in a PTC-100 thermocycler, Once the extension was done, a complementary sequence (cZipCode) to the 25 oligonucleotide, which has been coupled to a specific fluorescent microsphere, were hybridized to the extension product for 30-45 minutes at 54°C in presence of 1X TMAC. Streptavidin-phycoerythrin is conjugated to the biotin labeled extension product for detection. The reactions were read in a flow cytometer (Luminex 100), which detects each

microsphere by its unique fluorescent signal and the presence or absence of the SNP (streptavidin-SBE product). Data were analyzed with Masterplex GT (Miraibio Inc.) package in which the mean fluorescence intensity emitted by each of the samples is analyzed and used to define the SNP alleles belonging to each genotype.

Results

SNPs from *Phaseolus vulgaris*: 32 SBEP were designed from different CDs and introns. Only two of them belong to the same gene. From this 32 extension primers, 25 (78%) has been standardized using SBEP methodology on Luminex-100 with less than 5% of miscalling or nor signal. From this standardized set, only one SBEP was monomorphic between all ten controls and this result was reconfirm by checking the original sequence which showed low quality values in this base.

SNPs from soybean on *Phaseolus vulgaris*: 56 SBEP designed by Quigley at BARC-USDA were used on ten common bean genotypes in order to test them for genotyping and mapping on Luminex-100. From this 56 SBEP, 44 (79%) had been standardized on Luminex-100. All this set are polymorphic for DOR364 and G19833 and 22 standardized primers (from soybean) and eight primers (from bean) have molecular data for the progeny in order to be mapped.

SNPs in rice: Fourteen SBE primers were standardized for genotyping on Luminex-100 in two batches of 8 and 6 SBE reactions. Control materials matched very well with the published allele polymorphism. Four primers were polymorphic between BG90 and Rufipogon parentals, four polymorphic for Lemont and Barthii parentals and non-polymorphic primers were obtained for Caiapo and Glaberrima parentals. 87.5% of SBE primers tested were standardized and some of them can be used for mapping on different rice cruces. Additionally, there are 197 SBE primers already published which can be standardized on a Luminex-100 machine following the same procedure as used for this first test.

On-going Activities

- To continue standardizing *Phaseolus vulgaris* and Soybean SBEP
- To map SBEP from *Phaseolus vulgaris* and from Soybean on DOR364 x G19833

References

Chen, J., MA. Iannone, M. Li, J.D. Taylor, P. Rivers, A.J. Nelsen, K.A. Slentz-Kesler, A. Roses, and M.P. Weiner. 2000. A Microsphere-Based Assay for Multiplexed Single Nucleotide Polymorphism Analysis Using Single Base Chain Extension. Genome Res. 10: 549-557.

Cho RJ, Mindrinos M, Richards DR, Sapolsky RJ, Anderson M, Drenkard E, Dewdney J, Reuber TL, Stammers M, Federspiel N, Theologis A, Yang WH, Hubbell E, Au M, Chung EY, Lashkari D,

Lemieux B, Dean C, Lipshutz RJ, Ausubel FM, Davis RW, Oefner PJ (1999) Genome-wide mapping with biallelic markers in Arabidopsis thaliana. Nat Genet 23:203-207.

Gaitán, E. and J. Tohme. 2002. Discovery of SNPs in *Phaseolus vulgaris*. SB-2 Project, Annual Report 2002, CIAT. 105-106.

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post-genome era. Plant Physiol 129:440-450.

Rafalski A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Current Opinion in Plant Biology, 5:94-100.

Zhu, Y.-L, Q.-J. Song, D.L. Hyten, C.P. Van Tassell, L.K. Matukumalli, D.R. Grimm, S.M. Hyatt, E.W. Fickus, N.D. Young, and P.B. Cregan. 2003. Single nucleotide polymorphisms (SNPs) in soybean. Genetics 163: 1123-1134.

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

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CIAT

Funding: The Rockefeller Foundation and CIAT

Important Outputs

Molecular marker analysis of 1291 BC_2 progenies and identification of genotypes that combine resistance to CMD and CGM.
Testing of FTA paper leaf squashes as a replacement for the DNA isolation step in MAS.

Introduction

Perhaps the most powerful use of MAS in cassava breeding is in selection of recombinants that combine many genes, for example resistance to CMD and CGM, without the need for field trials. This could accelerate efforts to combine many traits into elite varieties. During the second year of the implementation of MAS at CIAT, we attempted to combine CMD and CGM resistance, derived from a wild relative of cassava, into genotypes that can serve as parents for introgression of the two traits. This year we also sought to address some of the most labor intensive steps of MAS, namely the tedious DNA isolation step and identification of markers that are useful in a wide range of germplasm. A low cost method for MAS could be PCR amplification of leaf squashes on FTA paper (Whatmann PLC, UK). An attempt was also made to evaluate the feasibility of this approach for MAS on cassava.

Methodology

Four inter-specific F_1 hybrids with resistance to CGM were crossed extensively to MTAI8, SM1741-1, SM1669-5, SM121-9, CM3306-4, and SM1460-1, elite parents of CIAT's cassava gene pools. BC_1 progenies having resistance to CGM, from field evaluations, were crossed to CMD donor parents at CIAT, a total of 1490 BC_2 sexual seeds from into 43 families were obtained. The sexual seeds were germinated from embryo rescue and multiplied (Activity 8.18, this report). DNA isolation and molecular marker analysis was with a SCAR marker obtained from the RAPD marker RME1 located at less than 4cM from the CMD resistance gene (CIAT 2003) or SSR markers, NS74, NS217 and NS260, associated with CGM and 1 or 2 *in vitro* plantlets as described earlier (Mba et al. 2001) .

To reduce the cost of marker genotyping, PCR amplification of leaf squashes on FTA paper (Whatmann Inc., UK) was tested. Fresh leaves were harvested from 15 *in vitro* plantlets, 15 plants in the screen house, and 15 plants from the field, all plants were 2-3 months old. Leaf squashes were made using 0.5-2g of leaf tissue on FTA paper and a 1mm disc excised using a FTA paper punch supplied by the manufacturer (Whatmann Inc., UK). The FTA paper disc was either used directly in the PCR or washed as follows: the disc was transferred to a 96-well PCR plate and 200ul of 70% isopropanol was added and mixed using a pipette, the wash was repeated with IX TE (Tris 10mM, EDTA 1mM) PCR amplifications were conducted with both SCAR and SSR markers as described above.

Results

A total of 1291 plants were successfully established by embryo rescue from the 1490 seeds. Molecular marker genotyping of 1291 progenies from 43 BC_2 families allowed the identification of 335 progenies that combine resistance to CMD and CGM. These progenies have been multiplied and shipped to Tanzania to serve as parents for molecular marker-assisted selection (MAS) introgression of CMD and CGM resistance into local cassava varieties (Activity 8.2, this report). The SCAR marker developed last year for CMD resistance revealed very good results in the analysis of the 1291 BC_2 progenies (Fig1).

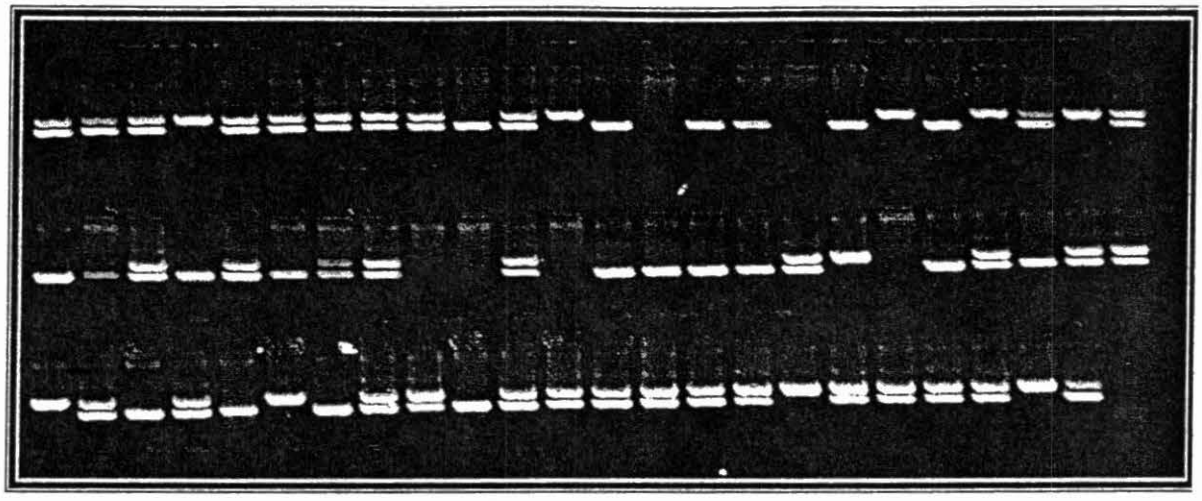

Figure 1. PCR amplification of BC_2 progenies using a SCAR marker developed from a RAPD marker RME1 located at less than 4cM from *CMD2*, the CMD resistance gene. The larger weight allele is associated with resistance

PCR amplification of FTA paper discs with leaf squashes from *in vitro* plants was 100% successful for both RAPD and PCR markers with or without the washing step. Leaf squashes using leaves from screen house or field plants were also 100% successful but only after inclusion of the washing step. Elimination of the washing step lead to a high number of failed PCR reactions suggesting that impurities from matured leaves was inhibiting the PCR reaction. This result suggests that FTA paper leaf squashes could replace cumbersome DNA isolation step.

Conclusion and Future Perspective

Molecular marker-assisted selection of 1291 BC_2 genotypes lead to the identification of 335 lines that combine resistance to CMD and CGM. A low cost method for MAS, PCR amplification of leaf squashes on FTA paper, was also evaluated and found to be feasible. We intend to extend these preliminary experiments to many more plants and also to see the effects PCR amplification of the storage of FTA paper leaf squashes at room temperature for extended periods.

References

CIAT 2003. Annual Report IP3. Improved cassava for the developing world. Pp8-68 to 8-70

Mba R. E.C., P. Stephenson, K. Edwards, S. Melzer, J. Nkumbira, U. Gullberg, K. Apel, M. Gale, J. Tohme, and M. Fregene (2001) Simple Sequence Repeat (SSR) Markers Survey of the Cassava (*Manihot esculenta* Crantz) Genome: Towards a SSR-Based Molecular Genetic Map of Cassava. Theor and Appl Genet 102:21-31

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

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Funding: The Rockeller Foundation

Important Outputs

1) Shipment to Tanzania of 191 genotypes with resistance to the cassava mosaic disease (CMD) and 335 genotypes that combine resistance to CMD and to the cassava green mites (CGM) (derived from a wild relative), up to 10 plants each per genotype. The plants were derived from embryo axes from mature sexual seeds

2) Molecular diagnostics of the introduced material for frog skin disease (FSD) and transfer of the introductions to the field in Tanzania

3) Collection and evaluation of local varieties in Tanzania for crosses to the introductions to introduce resistance to the cassava mosaic disease and green mites into the local varieties.

Introduction

The Tanzanian MAS project funded by the Rockefeller foundation seeks to massively improve farmer preferred varieties for CMD and CGM resistance by crossing introductions resistant to the above disease and pest to local varieties collected all over the country. The resulting large sized segregating populations are reduced with markers and the best genotypes, from the point of view of the farmer and breeder are selected over 2 cycles of parallel evaluations at the research stations and in farmer's field. We report here progress in the project this year. A total of 335 BC_2 progenies (AR lines) that combine resistance to CMD and to the cassava green mites (CGM), derived from a wild relative, and 207 genotypes (CR lines) obtained from crossing CIAT elite parents and CMD resistant lines were introduced from Colombia to Tanzania in three shipments this year. They were transferred to the screen house and while there evaluated for frog skin disease (FSD) and then transferred to the field. They will be evaluated later this season and no less than 60 genotypes selected based on evaluation of highly heritable agronomic traits to be crossed to 90 local varieties selected from all over the country. Molecular markers associated with CMD and CGM will be used to discard much of the resulting segregating populations so that the breeder and farmers can concentrate on a small number of progeny having

resistance to the principal pest and disease and farmer/end-user preferred traits. The Tanzanian MAS project seeks to transfer useful variability from the crop's center of diversity of cassava to Africa. The concept is already being extended to additional NARs in Africa, the AR and CR lines have been shipped to Uganda and Nigeria already in preparation for crossing to local varieties. Several concept notes have been prepared to fund the above efforts this year and if successful should begin next year.

Methodology

Following a decision made by CIAT management to permit direct transfer of cassava germplasm to African NARs without going through a third party, a committee was set up at CIAT to draw up guidelines for the safe transfer of cassava germplasm to Africa. The following recommendations were made by the committee:

1)A request for cassava materials with information regarding the quarantine requirements of the receiving country.

2) Only seeds from mother plants that are free of cassava frogskin disease (FSD) will be used. The mother plants will be inspected for root symptoms in the field. A significant sample of the mother plants will be tested using a diagnostic method appropriate for CFSD.

3) A record of the results of testing will be kept, and one copy will be sent to ICA quarantine officer.

4) Only plants that are placed *in vitro* through somatic embryo rescue will be exported.

5) Permission to export plants must be obtained from ICA.

6) All seed shipments from CIAT are accompanied by a Material Transfer Agreement (MTA).

7) The receiving country will have a quarantine period before the release of these materials in the field. The quarantine facilities should be insect proof in order to be sure that no biological agents from the receiving country are introduced during the quarantine period.

Two different sets of germplasm were shipped: first 191 F_1 genotypes derived from crosses of elite CIAT lines to CMD resistant parents followed by MAS for CMD resistance (CR lines) and 335 BC_2 genotypes obtained crossing CMD resistant lines to BC_1 derivatives of a wild close relative of cassava introgressed with elite CIAT parents (AR Lines). This second set of genotypes combines resistance to CMD and cassava green mites (CGM). Between 5 and 10 plants per genotype were shipped. The tissue culture plantlets were shipped in three batches, November 15 (CR plants), March 23 (AR first batch), and April 29 (AR second batch), to avoid over-loading facilities at ARI-Kibaha where the plants were sent to. On arrival in Dar es Salaam the plants were received by plant quarantine officials from the Tropical Pesticide Research Institute (TPRI, Arusha) and transferred to the tissue culture growth room of ARI-Mikocheni. After 7 days to allow the plants recover, they were moved to the screen house at ARI-Kibaha for hardening according to standard methods laid down at CIAT (Roca et al. 1984). The plants were inspected after one month in the screen house by TPRI officials and at 2 months just before transfer to the field. After the second inspection and further molecular diagnostics, the plants were

transferred to the field at the Alawi estate, a 4000ha sisal plantation owned by the Mohammed Enterprises who are now interested in producing cassava for starch.

In shipping germplasm to Tanzania, the conditions laid down by the CIAT committee on shipment of germplasm to Africa were strictly adhered to. Nevertheless molecular diagnostics for the presence of frog skin disease was carried out while the plants where in the green house to ensure that there was no escape in the germplasm. A molecular diagnostic method for the detection of CFSD based on hybridization of an FSD cDNA clone CFSV-S5 was used. The method is a modification at CIAT of the to dsRNA extraction the Morris and Dodds method (1993??). Briefly, three grams of young leaves or petioles were frozen with liquid nitrogen and homogenized with two volumes of extraction buffer (2X STE, 10% SDS, 1% bentonite, and 0.5% 2-mercaptoethanol) and 0.5 volumes of chloroform:pentanol (24:1). The extracts were centrifuged for 10 min at 8,000 G, and the aqueous phase was collected. Ethanol was added to a final volume of 16.5%, and for each gram of tissue 0.1 g CF-11 cellulose was added. The slurries were stirred for 30-60 minutes and poured into columns and drained completely. The columns were rinsed with 100 ml of 1X STE containing 16.5% ethanol. The column was rinsed with 0.1 ml of 1X STE, and the ds-RNAs were eluted using three 0.1 ml aliquots of 1X STE. The nucleic acids were precipitated with 2.5 volumes of absolute ethanol followed by centrifugation. The pellets were dried and then resuspended in sterile water.
The extracted products are run on 1% agarose gels using TAE 1X. The cDNA CFSD-S5 clone is run on the gels as the positive control. These are denatured in the gel by treatment with 0.05M NaOH and 0.15M, NaCl followed by neutralization in 0.1M Tris-HCl and 0.15M NaCl and transferred to nitrocellulose membranes using buffer 20X SSC. The labeling and detection was done with the Pierce chemiluminescent hybridization and detection kit with CSPD according to the manufacturer's instructions. The hybridization temperature is 42°C and highly stringent conditions are used to wash the filter (68°C), after which it was exposed to film for 15min to 1h.

Local cassava cultivars were this year collected from the principal growing regions by NARS partners in Tanzania. Germplasm collected from the Eastern zone, around Tanga, Kibaha and the coastal areas of Dar es Salaam were established at the Alawi estate in Kibaha. Collections from the south, Matwara, Lindi and Nachigwea districts were established at the Alawi estate in Kibaha, those from the Lake region around Geita, Musoma, Tarime, Muleba and Kasulu districts were established at ARI-Maruku. The experimental design of the trials was a random complete block design with 3 blocks and 10 plants per block. Farmers from the different regions will be invited during evaluations of these collections at harvest to determine the best local land races after their own criteria. Evaluations will be undertaken in September/October 2004. Selections will be planted in a crossing block early next year at the Alawi estate and ARI-Naliendele for genetic crossing.

Results

A total of 191 CR and 335 AR genotypes were successfully shipped to Tanzania. A description of the germplasm shipped and their parents are shown in Table 1 and 2. More than 85% of all plants, and 100% of all genotypes were successfully established in the screen house, a very high percentage of success. Molecular diagnostics carried out for all the introductions revealed that they were free of frog skin disease (Fig 1). Inspections by plant quarantine inspectors from TPRI also revealed an absence of pests and diseases in the plants growing in the screen house. The plantlets in the screen house were transferred to the field, the Alawi estate, in two batches, one set was moved in April 2004 and the second set was transferred in July 2004 (Fig 2). Some plants of 3 CR genotypes in the field showed some symptoms of purple/black discolorations (Fig 3) on the leaves but discussion with CIAT agronomist, Reinhardt Howeler and CLAYUCA agronomist Luis Fernando Cadavid revealed it might be a micronutrient deficiency due to boron or Iron. Application of liquid fertilizer with boron and zinc lead to the elimination of the trait. The introductions will all be harvested in the March/April period and evaluated emphasis will be placed on high heritability traits like dry matter content, harvest index, plant architecture, and production of quality planting materials. About 60 genotypes will be selected for establishment in a crossing block for genetic crosses to local land races.

A total of 80 varieties were collected from the Eastern coastal region, 90 from the southern region and 120 from the Lake region. The cultivars from the Eastern and Southern region were established at the Alawi estate and have been evaluated for morphological characteristics. Collections from the Lake region were established at ARI-Maruku and are yet to be evaluated. Harvest at both sites will be conducted in March next year and the varieties evaluated for dry matter yield, harvest index, plant type, dry matter content, and culinary quality. Farmer groups will also be invited for the harvest to take into considerations their criteria. At least 90 genotypes will be selected for crossing to the improved introductions from CIAT. Two crossing blocks, using a polycross design, will be established at the Alawi estate and at ARI-Naliendele for genetic crosses.

Conclusions and Future Perspectives

One hundred and ninety one genotypes with resistance to the cassava mosaic disease (CMD) and 335 genotypes that combine resistance to CMD and to the cassava green mites (CGM) (derived from a wild relative), were shipped to Tanzania this year for the MAS breeding project. Molecular diagnostics of the introduced material for frog skin disease (FSD) revealed the absence of the disease and the introductions were transferred to the field in Tanzania Collection and evaluation of local varieties in Tanzania for crossing to the introductions were also carried out. Future activities include evaluation of the introductions and local varieties in March next year and genetic crosses between the two groups of germplasm.

Table 1. List of CR F_1 genotypes from with resistance to CGM and CMD resistance introduced to Tanzania this year

Code	Female Parent	Male Parent	No. of Genotypes
CR11	MCOL 2206	C-127	15
CR14	C-4	CM523-7	6
CR15	CM523-7	C-33	2
CR20	CM3306-4	C-33	5
CR21	CM3306-4	C-243	3
CR24	CM7951-5	C-18	3
CR25	CM7951-5	C-33	1
CR26	CM7951-5	C-39	1
CR27	CM7951-5	C-243	8
CR34	SM1741-1	C-18	5
CR35	SM1741-1	C-33	5
CR36	SM1741-1	C-39	2
CR37	C-4	CM4574-7	3
CR41	C-18	MCOL 2056	3
CR42	C-18	MCOL 2206	5
CR43	C-33	CM4574-7	13
CR44	C-39	CM3306-4	3
CR45	C-39	CM4574-7	9
CR46	C-39	SM1219-9	1
CR49	C-243	CM4574-7	6
CR51	C-243	OW280-1	5
CR52	C-243	SM1219-9	15
CR53	C-243	MCOL 2206	1
CR54	C-243	MTAI 8	8
CR55	MBRA 12	C-18	2
CR57	MCOL 2206	C-18	4
CR58	MMAL 66	C-18	2
CR59	MTAI 2	C-18	6
CR62	MTAI 8	C-39	3
CR8	C-4	MCOL 2206	13
CR9	C-4	MTAI 8	33
Total			**191**

Table 2. List of AR genotypes from BC_2 families that combine resistance to CGM and CMD resistance introduced to Tanzania this year

Code	Female Parent	Male Parent	No. of Genotypes
AR1-1	C-127	CW27-12	85
AR11-2	C-243	CW259-43	6
AR23-1	C-39	CW259-43	1
AR26-2	C-413	CW259-43	1
AR41-2	C-19	CW259-42	1
AR37-1	C-33	CW259-42	41
AR38-1	C-377	CW259-42	5
AR40-3	C-39	CW259-42	13
AR42-3	C-413	CW259-42	3
AR16-1	C-33	CW259-3	12
AR22-1	C-39	CW259-3	1
AR36-5	C-127	CW259-10	9
AR34-2	C-19	CW259-10	1
AR32-1	C-33	CW259-10	3
AR33-1	C-39	CW259-10	1
AR17-1	C-33	CW258-17	14
AR21-2	C-39	CW258-17	1
AR30-3	C-413	CW258-17	3
AR9-2	C-243	CW257-12	43
AR15-1	C-33	CW257-12	9
AR20-1	C-39	CW257-12	1
AR35-1	C-243	CW257-10	2
AR14-1	C-33	CW257-10	7
AR6-1	C-4	CW235-72	9
AR7-4	C-127	CW234-2	25
AR4-1	C-19	CW234-2	1
AR8-3	C-243	CW234-2	1
AR12-2	C-33	CW234-2	30
AR2-3	CW236-14	C-4	6
Total			**335**

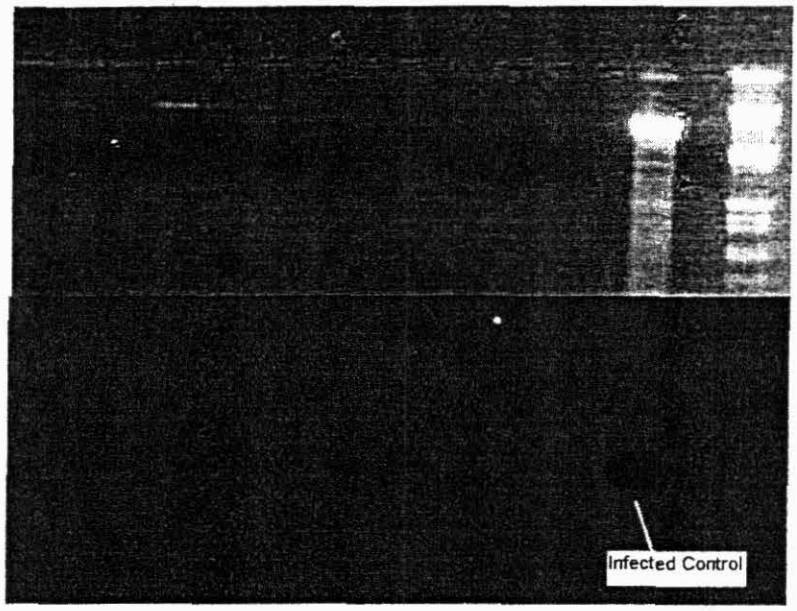

Figure 1. Agarose gel (upper picture) showing ethidium bromide stained double stranded RNA extraction from some AR plants (lanes 1 to 13), lanes 14 and 15 are extraction from frog skin disease (FSD) infected plants and the FSD cDNA probe (CFSD-S5). The lower picture is the Southern hybridization of CFSD-S5 hybridized to a Southern blot of the same gel.

Figure 2. **CR (background) and AR (foreground) plants in the field at the Alawi estate in Tanzania. The CR plants are 6 months old while the AR plants are 1 month old.**

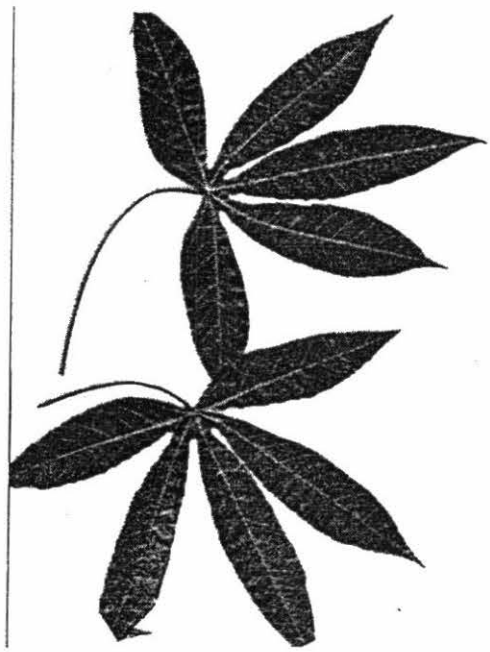

Figure 3. Leaves from 3 genotypes in the field showing purple/black discolorations symptoms due to micronutrient deficiency.

References

Roca,, W.M., Rodriguez, J.A., Mafla, G. Y Roa, J. (1984) Procedures for recovering cassava clones distributed *in vitro*. Centro Internacional de Agricultura Tropical (CIAT) 8 pp.

1.3.12 Genetic Mapping of Genes Involved in the Biosynthesis of Beta-carotene

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Funding: Harvest Plus Challenge Program

Important Output

1) Genetic mapping of the phytoene synthase gene using an S_1 mapping population (AM320) from the yellow variety MTAI8
2) There was no association between beta-carotene content and the phytoene synthase gene.

Introduction

The Harvest plus project in cassava seeks to improve, via conventional and genetic transformation methods, beta-carotene content in cassava and deploy these proVitamin A dense varieties in the fight against Vitamin A deficiency in the tropics. Naturally existing genetic variability for beta-carotene content in cassava is the basis for conventional improvement of beta-carotene content in cassava and a knowledge of functional diversity provides for a more rational exploitation and faster progress in breeding. A study was initiated last year to identify markers and genes in the biosynthetic pathway associated with beta-carotene content as a first step to analysis of functional diversity and development of markers for conventional breeding. Three SSR markers, SSRY251, NS980, and SSRY240 were identified associated with high beta-carotene content in the S_1 family AM320 obtained from MTAI8, a yellow cassava variety. This year the study was extended to genetic mapping, and searching for associations, with proVitamin A content, of 2 known biosynthetic genes, phytoene synthase and phytoene destarurase.

Methodology

The mapping population AM320 comprised of 100 S_1 plants obtained from selfing MTAI8, an elite cassava cultivar developed by the CIAT-Thailand breeding program. This population is also being used for genetic mapping of cyanogenic glucosides and dry matter content, two traits that are high in MTAI8. Two cDNA clones each for phytoene synthase and 2 phytoene desaturase had earlier been obtained from a cDNA library of the cassava variety MNG2 (Andrea et al. unpublished data; CIAT 2002). Genetic mapping of the cDNA clones was as restriction fragment length polymorphism (RFLP). First, a parental survey of polymorphism was conducted using the restriction enzymes *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, and *Dra*I. Parental survey filters were made using 10ug of cassava genomic from the MTAI8 parent and 4 S_1 progenies DNA digested with the enzymes mentioned above and separated on a 0.9% agarose gels as described earlier (Fregene et al.

1997). Progeny filters containing restricted DNA from the 100 S_1 plants, including DNA from the parent in the first lane, were prepared using the restriction enzyme that revealed polymorphism in the parental survey. The raw RFLP data was read as codominant markers and joined with 100SSR markers already evaluated in the S1 population. Linkage analysis and genetic mapping was as described earlier (Fregene et al. 1997) using a LOD score of 4.0 and a recombinantion fraction of 0.3. Association between the markers and beta-carotene content, earlier evaluated in the S_1 cross was by single marker analysis using simple regression.

Results

Of the 2 genes used in the parental survey only phytoene synthase revealed 2 alleles in MTAI8 that segregated in the 4 S_1 progenies in the expected model with the restriction enzyme HindIII, phytoene desaturase was monomorphic (Fig 1). RFLP data from Progeny hybridization with the same enzyme permitted the mapping of phytoene synthase in a linkage group different from that with SSRY251 a cDNA-SSR marker that was earlier found to be associated with beta-carotene content in the same AM320 population. Incidentally SSRY251 shows very high homology to pyroxidine synthase, a gene known to be involved in the biosythesis of vitamin B6. Single point marker analysis by simple regression between the phytoene synthase gene, as independent variable, and beta-carotene content, as dependent variable revealed no association with the gene explaining 30% of phenotypic variance for the trait.

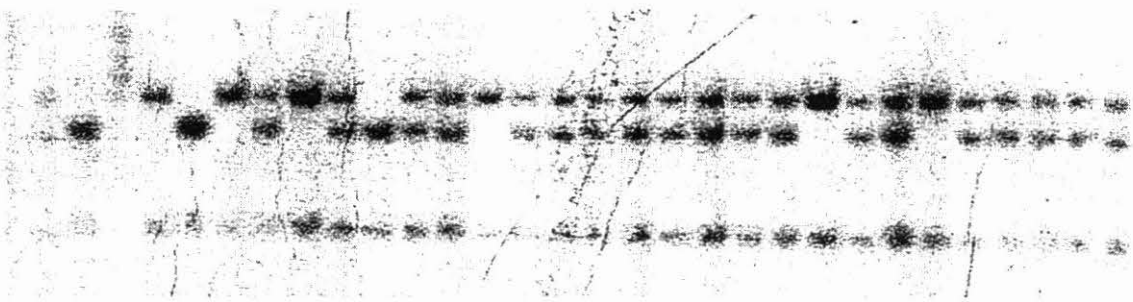

Figure 1. Southern hybridization of the phytoene synthase gene with *HindIII* digested DNA of progenies of AM320.

Conclusion and Perspectives

Genetic mapping of the phytoene synthase gene in an S_1 mapping population (AM320) from the yellow variety MTAI8 has been achieved. Mapping of another biosynthetic gene, phytoene desaturase, could not be achieved due to a lack of heterozygosity for this gene in MTAI8 with the five restriction enzymes employed. Current efforts are directed to search for olymorphisms using another panel of 5 restriction enzymes. The phytoene synthase gene was not associated with beta-carotene content in the AM320 cross and it explained

30% of phenotypic variance. The above results reveal that there are other genes that act to give the yellow color and need to be cloned for a complete understanding of the inheritance of beta-carotene content in cassava. Future activities include assessing SSR diversity of a collection of more than 200 yellow varieties and the combination of different alleles of the gene to assess the effect of combining different alleles of the gene.

References

CIAT 2003

Fregene, M.A., F. Angel, R. Gómez, F. Rodríguez, W. Roca, J. Tohme, and M. Bonierbale (1997). A molecular genetic map of cassava (*Manihot esculenta* Crantz). Theor. and Appl. Genet. TAG 95 (3) 431-441.

1.3.13 Progress in Genetic Mapping of Dry Matter Content (DMC) in Cassava

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Funding: Rockefeller Foundation, CIAT

Important Outputs

1) Analysis of dry matter content (DMC) in 23 F_1 families from a diallel experiment over a 3 year period
2) Putative markers SSRY160 and SSRY150 found to be associated with dry matter content (DMC) in the GM313 family are also linked with the trait in other families
3) Discovery of a inter-specific hybrid family with a very wide segregation for dry matter content and initiation of bulked segregant analysis (BSA) for the identification of markers

Introduction

In 2002 a diallel experiment was initiated to provide information on the genetics of traits of agronomic interest in cassava (CIAT 2003). Based upon GCA estimates in the parents and high standard deviation of dry matter content (DMC), 23 families were selected for further evaluation of dry matter content (DMC). Two families, GM313 and GM312, of the 23 were also used for bulked segregant analysis of DMC and 2 SSR markers SSRY160 and SSR150 were found to be associated with the trait (CIAT 2003). The utility of these markers in the other 21 families was tested in the past year. Due to the small sizes of families in the original diallel experiment, 30-50 progenies, that are inadequate for QTL mapping, larger sized families for 9 of the pair-wise combinations of parents were also generated for QTL mapping. A seedling trial of more than 1,500 genotypes from these

families is currently in the field this year. Also during the year, an inter-specific family CW208, a cross between MTAI8 and *Manihot tristis* was identified with a very wide segregation for DMC. This family is part of a large-scale evaluation of inter-specific between cassava and several wild *Manihot* species started in 2001. We describe here completion of evaluation of the 23 families of the diallel experiment, association of the SSR markers 160 and 150 with DMC in the other 21 families, and initiation of bulk segregant analysis (BSA) of CW208 using 600 SSR markers.

Methodology

Genotypes from 23 families selected based on large standard deviation for DMC in the 2002 harvest was planted in Santander de Quilichao in October 2002 and harvested in October 2003. The experiment consisted of six plants per genotype planted in a completely randomized block design. Plants were harvested and bulked per genotype for measurement of percent dry matter content (DMC) using the standard CIAT procedure of weighing in air and water and calculating specific gravity. Plants were observed for incidence of frog skin disease (FSD) and rated as absent (0) or present (1), all genotypes showing any signs of frog skin infection were discarded. In November 2003 these families were re-planted in a trial with 4 x 5 plant plots and four replications in a randomized block design. Harvesting was done in August 2004, the central eight plants from each plot were harvested and bulked. Data was taken as described above, this time severity of frog skin disease (FSD) was rated on a 0-5 scale with 0 signifying no observed symptoms and 5 very severe. Data was subjected to ANOVA having as sources of variation genotypes and replication, a Generalized Linear Model (GLM) analysis was also carried out using the 3 year data, due to uneven number of observations across years; variation due to years was included as a source of variation.

Randomly amplified polymorphic DNA (RAPD) was employed in bulked segregant analysis in a search for more markers associated with DMC in the families GM313 and GM312. A total of 492 10-nucleotide random primers available at CIAT Cassava Genetics laboratory were evaluated in two sets of bulks, high and low for DMC respectively, from the families GM 312 and GM 313. RAPD analysis was as described earlier (CIAT 2003). PCR product was run on 1.5% agarose gels at 240 volts for about one hour, stained with ethidium bromide and photographed. Polymorphic markers in the bulks were analyzed in individuals of the bulks, and markers that remained polymorphic between individuals high and low in dry matter content were analyzed in all genotypes of the cross.

The SSR marker SSR 160 and SSR150 were also analyzed in the remaining 21 families, to assess the utility of this markers in different genetic background. DNA samples from all genotypes was extracted from 1g of oven dried (48h at 50°C) leaves using a mini-prep version of the Dellaporta *et al.*, (1983) protocol. 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. SSR analysis was as described earlier

by Mba et al. (2001). Bulk segregation analysis (BSA) of DMC in the inter-specific family CW208 was also carried out using SSR markers as described earlier (CIAT 2003).

Results

2003 harvest

During the 2003 harvest number of tubers per plant ranged from 0.30 to 31.00 in GM 283-8 and GM 267-5 respectively with an average of 9.49 (Table 1). They tended to be affected by FSD as infected plants had many undeveloped tubers. Fresh root yield greatly varied with the lowest, GM 306-21 having as low as 0.2 ton/ha and the highest CM 9642-26, 111.8 ton/ha. Percent dry matter content had values ranging from 16.09 in GM 284-23 and GM 309-9 and as high as 51.07% and 69.07% in GM 313-23 and GM 311-15 respectively, while dry yield values ranged from 0.08 ton/ha in GM 306-21 and GM 309-39 to 39.26 t/ha in CM 9642-26.

High incidence of frog skin disease was observed with 23% of the genotypes showing symptoms, simple correlation analysis in infected genotypes revealed significant negative estimates with all the yield parameters. Highest estimate was obtained with percentage dry matter (-0.33) followed by harvest index (-0.24), dry tuber yield and number of commercial roots (-0.23), fresh tuber yield and number of tubers (-0.20). Correlation between the 2002 and 2003 percent dry matter estimates revealed a low positive value of 0.12 suggesting that frog skin disease pressure had affected evaluations in 2003.

Table 1. Simple statistics of yield components estimated from clones harvested in October 2003 in Quilichao.

	Minimum	Maximum	Mean	Std Dev[a]
Stand count	1	6	4.32	1.73
FSD[b]	0.00	1.00	0.23	0.42
Foliage weight[c]	0.10	62.70	10.19	7.86
ComRt[d]	0.00	65.00	17.23	13.58
TbNo[e]	0.30	31.00	9.49	4.63
Harvest Index	0.02	0.92	0.51	0.16
Fyield[f]	0.20	111.80	21.96	16.74
DM[g]	16.30	69.07	31.10	5.06
Dyield[h]	0.08	39.26	7.06	5.73

[a]standard deviation; [b]Frog skin disease, 0=absent, 1=present; [c]Vegetative yield per plot(kg); [d]Number of commercial roots per plot; [e]Number of tubers per plant; [f]Fresh tuber yield (t/ha); [g]Percent dry matter content; [h]Dry tuber yield (t/ha)

2004 harvest

A high incidence of frog skin disease was again observed in the 2004 harvest, 36.9% of the genotypes showed symptoms although with low severity, most affected plants showed

average severity of 1 or less. The low level of severity could be a result of discarding infected plants from the previous year therefore avoiding inoculum buildup. Fresh and dry root yield was significantly lower in the 2004 harvest (Table 2). Analysis of variance (ANOVA) showed differences amongst genotypes to be highly significant (P=0.001) for all the yield parameters (Table 3). Replication was highly significant (P=0.001) for all the parameters except DMC, which was significant at 0.05, indicating that DM is the most stable of yield parameters, this is supported by the low CV value (11.28%). GLM was performed on the three sets of data (2002, 2003 and 2004) and results are shown in Table 4. Genotype and year were highly significant (P=0.001) for dry matter yield, most likely due to the variable climatic conditions in Santander de Quilichao. Percent dry matter showed the lowest coefficient of variation (9.65%) implying that it is stable across years and that a single year data is sufficient for evaluation of DMC.

Table 2. Simple statistics of yield components estimated from clones harvested in August 2004 in Santander de Quilichao

	Minimum	Maximum	Mean	Std Dev[a]
PltHa[b]	1.00	8.00	4.9	1.66
FSD[c]	0.00	3.00	0.3	0.53
ComRt[d]	0.00	30.50	8.2	5.59
TbpIt[e]	0.29	12.00	3.5	1.63
FolWt	0.08	13.90	4.75	2.56
HI[f]	7.89	90.74	41.1	13.24
Fyield[g]	0.23	25.06	5.8	3.95
DM[h]	18.66	40.40	30.2	3.51
DYield[i]	0.04	8.82	1.8	1.32

[a]Standard deviation bNumber of plants harvested [c]Frog skin disease scores on a scale of 1-5 (1 no symptoms detected, 5 very severe), [d]Number of commercial roots per plot, [e]Number of roots per plant, [f]Harvest index, [g]Fresh tuberous root yield (tons/ha), [h]Percent dry matter content, [i]Dry root yield (tons/ha).

Table 3. Analysis of variance (ANOVA) table of yield parameters evaluated at harvest in CIAT, Quilichao in August 2004.

Source of Variation		M E A N S Q U A R E S							
	df[a]	FSD[b]	TbNo[c]	ComRt[d]	TbPlt[e]	HI[f]	Fyield[g]	DM[h]	Dyield[i]
Genotype	430	0.75***	228.77***	88.39***	6.50***	418.75***	45.92***	32.51***	5.05***
Replication	3	1.86**	7947.54***	2313.45***	60.92***	1909.87***	783.54***	3.43*	71.03***
Error	757	0.39	106.08	37.88	4.09	132.63	19.76	11.58	2.12
CV[j]			61.3	74.9	57.56	28.02	75.83	11.28	79.7

[a]Degree of freedom; [b]Frog skin disease; [c]Number of tubers per plot [d]Number of commercial roots per plot [e]Number of tubers per plant; [f]Harvest index [g]Fresh tuberous root yield (t/ha); [h]Percent dry matter content, [i]Dry tuberous root yield; [j]Coefficient of variation

Table 4.Analysis of variance (ANOVA) table of yield parameters taken over 3 years at CIAT, Quilichao

Source of Variation	df	MEAN SQUARES				
		FSD	HI	DM	Fyield	Dyield
Genotype	345	0.11	0.02***	19.70***	336.42***	46.21***
Year	2	9.74***	1.90***	2795.22***	165359.37***	22200.14***
Error	428	0.11	0.01	9.78	255.65	32.81
CV (%)		276.20	20.98	9.65	64.6	67.35

A total of 70 primers were polymorphic in one or both of the bulks (16 were polymorphic in both). When run on *open bulks* most of the primers were false positives. However, eight primers:- AB15, C18, H09, K10, O01, O14, H09, AH09 and AO14 continued to be polymorphic (Fig 1) and were tested on the whole populations. Simple regression of DM phenotypic data on the RAPD marker genotype classes produced very low values regression coefficients, 1.05 to 1.15%, eliminating the utility of these RAPD markers.

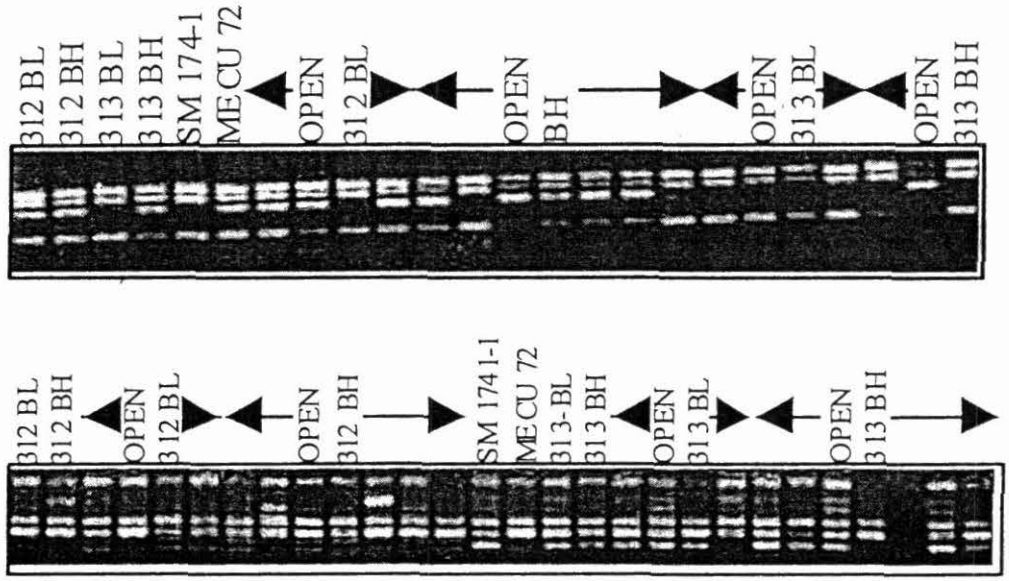

Figure 1.- Ethidium strained agarose gel showing PCR amplification of two RAPD primers AB 15 and K10 of parents, bulks and individuals constituting the respective bulks of family GM313 and GM312. BL and BH signify bulk high and bulk low respectively.

Evaluation of the markers SSRY160 and SSRY150, earlier observed to be associated with DMC in the family GM313, in the other 21 families of the diallel experiment revealed association with the trait in several other families, for example all families that have SM1741-1 as parent showed a strong association and high regression coefficients between SSR marker 160 and DMC. (Table 5). This suggests that this marker is associated with a favorable allele for DMC found in a specific genetic background. Efforts are now directed to evaluating this marker in larger sized families having SM1741-1 as one of the parents generated last year and planted in the field this year to obtain a more accurate value of regression coefficient in preparation of its use in marker assisted selection (MAS) of DMC.

Segregation of dry matter content in the inter-specific family CW208 is maybe the highest found to date. Distribution of the trait in this family reveals a non-normal distribution that suggests large QTLs genes might be involved (Fig 2). Bulked segregant analysis with 600SSR markers is ongoing and should lead to identification of the responsible genes.

Table 5. Simple regression coefficients of DMC of the SSR markers SSR150 and SSRY160 in 21 families obtained from a diallel experiment

Family	Mother	Father	R^2 (SSRY 150)	R^2 (SSRY 160)
GM 257	SM 1219- 9	SM 1636- 24	17.2	5.3
GM260	SM 1219- 9	SM 1673- 10	ND	11.98
GM265	SM 1219- 9	MPER 183	4.8	0.5
GM268	SM 1278- 2	SM1673- 10	4.2	12.6
GM269	SM 1278- 2	SM 1741- 1	3.2	25.7
GM283	SM 1636- 24	SM 1673- 10	1.5	ND
GM284	SM 1636- 24	SM 1741- 1	9.8	28.89
GM285	SM 1636- 24	HMC 1	ND	0.56
GM286	SM 1636- 24	MPER 183	12.08	11.72
GM293	SM 1673- 10	HMC 1	0.3	2.77
GM294	SM 1673- 10	MPER 183	0.1	5.3
GM306	MECU 72	MPER 183	0	8.07
GM309	MECU 72	SM 1219- 9	5.98	2.25
GM310	MECU 72	SM 1278- 2	0.8	11.36
GM313	MECU 72	SM 1741- 1	18.1	29.3
GM311	MECU 72	SM 1636- 24	0.6	0.4
GM314	MECU 72	HMC 1	0.4	10.23
CM9642	CM 6740- 7	MPER 183	6.29	1.84
CM977	HMC 1	MPER 183	36.56	0.46
CM9901	CM 6740- 7	SM 1219- 9	7.69	ND

ND: Not determined

Conclusion and Future Perspectives

Analysis of dry matter content (DMC) in 23 F_1 families from a diallel experiment over a 3 year period revealed the profound effect of different seasons (years) on DMC, the effect of replication was of lesser importance. Putative markers SSRY160 and SSRY150 found earlier to be associated with dry matter content (DMC) in the GM313 family was also linked to the trait in other families. Discovery of markers for DMC have also been extended to a inter-specific hybrid family with a very wide segregation for dry matter content. Future perspectives are completion of bulked segregant analysis (BSA) in the inter-specific hybrid cross and evaluation of markers identified until now in larger sized families generated for QTL mapping.

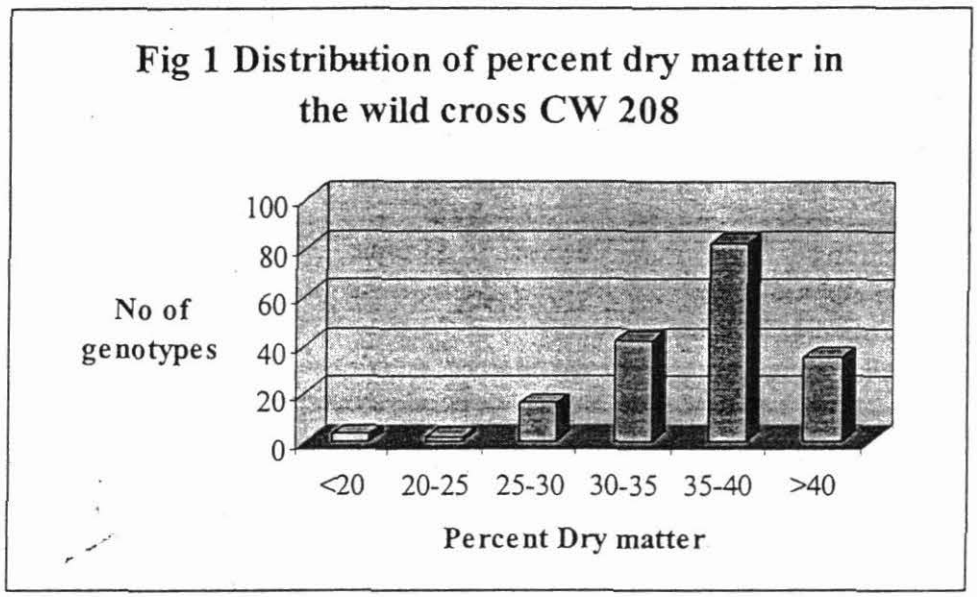

Figure 2. Frequency distribution of different classes of dry matter content in an inter-specific family CW208 obtained from a cross between MTAI8 and *M. tristis*

References

CIAT, 2003

Dellaporta, S. L., J. Wood, J. B. Hicks. 1983. A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1 (14): 19-21.

Mba R. E.C., P. Stephenson, K. Edwards, S. Melzer, J. Nkumbira, U. Gullberg, K. Apel, M. Gale, J. Tohme, and M. Fregene (2001) Simple Sequence Repeat (SSR) Markers Survey of the Cassava (*Manihot esculenta* Crantz) Genome: Towards a SSR-Based Molecular Genetic Map of Cassava. Theor and Appl Genet 102:21-31

1.3.14 QTL Mapping of Cyanogenic Glucoside Content in a S_1 Population derived from MTAI8 and Candidate Gene Mapping of Two Cytochrome P-450 Biosynthetic Genes (CYP79D1 Y CYP79D2)

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Funding: Bioearn, SLU, Uppsala, Sweden, CIAT

Important Outputs

1) Development of a partial map of the cassava genome using SSR and DArT markers and a S_1 population derived from MTAI8 and genetic mapping of a cytochrome P450 gene
2) Establishment of a replicated trial of 200 S_1 progenies to measure cyanogenic potential in leaves and roots.

Introduction

The presence of cyanogenic glucosides in cassava roots is a nutritional deficiency and a potential health problem for human and animal consumers. There has therefore been a considerable amount of interest in understanding the biosynthesis of the two cyanogenic glucosides, Linamarin and Lotaustralin, produced in cassava and ways of reducing or eliminating them all together in the roots. In 2000, the enzyme that catalyzes the rate limiting the most important step in the biosynthesis of the cyanogenic glucosides, the conversion of amino acids to oxime, was cloned and identified as a cytochrome P-450 gene, 2 cDNAs (CYPD1 and D2) with about 80% homology were identified (Anderson et al 2000). In collaboration with the group of Prof Moller that cloned the CYP genes and a doctoral student from the Swedish Agricultural University (SLU), Uppsala, an attempt was made to associate the genes with cyanogenic glucoside content and also identify other QTLs controlling the trait in cassava. We describe here genetic mapping of the CYP genes, as RFLP markers, 70 SSR and 150 Diversity Array Technology (DArT) markers in an S_1 family derived from MTAI8, a Thai variety with high cyanogenic glucoside content. We also report a field experiment to measure the trait in the S_1 family. The identification of markers associated with cyanogenic potential (CNP) in cassava will provide tools to accurately identify the trait in an effort to breed for low CNP cassava varieties.

Methodology

The S_1 family (AM320) consisted initially of 104 individuals but was increased to 200 from new selfs made with MTAI8 this year. DNA was isolated from all 200 genotypes using a mini-prep method of the Dellaporta extraction protocol (1983). SSR markers for

genetic mapping were 600 SSR markers developed earlier in cassava, they were screened in the MTAI8 parent and 5 other S_1 progenies as described earlier by Mba et al (2001). A previously constructed DArT chip of about 1000 polymorphic markers (Liu et al. 2004) was the source of DArT markers for evaluating the AM320 population. The cytochrome P-450 genes CYPD1 and D2 were evaluated in the MTAI8 parent along with 4 S_1 progenies for restriction length polymorphisms (RFLPs) using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI*. Preparation of parental and progeny filters, and Southern hybridization of the filters were as described by Fregene et al. (1997). Polymorphic SSR and RFLP markers were evaluated in the entire S_1 progeny. A chi-square test at a confidence level of 0.05 and 0.01 was used to test goodness of fit of the segregation date with the expected model of 1:2:1 ratio for co-dominant markers and 3:1 ratio for dominant markers. Linkage analysis of the raw segregation marker data was done using the Mapmaker linkage analysis software (Lander et al. 1993) and a LOD of 5.0 and recombination fraction (theta) of 0.3 for the dominant markers and a LOD of 9.0 and theta of 0.2 for the dominant markers. Map distances were calculated by the Kosambi method that takes into account double-crossovers. Initial linkage analysis was carried out with the co-dominant SSR and RFLP markers combined with the dominant DArT markers, these were later separated due to difficulties in placing the DArT markers, separate maps were therefore developed.

A preliminary evaluation of CNP in leaf tissue and roots was conducted in the AM320 S_1 family last year based upon 3 plants and a single replication. Evaluations were conducted on a single root and leaf tissue harvested from each of the plants and CNP determined according to the enzymatic protocol developed by Cooke (1978) and modified by O'Brien (1991). This year 200 S_1 progenies of the AM320 family were re-established in single plant plots replicated eight times in CIAT, Palmira, for evaluation of the CNP phenotype. The trial will be harvested piece-meal at 4, 6 and 10 months after planting to measure the accumulation of CNP over a range of growth period.

Results

So far, a total of 100 SSR markers have been found to segregate in the AM320 S_1 family, while 208 polymorphic DArT markers were found. Less than 20 of the SSR markers and more than 90% of the DArT showed segregation distortion at a 0.05% confidence level in the Chi square test. Seventy-four of these SSR markers were organized into 23 linkage groups that covered 819.5cM of the cassava genome by linkage analysis while 26 markers remained unlinked. The 2 cytochrome P-450 gene CYPD1 was polymorphic with the restriction enzyme *Eco*RI used in the RFLP parental survey but CYPD1 was monomorphic with the 5 enzymes used (Fig 1). RFLP segregation data for CYPD1 revealed possible duplicated loci, one segregating at the expected ration of 1:2:1, at a 0.05% confidence level in the Chi square test, and the second at a ratio of 3:1. Linkage analysis permitted the mapping of both loci to linkage group 3. Efforts are ongoing to use many more restriction enzymes to look for RFLPs with CYPD2 to enable genetic mapping of this gene.

Preliminary results of cyanogenic glucoside content in the AM320 family revealed a wide segregation for the trait and the appropriateness of this family for mapping the trait. In leaves, 5% of the progenies had below 1075ppm, 85% had a range of 1075 -3048, while 10% had between 3049 -5071ppm. In the roots, 11% of the family had below 258ppm, 76% had between 259 and 878ppm, while 13% had higher than 1294ppm. The distribution of the trait in both leaves and roots was normal suggesting a quantitative trait. However, the above data is based upon a single replication and cannot be said to be an accurate representation of CNP in these genotypes.

Conclusions and Perspectives

A partial molecular genetic map of cassava has been constructed using SSR and RFLP markers and the cytochrome P-450 gene CYPD1, in the S_1 family AM320 derived from MTAI8. Preliminary evaluation of cyanogenic glucoside content in this family revealed wide segregation of the trait. A proper evaluation of the trait is being carried out this year in preparation for association of cyanogenic glucosides content with the biosynthetic genes and QTL mapping. Work is also ongoing to identify RFLPs for the second gene, CYPD2 so that it can also be placed on the genetic map.

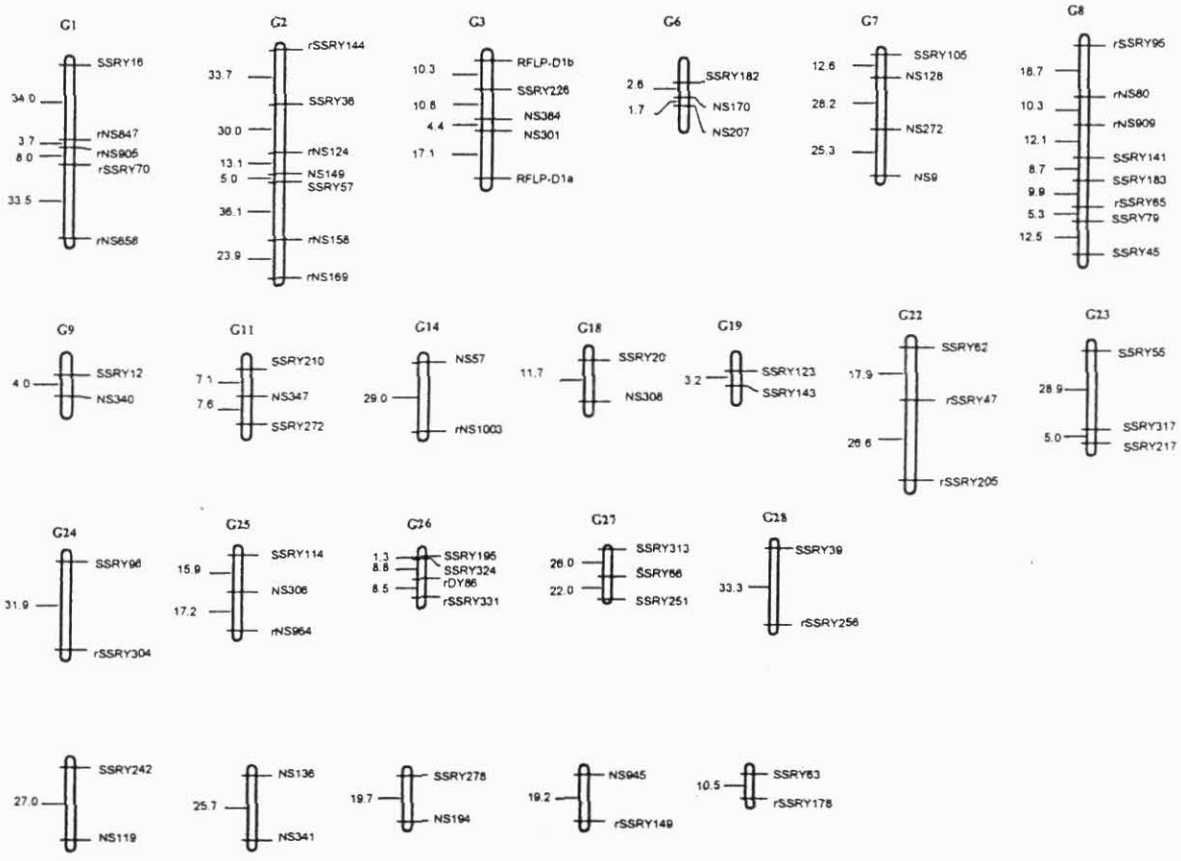

Figure 1. A SSR and RFLP genetic map of the AM320S1 family

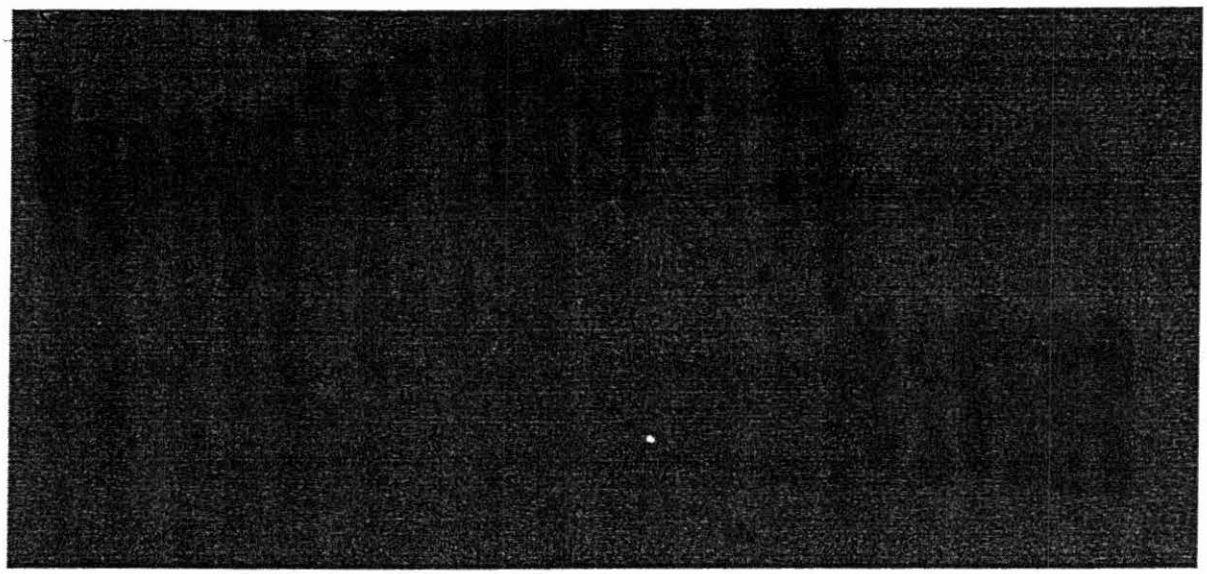

Figure 2. Southern hybridization of the cyanogenic glucoside biosynthetic gene CYP79D1 with MTAI 8 and 4 S_1 progenies with the restriction enzymes, from left to right, *EcoRI*, *EcoRV*, *HindIII*, and *HaeIII*

References

Andersen,M.D., P.K.Busk, I.Svendsen, B.L.Moller. 2000. Cytochrome P-450 from Cassava (*Manihot esculenta* Crantz) Catalyzing the First Steps in the Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin. The Journal of Biological Chemistry 275 (3): 1966-1975.

Cooke,R.D. 1978. An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). J. Sci. Food. Agric. 29: 345-352.

Dellaporta, S. L., J. Wood, J. B. Hicks. 1983. A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1 (14): 19-21.

O'Brien, G.M., A. J. Taylor, N. H. Poulter. 1991. Improved enzymic assay for cyanogens in fresh and processed cassava. J. Sci. Food Agric. 56: 277-289.

1.3.15 Development of Mapping Populations for Gene Tagging of Post Harvest Physiological Deterioration (PPD), Resistance to Hornworms and Whiteflies Found in Wild Relatives of Cassava

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Funding: CIAT

Important Outputs

Development of 8 populations for QTL mapping of post-harvest deterioration (PHD), resistance to whiteflies and hornworm.

Introduction

Post harvest physiological deterioration (PPD) and arthropod pests are severe marketing and production constraints respectively in cassava. It has been estimated that cassava farmers, typically resource-poor farmers, lose 48 million tons of fresh root valued at US$1.4billion every year to pests, diseases, and PPD; some 30% of total world production. Wild relatives of cassava are important sources of genes for resistance to pests and diseases and longer shelf life. The only source of dramatically delayed PPD has been identified in an inter-specific hybrid between cassava and *Manihot walkerae* (Sanchez et al. 2003, unpublished data). The delayed PPD trait, originally from the wild *Manihot* parent, was successfully transferred to an F_1 inter-specific hybrid suggesting a dominant or additive gene action of gene(s) involved. The only source of resistance to the cassava hornworm and a widely deployed source of resistance to the cassava mosaic disease (CMD) were identified in 4th backcross derivatives of *M. glaziovii* (Chavariagga et al. 2004). Moderate to high levels of resistance to white flies have been found in inter-specific hybrids of *M. esculenta* sub spp *flabellifolia* (CIAT, unpublished data). Again, resistance was recovered easily in F_1 inter-specific hybrids, suggesting a simple inheritance of the trait. For several years now molecular marker tools and a modified Advanced Back Cross QTL (ABC-QTL) scheme have been tested for cost-effective pyramiding of useful genes from cultivated and wild gene pool through the elimination of phenotypic evaluations in each breeding cycle. We describe here the development of populations for QTL mapping of post-harvest deterioration (PHD), resistance to whiteflies and hornworm.

Methodology

Segregating populations for the identification of molecular markers for the introgression of delayed PPD, resistance to the cassava hornworm and white flies (presently as sexual seeds) include BC_1 as well as S_1 families to enable identification of recessive genes. The inter-specific hybrid from *Manihot walkerae* with delayed PHD, CW429-1, was crossed extensively to the elite cassava genotypes MTAI8, CM523-7, and SM909-25 to create 3 BC_1

families (BC_1 only in the sense of crosses to cassava). This genotype, CW429-1, was also selfed to generate an S_1 family. The variety MNG11, a BC_4 derivative of *M.glaziovii* with cassava as recurrent parent, having resistance to the hornworm was also crossed to MTAI 8 and selfed to produce BC_1 and S_1 families respectively. The inter-specific hybrid CW251-3, a progeny of *M.esculenta* sub spp flabellifolia (OW189-1) and a high beta-carotene cassava land race CM1734, showing a high level of resistance to white flies was crossed to MTAI 8 and selfed to produce BC_1 and S_1 families respectively. All the above-mentioned crosses were done in the 2003/2004 season.

Results

Between 50 and 150 crosses per cross combinations have been made for the development of BC_1 and S_1 gene tagging populations for PHD, resistance to whiteflies and hornworm (Table1). Sexual seeds will be harvested later in the season in preparation for *in vitro* establishment next year. At least 200 progenies, including reciprocals, of each BC_1 and S_1 populations will be established *in vitro* from embryo axes and multiplied to obtain 10 plants per genotype. The tissue culture plantlets will be transferred to the screen house and then to the field as a single row trial (SRT) of ten plants.

Table 1. List of crosses made to date for development of populations for QTL mapping pf PDH, resistance to whiteflies and hornworm

Female	Male	Seeds available or expected
PHD		
MTAI – 8	429 - 1	75
429 - 1	MTAI- 8	127
CW 429 - 1	SM 909-25	157
CW 429 - 1	CM 523-7	143
429 - 1	429 - 1	270
White flies		
CW 251-3	MTAI- 8	120
MTAI- 8	CW 251-3	43
CW 251-3	CW 251-3	165
Horn worm		
MNIG19	TAI - 8	87
TAI - 8	MNIG19	136
MNIG19	MNIG19	124

The following year progenies will be re-established in a QTL mapping trial of single row plots of 8 plant with 5 replications in one location. Great efforts will be made to ensure that the trials are kept free of weeds, pests, diseases, and nutritional deficiencies to minimize environmental variation.

Conclusions and Future Perspectives

Eight populations have been developed for QTL mapping of post-harvest deterioration (PHD), resistance to whiteflies and hornworm. The segregating populations will be established early next year from embryo axes and multiplied in preparation for field evaluations of PHD and green house evaluations of hornworm and whiteflies resistance. Based on the results of the phenotypic evaluations, bulks of extreme phenotypes will be made for bulk segregant analysis (BSA) with 600 SSR and RAPD markers as described earlier. Polymorphic markers will be evaluated in individuals of the segregating populations and strength of association measured by simple regression. Should BSA fail to identify markers, then a standard QTL procedure, including development of a genetic map with SSR markers, will be followed.

References

Chavarriaga P.; S. Prieto; C.J. Herrera; D. López; A. Bellotti[1]; J. Tohme (2004). Screening transgenics unveils apparent resistance to hornworm (*E. ello*) in the non-transgenic, African cassava clone 60444. In: Alves and Tohme. Adding Value to a Small-Farmer Crop: Proceedings of the Sixth International Scientific Meeting of the Cassava Biotechnology Network. 8-14 March 2004, CIAT, Cali Colombia. Book of Abstracts Pp4.

1.3.16 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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Funding: CIAT core funds

Important outputs

1) Generation of back cross families for QTL mapping and development of improved varieties with high protein content.
2) Evaluation of protein content in Ghana of varieties from Central America confirms the high protein content observed earlier
3) Standardization of a SDS-PAGE analysis method towards a proper characterization of the proteins contained in high protein content cassava and inter-specific genotypes

Introduction

As a major staple throughout the tropics, cassava can serve as a cheap means of deploying adequate protein requirement amongst poor people as well as for animal feeds. An

advanced back cross QTL (ABC-QTL) to introgress high protein content genes from wild relatives into cassava is in its third year at CIAT. Similarly high protein content cassava varieties mostly from Central America were re-evaluated in another environment, Wenchi, Ghana, this year. The cassava varieties have also been re-established in the field at CIAT for a second year of evaluation. Genetic crosses of these high protein varieties are being made to elite parents of the CIAT cassava gene pools for breeding for high protein content and for QTL mapping studies. Other activities continued this year include standardization of the SDS-PAGE methodology for the determination of kind and size of proteins found in high protein accessions.

Methodology

High protein inter-specific lines with between 6 and 10% of crude protein in the roots, from evaluations conducted in June 2002 and repeated August 2003, were established in a crossing block in September 2003 for genetic crosses to the elite line MTAI 8, a yellow variety. It has been observed that earlier crosses of wild relatives with high protein content in the roots to MTAI 8 and other yellow varieties always gave families with a high average of root proteins, confirming earlier observation that beta-carotene in cassava roots is associated with some storage proteins (Carvalho 2003, personal communications harvested in). Thirty three varieties of cassava identified earlier to have high proteins in the roots (CIAT 2003) were shipped as *in vitro* plantlets to Ghana for genetic crosses to local varieties, part of the Ph.D. degree of Ms Elizabeth Okai. The plants were held for one week at the tissue culture facility of the Botany Department of the University of Legon, Accra, then hardened and transferred to a crossing block at Wenchi. Between 5-10 plants per variety were transferred to the field at Wenchi.

Root flour from 49 inter-specific hybrids with 6-10% of crude protein in their roots from evaluations conducted in August 2003 and May 2004 were selected for SDS-PAGE gel analysis of protein content (Table1.). Root flour from two cassava varieties, a high (MGUA 10) and low (CM 523-7) protein content, were used as controls. Starch granules were isolated as described by Jasso (1986). The protein compositions of starch granules were analyzed on SDS-PAGE. Dry starch granules (the pellet) was diluted to a 1:1 ratio with denaturation buffer (2% SDS and 5% ß-mercaptoethanol) and extracted by boiling for 10 minutes (Vos-Scheperkeuter, et al. 1986) The samples were centrifuged at 14 000 rpm for 20 minutes and the resulting supernatant (crude extract) was subjected to SDS-PAGE denaturing electrophoresis as described by Laemmli (1970) with some modifications (CIAT 2003). Ten µl of each sample was loaded per lane. Constant voltage of 100V 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 I) was used as a check in the protocol assay and some cassava varieties with high (MGUA 10) and low (CM 523-7) protein content were used as controls. A Bio-Rad low molecular weight (20 to 113 KD) (Bio-Rad's Prestained SDS-PAGE Standards, low range) protein marker was used to determine approximate protein size.

Table 1. Percent protein content of 49 inter-specific lines selected for SDS-PAGE análisis

	Name	Genotype	Maternal parent	Paternal parent	P.C. (%)
1	1	CW 178-1	OW 132-2	CW 48-1	6.39
2	2	CW 178-2	OW 132-2	CW 48-1	6.44
3	3	CW 179-1	OW 132-2	MTAI 8	7.02
4	4	CW 179-2	OW 132-2	MTAI 8	7.86
5	8	CW 179-6	OW 132-2	MTAI 8	6.72
6	14	CW 179-12	OW 132-2	MTAI 8	7.8
7	23	CW 205-4	OW 231-3	MTAI 8	7.62
8	24	CW 205-5	OW 231-4	MTAI 9	7.25
9	28	WW 3-1	OW 132-2	OW 240-6	7.31
10	31	WW 3-4	OW 132-2	OW 240-6	6.33
11	33	WW 3-6	OW 132-2	OW 240-6	7.32
12	34	CW 177-1	OW 132-2	CM 1585-13	7.19
13	35	CW 177-2	OW 132-2	CM 1585-13	6.55
14	40	CW 177-7	OW 132-2	CM 1585-13	6.84
15	42	CW 177-9	OW 132-2	CM 1585-13	6.81
16	43	CW 177-10	OW 132-2	CM 1585-13	7.43
17	59	CW 177-26	OW 132-2	CM 1585-13	8.52
18	61	CW 177-28	OW 132-2	CM 1585-13	6.7
19	70	CW 177-37	OW 132-2	CM 1585-13	7.73
20	84	CW 177-51	OW 132-2	CM 1585-13	7.05
21	87	CW 177-54	OW 132-2	CM 1585-13	7.17
22	89	CW 177-56	OW 132-3	CM 1585-14	6.77
23	97	OW 253-3	OW 132-2	CM 1585-13	8.65
24	29	CW 99-30	CW 30-29	OW 280-1	7.66
25	32	CW 160-3	CW 56-5	OW 181-2	7.86
26	33*	CW 161-1	CW 56-5	OW 189-1	8.03
27	35*	CW 161-3	CW 56-5	OW 189-1	7.42
28	37	CW 183-1	OW 180-1	CW 48-1	6.05
29	39	CW 185-1	OW 180-1	MTAI 8	7.58
30	65	CW 198-23	OW 230-3	CW 30-65	6.77
31	81	CW 201-1	OW 230-3	CW 56-5	7.1
32	92*	CW 203-3	OW 230-4	CW 48-1	6.37
33	93	CW 204-1	OW 231-3	AM 244-31	6.25
34	104	WW 14-5	OW 181-2	OW 280-2	9.18
35	134	WW 14-37	OW 181-2	OW 280-2	9.23
36	249	WW 22-76	OW 231-3	OW 240-8	7.73
37	256	WW 24-1	OW 231-3	OW 280-2	7.8
38	280	WW 24-25	OW 231-3	OW 280-2	7.66
39	290	WW 39-1	OW 280-1	OW 280-2	7.27
40	324	WW 40-12	OW 284-1	OW 280-2	8.78
41	382	WW 40-74	OW 284-1	OW 280-2	9.28
42	377	WW 40-68	OW 284-1	OW 280-2	10.46
43	409	WW 41-25	OW 284-1	OW 146-1	8.7
44	410	WW 41-26	OW 284-1	OW 146-1	8.41
45	442	CW 184-5	OW 180-1	MCOL 1734	6.27
46	447	CW 212-1	OW 284-1	MCOL 1734	6.53
47	455	CW 251-2	MCOL 1734	OW 189-1	6.61
48	457	CW 251-4	MCOL 1734	OW 189-1	6.46
49	459	CW 256-1	MCOL 1734	OW 280-1	7.2

The protein molecular weight markers in Bio-Rad's Standard were: Phosphorylase b (103.000 Da.), albumin serum (BSA) (77.000 Da.), Ovalbumin (50.000 Da.), Carbonic anhydrase (34.300 Da.), Soybean trypsin inhibitor (28.800 Da.), and Lysozyme (20.700 Da.).

After electrophoresis, gels were stained in a solution containing 50% Methanol, 10% Acetic Acid and 0.25% Coomassie Brilliant Blue R, over night at RT. Gels were washed with water and distained by two changes of distaining solution first with 20% methanol and 10% Acetic Acid, and second with 5% methanol and 7% Acetic Acid.

Results

Back crosses were started in April 2004, a total of 5,980 crosses have been made so far and will continue until the first week of November. Table 2 shows the back cross families and the available or expected seeds from this year's crosses. Currently, 433 seeds have been sent to the tissue culture lab for the embryo rescue and establishment of the BC_1 populations. After embryo rescue the plantlets will be multiplied and 10 plantlets sent to the screen house for hardening and transfer to the field. The crosses will be established next year in single row plots of 8 plants with one replication.

Table 2. BC_1 families for QTL mapping of high protein content generated in 2004

	BC_1 donor parent	Mother	Father	%Protein Content	BC_1 cassava parent	Seeds available or expected
1	CW 73 – 2	CM 1585 - 13	OW 284 - 1	15.94	MTAI 8	300
2	CW 187 - 222	OW 181 - 2	CW 48 - 1	12.71	MTAI 8	200
3	CW 198 - 11	OW 230 - 3	CW 30 - 65	11.28	MTAI 8	340
4	CW 201 - 2	OW 230 - 3	CW 56 - 5	10.2	MTAI 8	105
5	CW 205 - 2	OW 231 - 3	MTAI 8	10.54	MTAI 8	255
6	CW 188 - 4	OW 181 - 2	MCOL 1734	9.62	MTAI 8	145
7	CW 151 - 38	CW 48 - 1	OW 280 - 1	8.18	MTAI 8	170
8	CW 198 - 28	OW 230 - 3	CW 30 - 65	8.37	MTAI 8	200
9	CW 201 - 3	OW 230 - 3	CW 56 - 5	8.25	MTAI 8	50
10	CW 205 - 4	OW 231 - 3	MTAI 8	8.4	MTAI 8	28
11	CW 179 - 2	OW 132 - 2	MTAI 8	7.92	MTAI 8	406
12	CW 198 - 10	OW 230 - 3	CW 30 - 65	7.3	MTAI 8	124
13	CW 198 - 17	OW 230 - 3	CW 30 - 65	7.07	MTAI 8	21
14	CW 203 - 3	OW 230 - 4	CW 48 - 1	7.05	MTAI 8	200
15	CW 251 - 4	MCOL 1734	OW 189 - 1	7.99	MTAI 8	120

Crude protein content for the high protein varieties evaluated in Ghana revealed a range of 5-7% and a 0.9 correlation with previous evaluations at CIAT. These results confirm earlier results for these varieties and suggest that protein content in cassava roots is a stable

trait. Based on this, genetic crosses have been started this year between these varieties and elite CIAT parental lines, more than 500 crosses have been made this year.

The SDS-PAGE profiles of the root proteins in the crude extracts from the 49 cassava genotypes, showed different protein bands (Fig. 1). The difference among polypeptide profiles is in both the number and relative intensity of the bands. The highest molecular weight protein group (2 bands) observed was between 103,000 and 77,000 Da and the lowest band 20,700 Da. There are reports of cassava proteins showing bands at 14,000-80,000 Da of molecular mass (Glaucia, 2001), using two-dimensional gel electrophoresis. Souza *et al*, 1998 reported the isolation of a major protein of MW of about 22,000, which was restricted to the parenchyma rather than the peel of the tuber. These results show that the patterns observed in our study are between the reported ranks (22,000-80,000 Da.).

Proteins in cassava roots showed six main groups of bands between 103,000 and 20,700 Da. Group one contained two bands between 103,000 and 77,000 Da (Table 3). Group 1a contained one band of 77,000 Da, present in the sample 2C (control). Group two contained one band of 50,000 Da, which coincided with the ovalbumin pattern and the band of I Phaseolin (*Phaseolus vulgaris* control). Group three contained two bands of 50,000 present only in the sample 4C (control), that coincided with the bands of I phaseolin.

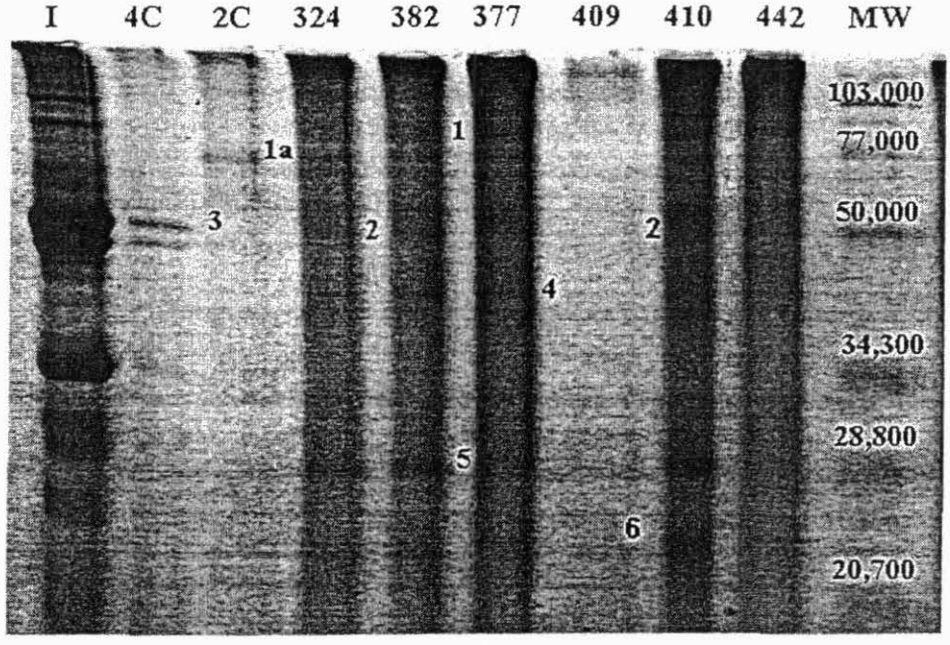

Figure 1. One-dimensional, SDS-PAGE gel electrophoresis of total proteins from root of eight cassava cultivars. The samples 4C and 2C are cassava varieties with high and low protein content respectively, the first late (I) is a protein extraction from *Phaseolus vulgaris* used as reference. Bands 1a and 3 only seen in the cassava controls.

Table 3. Groups of bands found SDS-PAGE protein electrophoresis of 49 high protein lines.

Sample Number	Genotype	Group 1	Group 1a	Group 2	Group 3	Group 4	Group 5	Group 6
1	CW 178-1	+		+		+		
2	CW 178-2	+		+		+		
3	CW 179-1	+		+	+	+	+	+
4	CW 179-2*	-		-	-	-	-	-
8	CW 179-6*	-		-	-	-	-	-
14	CW 179-12*	-		-	-	-	-	-
23	CW 205-4		+					
24	CW 205-5		+			+		
28	WW 3-1		+			+		
31	WW 3-4	+		+				+
33	WW 3-6	+		+		+		+
34	CW 177-1	+	+					
35	CW 177-2		+			+		
40	CW 177-7	+		+		+		
42	CW 177-9	+	+					
43	CW 177-10	+		+		+	+	+
59	CW 177-26	+		+	+	+	+	+
61	CW 177-28	+		+		+	+	+
70	CW 177-37	+	+					
84	CW 177-51		+			+		
87	CW 177-54		+			+		
89	CW 177-56*	-		-	-	-	-	-
97	OW 253-3	+		+	+	+	+	+
29	CW 99-30			+				+
32	CW 160-3			+		+		
33*	CW 161-1			+		+	+	+
35*	CW 161-3*	-	-	-	-	-	-	-
37	CW 183-1*	-	-	-	-	-	-	-
39	CW 185-1			+		+		+
65	CW 198-23					+	+	
81	CW 201-1			+		+	+	
92*	CW 203-3			+		+	+	
93	CW 204-1	+		+		+		
104	WW 14-5	+		+				
134	WW 14-37*	-	-	-	-	-	-	-
249	WW 22-76			+				+
256	WW 24-1						+	+
280	WW 24-25*	-	-	-	-	-	-	-
290	WW 39-1*	-	-	-	-	-	-	-
324	WW 40-12	+		+		+	+	+
382	WW 40-74	+		+			+	+
377	WW 40-68	+		+		+	+	+
409	WW 41-25*	-	-	-	-	-	-	-
410	WW 41-26	+		+		+	+	+
442	CW 184-5	+		+		+	+	
447	CW 212-1						+	+
455	CW 251-2*	-	-	-	-	-	-	-
457	CW 251-4		+			+		
459	CW 256-1		+			+		
C4	MGUA 10				+			
C2	CM 523-7	+	+					

* Genotypes that did not show a pattern of bands

Group four contained two bands between 34,300 and 50,000 Da. Group five contained one band of 28,800 Da with the same weight as the soybean trypsin inhibitor. Group six contained one band between 28,800 and 20,000 Da. (Table 3.) (Fig.1).

Conclusions and perspectives

Generation of backcross families for QTL mapping and development of improved varieties with high protein content were initiated this year. Evaluation of protein content in Ghana of varieties from Central America conducted this year confirmed the high protein content observed earlier at CIAT. SDS-PAGE protein analysis made possible identification of polypeptides that could be isolated in future for more detailed characterization. Future perspectives include the establishment and evaluation of the BC_1 QTL mapping populations and genetic crosses between the high protein cassava varieties and elite parents of CIAT's cassava gene pools.

References

Cabral,G.B., Carvalho,L.J. 2001. Analysis of proteins associated with storage root formation in cassava using two-dimensional gel electrophoresis. R. Bras. Fisiol. Veg., 13(1):41-48.

Carvalho, L.J.C., Agostini, M.A.V., Bloch, C.J. 2004. Chromoplast associated proteins from color storage root diversity in cassava (*Manihot esculenta* Crantz). Six International Scientific Meeting of the Cassava Biotechnology Network. CIAT, International Scientific Meeting of the Cassava Biotechnology Network. CIAT, CBN.

Hovenkamp-Hermelink, J.H.M , Jacobsen, E., Ponstein, A.S., Visser, G.F., Vos-Scheperkeuter, G.H., Bijmolt, E.W., de Vries J.N., Witholt, B. and Feenstra, W. J. 1987.Isolation of an amylase-free starch mutant of the potato (*Solanum tuberosum* L.). TAG 75:217-221

Jasso, D., Romero, J., Rodríguez, R. and Angulo J.L. 2002. Characterization of Proteins from Sunflower Leaves and Seeds: Relationship of Biomass and Seed Yield. Reprinted from: Trends in new crops, and new uses. ASHS press.

Laemmli, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature Vol. 227: 680-685

Shewry, P.R. 1995. Plant storage proteins. Biological Review 70:375-426.

Souza PAS, Gomes E., Compos F.A.P. 1998. Tissue distribution and deposition pattern of a cellulosic parenchyma-specific protein from cassava roots. Brazilian Archives of biology and Technology 41:1-9.

Vos-Scheperkeuter, G.H., De Boer, W., Visser, R.G.F., Fenstra, W.J. and Witholt, B. 1986. Identification of Granule-Bound Starch Synthase in Potato tubers. Plant Physiol 82, 411-416.

Yeo H.H., Chew, M.Y., 1977. Protein content and acid composition of cassava seed and tuber. Malaysian Agricultural Jopurnal 51:1-6.82, 411-416.

1.3.17 Identification of Naturally Occurring and Irradiation-Induced Mutant GBSSI Alleles of Cassava in a Heterozygous Genetic background

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Funding: IAEA, CIAT

Important Outputs

1) Development of allele specific markers for mutant waxy alleles (GBSSI alleles) from an accession each of the *Manihot* species *M. crassisepala* (MCRA013) and *M. chlorostica* (CW14-11); initiation of genetic crosses
2) Discovery of a possible duplication for the GBSSI locus in cassava and efforts to map the gene in the cassava genome.

Introduction

A change in starch quality, such as the elimination of amylose (waxy starch), via a natural, GMO or irradiation-assisted 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. The GBSSI gene is directly implicated in the synthesis of amylose, and the identification of naturally occurring or irradiation-induced mutant alleles has great importance for the cassava breeding program. The waxy trait is a recessive one, the function of all GBSS alleles have to be eliminated for the trait to be expressed. The elimination of the GBSSI genes has been attempted via genetic engineering, but a search for natural or irradiation-induced mutant alleles of the GBSSI gene has also been initiated (CIAT 2003). An accession each of the wild *Manihot* species *M. crassisepala* and *M. chlo*rosticta are the only genotypes from the primary and secondary gene pool of the crop discovered to possess the waxy phenotype (CIAT 1995, 2002, unpublished data). Irradiation-induced mutation of the GBSS gene has also been attempted using both gamma rays and fast neutrons irradiation of sexual seeds (This report). The heterozygous nature and possibilities of a duplicated GBSSI loci in cassava implies that mutant alleles implies that the identification of a waxy phenotype requires selfing thousands of plants or development of molecular markers for identification of mutant alleles in a heterozygous background. We describe here the development of molecular markers for the mutant allele in the wild waxy relatives and the initiation of genetic crosses to transfer this trait into cultivated gene pools around. The marker tools developed will be critical to track down these mutant alleles genes to efficiently move these genes around the different cassava gene pools defined by agro-ecologies. We also describe preliminary molecular work on the search for mutant alleles in the irradiated seeds.

Methodology

Plant material for the development of markers for mutant GBSSI alleles were two wild *Manihot* accessions MCRA-013 and CW14-11 identified earlier with amylose content between 3-5%. Two cultivated cassava MCOL 1508 and MCOL 2269 were included as reference for normal alleles (Table 1). The Codon Optimized to Discover Deleterious Lesions (CODDLE) program was used to identify regions of the GBSS genes where mutations are most likely to lead a loss of function in the protein.

Table 1. Wild *Manihot* and cassava accessions used for the development of markers for mutant GBSS alleles

Cassava accessions	Origin	Species	% Amylose
CRA-013 (Wild)	Mexico	*M. Crassisepala*	3.08
CW14-11	CIAT	Hybrid of *M. Clorostrictha* with unknown parent	4.97
MCOL-1508	CIAT	*M. esculenta*	23.29
MCOL-2269	CIAT	*M. esculenta*	10.55

Primers were designed to amplify the 3' and 5' of the Glycosyl Transferase region (GT1 and GT2) of the GBSSI gene, between 300-400bp, primer sequences are shown below (Table 2). PCR template was both genomic DNA and cDNA (RT-PCR) from the wild and cassava accessions and amplification was as described earlier by Salehuzzaman *et al* (1993). A full length GBSS cDNA clone, Clone 3, isolated from a cassava root cDNA library was used as positive control.

PCR product was cloned into pGMT (Promega, Madison WI) and 5 clones each from each PCR fragment and each clone were sequenced by big dye (Perkin Elmer, Alamo, CA) cycle sequencing according to the manufacturer's instruction. Sequence information was aligned using the CLUSTAL software program and point mutations identified. An attempt was also made to map the GBSSI gene, as an RFLP marker, on to a molecular

Table 2 Primer sequences designed to amplify the 3' (GT1) and 5' (GT2) end of the Glycosyl Transferase regions of the GBBSSI gene

GBSSI region	Primer sequence
GBSSI(GT1) Forward Primer	5' CAGCTATTTCCCAATTGGTTG 3'
GBSSI(GT1) Reverse Primer	5' GCGGTAGCATAAGTGCCAAG 3'
GBSSI(GT2) Forward Primer	5' GCAGGTTTGGATTACCAGGA 3'
GBSSI(GT2) Reverse Primer	5' CAGCAATGCCAGTTTTACGA 3'

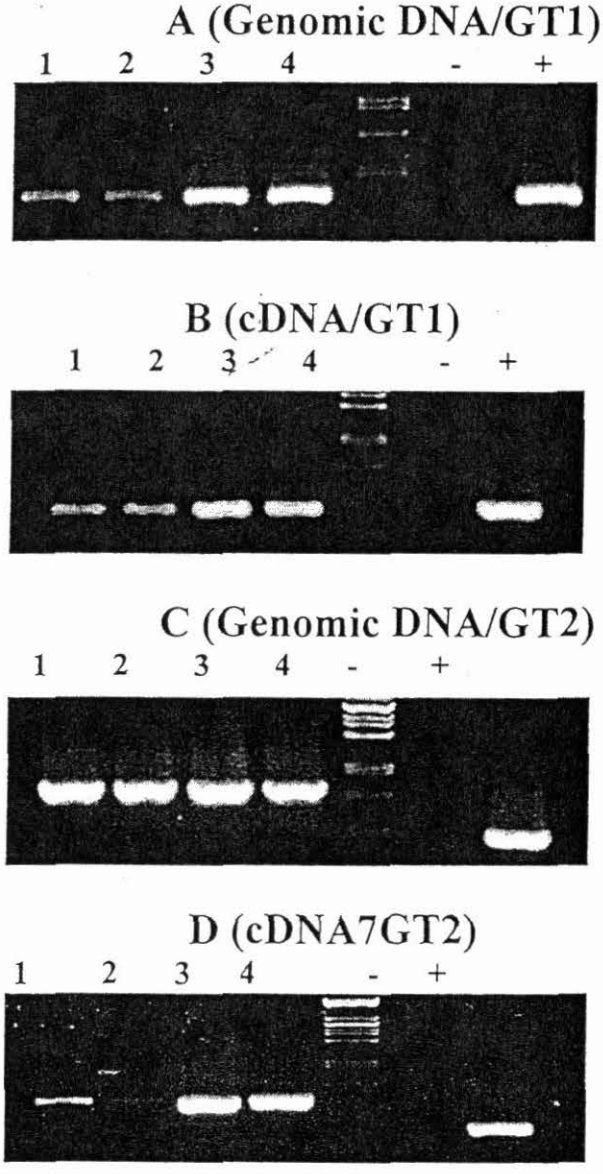
genetic map of cassava being drawn in a S_1 family (AM320) from the cassava variety MTAI8. Parental survey filters were made using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI* using the following DNA samples: MTAI8, 4 S_1 progenies, MCRA-013, CW14-11, MCOL 1508, and MCOL 2269. RFLP probe was a full length GBSSI cDNA clone (clone-3) from a root cDNA library from TMS30572.

Preparation and Southern hybridization of the filters were as described by Fregene et al. (1997).

About 2000 sexual seeds from cassava genotypes tolerant to inbreeding shipped to the International Agency of Energy Atomic (IAEA), Vienna, Austria, for irradiation using gamma rays (a Cobalt-60 source) and fast neutrons, 1000 seeds each for both irradiation methods. The level of irradiation with gamma rays was 200Gy. They were sent back to CIAT and germinated in seedling nursery and transferred to the field, about the 1600 seeds could be transferred to the field, the other seeds suffered from lethal mutations that affected their ability to germinate.

Results

Amplification of primers flanking the GT1 and GT2 regions from genomic DNA and cDNA (RT-PCR) revealed amplification of similar size fragments for all accessions per primer pair although the wild genotypes had a lower intensity of PCR products with the exception of PCR products from genomic DNA using the GT2 primer pair (Fig 1). The lack of size polymorphisms in the PCR products suggests that there are no INDELs (insertions-deletions) type polymorphisms in the wild accessions for this region of the GBSSI gene. The sequence data from DNA clones of the different accessions were aligned and four single point polymorphisms (SNPs) mutations were found that differentiated the wild accessions from the cultivated, two were common to both wild species, while two mutations distinguished *M. chlorosticta* from *M. crassisepala*. The SNPs were sense mutations that led to amino acid changes in the GBSS protein. Allele specific primers were designed for all 4 SNPs as described by Hayashi et al (2004) and have been ordered (Table 3). This PCR-based molecular marker could be used to follow the alleles in back crosses with elite cassava parents.



1. *M. Crassisepala*
2. CW14-11
3. MCOL1508
4. MCOL2269
- COCKTAIL
+ GBSSI cDNA (CLON 3)

Figure 1. PCR amplification of genomic DNA and cDNA from the 2 wild accessions MCRA013 and CW14-11 and 2 cassava varieties MCOL1508 and MCOL2269 with primers from the GT (A and B) and from the SS region (C and D) of the GBSS gene

Southern analysis of MTAI8, 4 S_1 progenies, the 2 wild accessions, and 2 cassava genotypes revealed 2 or more fragment of different intensities for all 5 restriction enzymes (Fig 2). This result suggests that there is more than one copy of the GBSSI gene in

cassava. Previous studies have revealed 2 GBSS cDNA clones in cassava with <80% homology (Baguma et al. 2003).

The Southern hybridization also revealed polymorphism between the wild and the cultivated confirming previous results of SNPs in the wild species. These SNPs are no doubt associated with the low content of amylose in the two wild genotypes.

Table 3. Allele specific primers designed to amplify mutant alleles from MCRA013 and CW14-11

Source of mutant allele	Primer sequence
MCRA013 and CW14-11 mutant allele (1)	5'AGAAATTTGAGAAGCAGATTGAGCAG 3'
MCRA013 and CW14-11 mutant allele (2)	5' TCAATTTTGTCACATTCAACGAGC 3'
CW14-11 mutant allele (1)	5' CTGACAAGGCAAGAGGAGTTGTT 3'
CW14-11 mutant allele (2)	5' ACAGCTGGTGCAGACTTTATGCTTA 3'

Polymorphisms in a fragment of lower intensity were found with the restriction enzymes DraI and Hae111 in progenies of an S_1 mapping population (Fig. 2). These enzymes will be used to prepare progeny filters for mapping the gene GBSSI in the S_1 mapping population derived from MTAI 8.

There are plants of MCRA013 and CW14-11 growing in the field for genetic crosses to cassava. So far no flowering has been obtained with MCRA013, but several male and female flowers have been obtained with CW14-11. More than 30 crosses were made to elite parents of CIAT cassava gene pools but only 2 of these crosses have been successful to date. Additional crosses are continuing while the 2 fruits obtained till date will be harvested at the end of the crossing season.

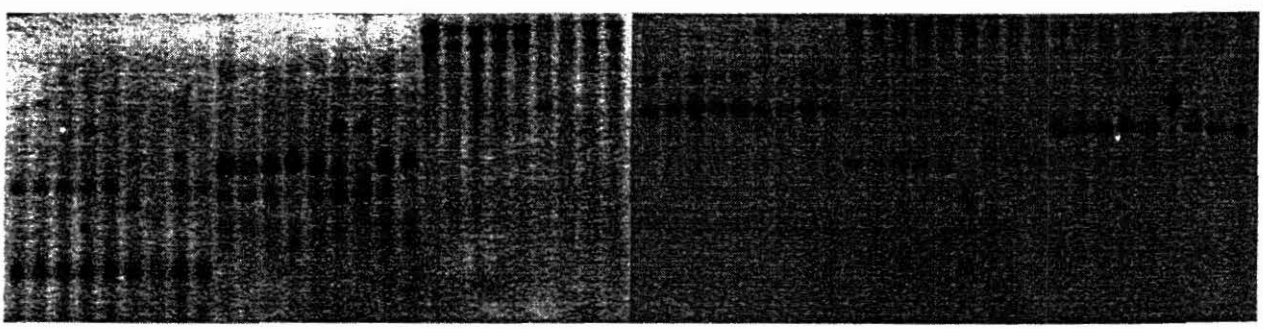


Figure 2. Southern blot of restriction enzyme digested cassava DNA from MTAI-8 and 4 of its S_1 progenies (lanes 1-5), MCRA013, CW14-11, MCOL1508, and MCOL2269 (lanes 6-9) probed with a ^{32}P labeled full-length GBSSI cDNA clone (clone 3). Ten microgram of digested DNA was loaded per lane.

The effect of fast neutrons on irradiated seeds was quite severe and less than 200 plants could be established in the field. At the moment there are 943 surviving plants from both

gamma and fast neutron irradiation in the field. Morphological characterization of these plants has been initiated. Molecular analysis of these plants to identify mutant GBSS alleles has also been started. Briefly, 8 young shoots were harvested from all over

individual plants and bulked for DNA isolation. Plants from irradiated sexual seeds are expected to contain chimeras and bulking the leaf tissue from all over the plant eliminates this problem. All DNA samples will be amplified with primers from the glycosyl transferase region and analyzed by gel electrophoresis to identify any insertions/deletions (INDELS). Amplified PCR products will then be bulked, at least, 20 genotypes per bulked, and used as template for sequencing to identify SNPs. Phenotypic evaluations for amylose content will also be conducted to identify mutants, although improbable, at all possible 4 GBSSI alleles.

Conclusion and future perspectives

Sequence and Southern hybridization analysis have identified SNPs in an accession each of *M.crassisepala* and *M.chlorosticta*. The SNPs from sequence data are sense mutations that are most likely associated with the waxy phenotype found in these genotypes. Allele specific primers have been designed from the SNPs for use in following the alleles in introgression of the waxy trait into elite gene pools. Southern analysis also revealed a high probability of duplication of the GBSSI loci in cassava. Preliminary molecular analysis of plants obtained from irradiation of cassava seeds with gamma and fast neutrons have also been initiated. Future perspectives include:

- Evaluations of allele specific primers in the wild and cassava accessions.
- Genetic mapping of the gene GBSSI in the S_1 population from MTAI8
- Crosses of the wild genotypes to elite cassava cultivars to transfer the mutant alleles.
- Molecular characterization of the irradiated population to identify GBSSI mutants

References

Salehuzzaman,S.N., Jacobsen,E. and Visser,R.G. (1993) Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta* Crantz) and its antisense expression in potato. Plant Mol. Biol. 23 (5), 947-962

1.3.18 Mining the Primary Gene Pool: Green Mites (CGM) Resistance Genes from *Manihot* tristis.

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CIAT

Important outputs

1. Identification of additional sources of resistance to green mites in an F_1 inter-specific hybrid between cassava and *Manihot tristis*
2. Development of BC_1 families to introgress the high level of green mites resistance into cassava

Introduction

A high level of resistance to the cassava green mites (CGM) was found 2 years ago in 4 F_1 inter-specific hybrids between cassava and *Manihot esculenta sub spp Fabellifolia.* An attempt was made to identify SSR markers linked to CGM resistance genes as well as introgress the resistance into cassava by the generation of 45 BC_1 families (CIAT 2003). This year another inter-specific family CW208, a cross between MTAI8 and OW132-2 *Manihot tristis*, was also observed to have a wide segregation for resistance/susceptibility to CGM. This same inter-specific cross also has a wide segregation for dry matter content. We describe here evaluation of this cross for resistance to CGM and efforts to develop gene tagging and breeding populations for increased resistance to CMD.

Methodology

The CW208 family is an F_1 inter-specific hybrid cross with a total of 120 genotypes. A single row trial (SRT) of the family was planted August 2003 at CIAT, Palmira. It was evaluated in April this year for resistance to mites according to a 3 class score of 1: means no visible mite damage, 3: moderate damage to mites, and a score of 5: severe damage and loss of all apical meristems due to mite damage. Trait score data was entered into Microsoft Excel and a frequency distribution of score classes derived.

Results

Frequency distribution of 3 score classes revealed a close to normal distribution pattern for resistance to green mites found in the family CW208 (Fig1). The large number of highly resistant and highly susceptible genotypes makes this family an ideal one for bulked segregant analysis (BSA) to identify genes for green mite resistance. Bulks have been made with genotypes from both resistant and susceptible extremes and will be analyzed shortly with over 600 SSR markers. Backcrosses are also being made using selected

progenies with resistance to CGM to MTAI 8, an elite cassava genotype that is highly susceptible to green mites.

Conclusions and Future Perspectives

An additional source of resistance to green mites has been identified in an inter-specific hybrid family between cassava and *Manihot tristis*. The family is currently being used for BSA for genes controlling resistance to CGM and for the development of BC_1 families to introgress the high level of green mites resistance into cassava

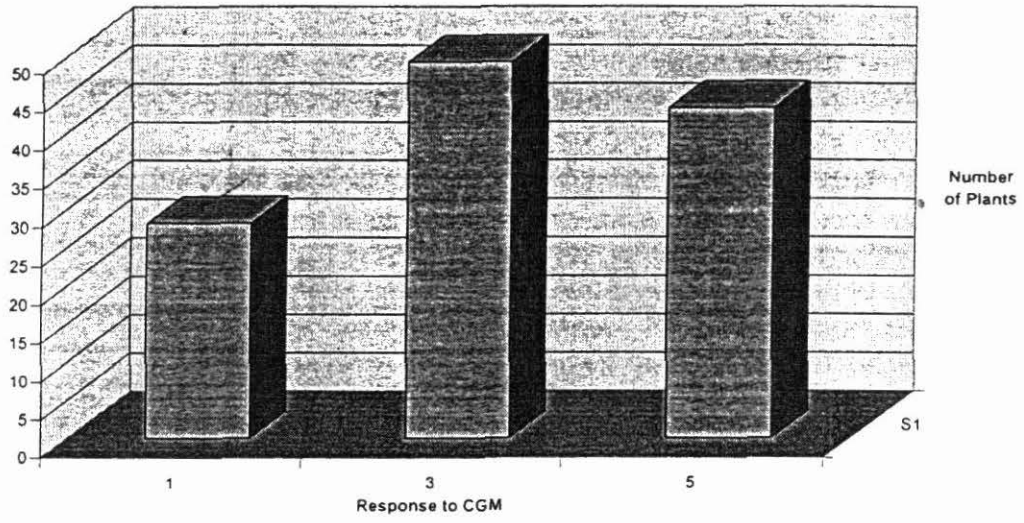

Figure 1. Frequency distribution of response to the cassava green mites in the inter-specific family CW208

References

CIAT 2003. Annual Report IP3. Improved cassava for the developing world. Pp8-65 to 8-68

1.3.19 Identification of QTLs for yield and yield components in a Backcross Population 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 as reported in the literature. Likewise, chromosomes 3 and 8 are important for determining panicle traits, perhaps one of the most relevant yield components found in advanced breeding lines derived from the Lemont/*O.barthii* cross. Marker RM42 on chromosome 8 is associated with a region derived from *O.barthii*. Marker RM184 on chromosome 10 was associated with days to heading; however, this is a very complex trait as has been reported by others. Advanced lines with different flowering dates have been selected 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 world. 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 the identification of quantitative trait loci (QTL) associated with yield and yield components in a BC3F2 population derived 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 presented. Marker data were used to identify QTLs associated with yield and yield components.

Materials and methods

Lemont was used as the recurrent parent in this study. *O. barthii* (Accession # 104119), which served as donor parent, is a relative of *O. glaberrima*. 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. 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 this population. Markers used in the evaluations and the QTL analyses were selected from the rice molecular framework linkage map (Causse et al., 1994; Chen et al., 1997).

This 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 nine agronomic traits including days to heading (*dth*), plant height (*ph*), panicle length (*pl*), percent sterility (*ps*), yield (*yld*), panicle number (*ppl*), tiller number (*tpl*), 1000-grain weight (*gw*), and number of grains per panicle (*gpp*) 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 was done and significance thresholds were estimated by permutation tests. Moreover, permutations allow the estimation of experiment-wise 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.

Results

Data showed that in chromosomes 2, 4, 5, 6 and 9, neither marker was associated with neither of the traits; while, in chromosomes 1, 3, 7, 8, 10, 11 and 12, there were several genetic regions associated with more than one trait. In some cases SPA did not detect QTLs that were associated for IM and CIM (low potency, type II-error) (Table 1).

Table 1. QTLs not detected for SPA analysis.

Trait	Chromosome	Marker
Plant height (*ph*)	3	RM60
	7	RM82
	8	RM42
Days to heading (*dth*)	10	RM304
Percent sterility (*ps*)	8	RM42
Panicle length (*Pl*)	8	RM42

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 ppl , *tpl, ph, pl* and *ps* but not with *yld* (Table 2) . On chromosome 1, *yld* was associated with microsatellite RM5 (Fig. 1).

Table 2. Microsatellite marker associated with different agronomical traits.

Trait	Chromosome	Marker
Plant height (*ph*)	8	RM42
Percent sterility (*ps*)	8	RM42
Panicle number (ppl)	8	RM42
Number of tiller (*tpl*)	8	RM42
Panicle length (*Pl*)	8	RM42

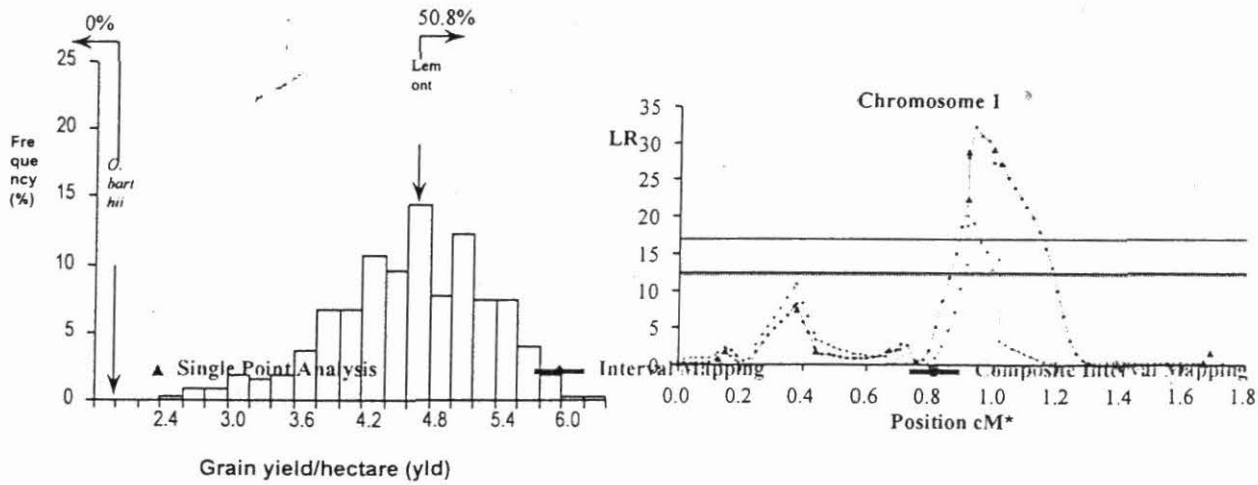

Figure 1. QTL identification and segregation histograms for yield in the Lemmont / *O.barthii* cross.

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.

Variable	Chro.	SSR	SPA	Position	Interval Mapping				Composite Interval Mapping			
					LR	LOD	R2	A	LR	LOD	R2	A
Number panicle (*mtn*)	11	RM167	*	0.1934	14.7488	3.2034	0.0441	0.8991				
	11	RM21	*	0.4429	29.2515	6.3534	0.0856	1.2211	17.9994	3.9095	0.0416	0.8752
Number of tiller (*tpl*)	3	RM60		0.4001					16.7752	3.6436	0.0395	0.8461
	3	RM81B	*	0.4006	29.7686	6.4657	0.0887	1.2186				
	8	RM25	*	0.0461	16.3878	3.5594	0.0507	-1.6625				
	8	RM42	*	0.1074	37.5531	8.1565	0.1108	-2.3873	31.1556	6.7670	0.0739	-1.9998
Number of fully grains per panicle (*gpp*)	2	RM236	*	0.2499	16.2147	3.5218	0.0534	-19.6958				
	2	RM211	*	0.2730	16.2113	3.5211	0.1147	-40.3618				
	2	RM208	*	0.9979	22.7710	4.9459	0.0675	-15.4639				
	2	RM207	*	1.6861	15.5634	3.3804	0.0490	-13.5627				
Plant height (*ph*)	1	RM1		0.2884					180.3829	39.1792	0.6379	-22.1183
	1	RM243	*	0.5405					170.4507	37.0219	0.6403	-22.0876
	3	RM60		0.2601	131.2772	28.5134	0.6746	-21.6798	207.9528	45.1674	0.5970	-21.5614
	3	RM231		0.6899					132.9728	28.8817	0.5876	-21.7906
	12	RM247		0.2814					168.2731	36.5489	0.5870	-21.9436
	12	RM117		0.5381					192.9978	41.9191	0.5887	-21.8944
Panicle length (*pl*)	3	RM168		1.1247	16.2683	3.5335	0.0915	-1.0555				
	3	RM227	*	1.3431	52.8798	11.4855	0.1931	-1.2194				
	3	RM114	*	1.3540					70.8607	15.3909	0.1563	-1.0630
Yield per plant (*yld*)	1	RM5	*	0.9363	32.1769	6.9888	0.1255	725.9218	20.0073	4.3456	0.0510	408.6923
	1	RM140	*	1.0134					14.0609	3.0540	0.0390	443.0055

Data analyzed in global form suggested 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 suggested a wide region that must be confirmed (Table 4).

Table 4. Genetic regions for confirming QTLs

Variable	Chromosome	Marker	SPA	Position	Interval Mapping				Composite Interval Mapping			
					LR	LOD	R2	Origin	LR	LOD	R2	Origin
Number of panicles	3	RM81B	***	0.401	36.144	7.851	0.106	1.142	22.900	4.974	0.055	0.851
	8	RM42	***	0.107	26.963	5.856	0.081	-1.745	19.827	4.306	0.047	-1.369
	11	RM21	***	0.443	29.251	6.353	0.086	1.221	17.999	3.909	0.042	0.675
Number of tiller	8	RM42	***	0.107	37.553	8.157	0.111	-2.387	31.156	6.767	0.074	-2.000
	11	RM21	***	0.443	29.280	6.360	0.086	1.427	18.466	4.011	0.042	1.026
Number of fully grains per panicle	12	RM19	***	0.059	29.675	6.445	0.093	29.747	23.990	5.211	0.069	26.363
1000 grains weight (gr)	3	RM114	***	1.374	21.229	4.611	0.078	0.687	20.501	4.453	0.070	0.657
Days to heading	10	RM184	***	0.013	99.533	21.618	0.260	15.941	99.936	21.706	0.219	14.326
Panicle length (cm)	8	RM210	***	0.570	35.383	7.685	0.146	1.142	16.840	3.658	0.055	0.742
	8	RM256	***	0.989	42.172	9.160	0.159	1.261	36.825	7.998	0.098	1.045
Yield (t/ha)	1	RM5	***	0.936	32.177	6.989	0.126	725.922	20.007	4.346	0.051	408.692

This preliminary analysis indicates that certain regions associated with important agronomic traits (putative QTLs) in the Lemont/*O. barthii* cross 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 (Fig. 2), 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 heading; 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.

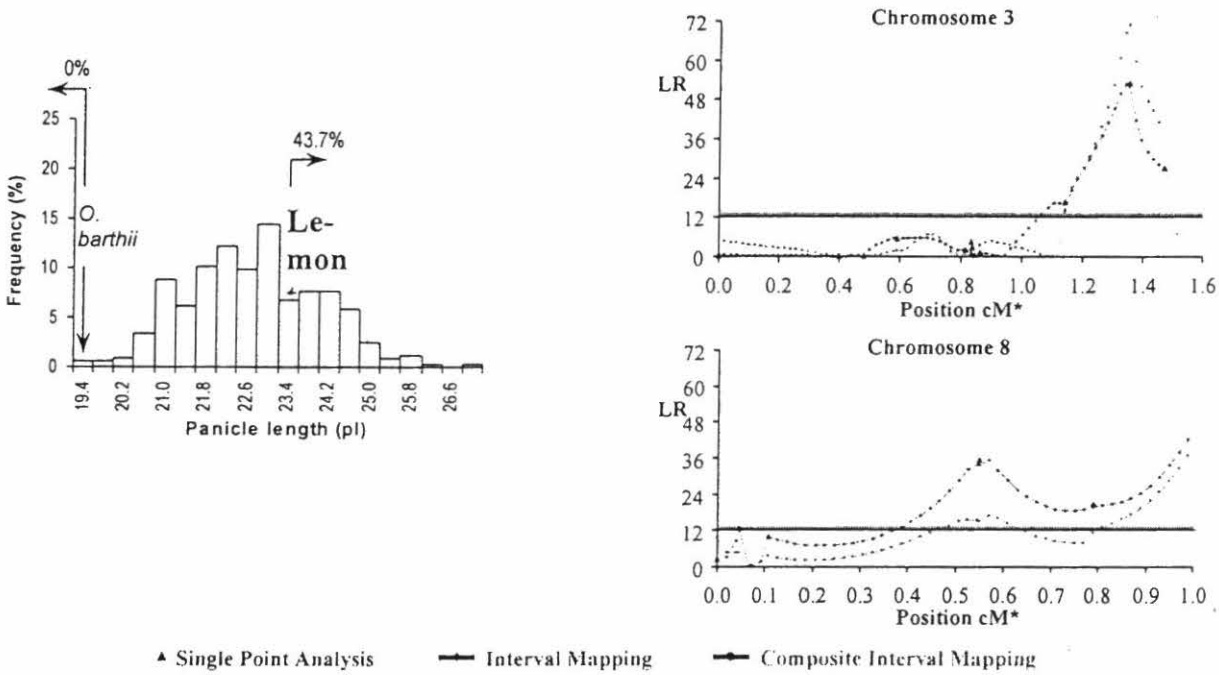

Figure 2. QTL identification and segregation histograms for panicle length in the cross Lemmont / *O.barthii*

Ongoing activities

Cross: O. barthii and Lemont

- Complete the characterization of agronomic and molecular data and QTL analysis 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 (chromosomes 1, 5, and 10) to elucidate QTLs associated with yield increase and verify which of the *O. barthii* alleles was introgressed in this population.

Other projects

- Complete the characterization of agronomic and molecular data and QTL analysis 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 lines derived from the Bg90-2/*O.rufipogon* cross to identify QTLs associated with yield increase and verify which of the *O. rufipogon* alleles were introgressed into this population.
- Initiate the analysis of agronomic and molecular data from different generations obtained from the Bg90-2/*O. rufipogon* cross to determine gene introgressions from the wild species.

References

Causse, M.A.; Fulton, T.M.; Cho, Y.G.; Nag Ahn, S.; Chunwongse, J.; Wu, K.; Xiao, J.; Yu, Z.; Ronald, P.; Harrington, S.E.; Second, G.; McCouch, S.R. and Tanksley, S.D. 1994. Saturated molecular map of the rice genome based on an inter-specific backcross population. Genetics 138:1251-1274.

Chen, X.; Temnykh, S.; Xu, Y.; Cho, Y.G.; and McCouch, S.R. 1997. Development of a microsatellite framework map providing genome-wide coverage in rice (*O. sativa L.*). Theor. Appl. Genet. 95:553-567.

Delseny M., Salses J., Cooke R., Sallaud C., Regad F., Lagoda P., Guiderdoni E.,Ventelon M.,Brugidou C.,Ghesquière. 2001. Plant Physiol. Biochem. 39:323-334.

McCouch, S.R.; Kochert, G.; Yu, Z.; Wang, Z.Y.; Khush, G.S.; Coffman, W.R. and Tanksley, S.D. 1988. Molecular mapping of rice chromosomes. TAG 76:815-829.

Temnykh, S.; Park, W.D.; Ayres, N.; Cartinhour, S.; Hauck, N.; Lipovich, L; Cho, Y.G.; Ishii, T. and McCouch, S.R. 2000. Mapping and genome organization of microsatellite sequences in rice (*O. sativa L.*). Theor. Appl. Genet. 100:697-712.

1.3.20 Evaluation of segregation of carotene content in self-pollinated progenies from selected clones.

Introduction

In reports from previous years the results of self-pollinations of the elite clone MTAI 8 (Rayong 60) illustrated the advantages of inbreeding cassava for research purposes. One of the surprises of this early work was the apparent recessive behavior of carotene content in cassava roots. Further evaluation of self-pollinated progenies was therefore, pursued.

Specific Objectives

a) Produce self-pollinated progenies from elite cassava clones.
b) Evaluate these progenies for carotene content in their roots.

Results

Self-pollinated progenies from three elite clones were harvested. The most profusely sampled genotype was MTAI 8 (slightly colored roots) with a total of 181 S_1 plants, followed by yellow-rooted clones CM 2772-3 and CM 4919-9 with only 12 S_1 plants each. At this point only ratings for color intensity can be provided because the carotene-quantification equipment if fully used for other research activities. Root samples have been stored for analysis when the analytical laboratory can proceed. However, as described in Output 1, there is an excellent correlation between color intensity and carotene content. Therefore, the results presented preliminary as they are, will suffice to illustrate the kind of segregations obtained.

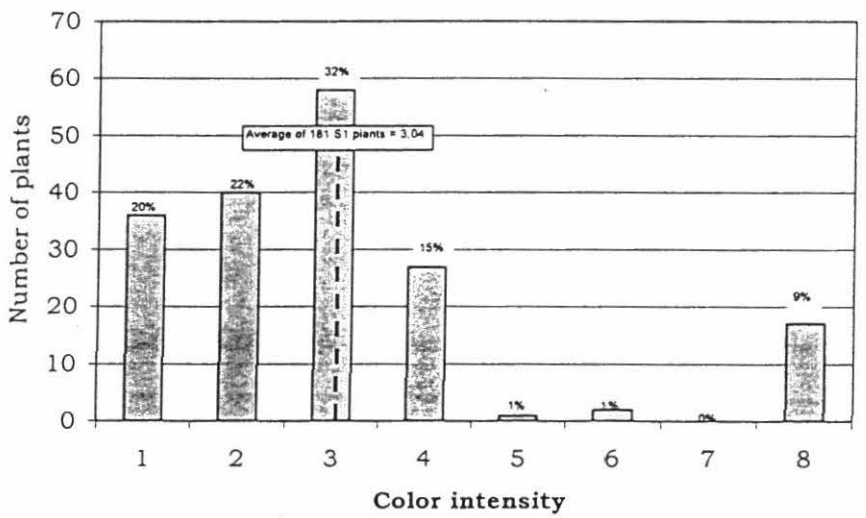

Figure 3. Segregations for color intensity in 181 S_1 plants from MTAI 8 (Scale 1= white; 5= deep yellow; 8= pinkish roots).

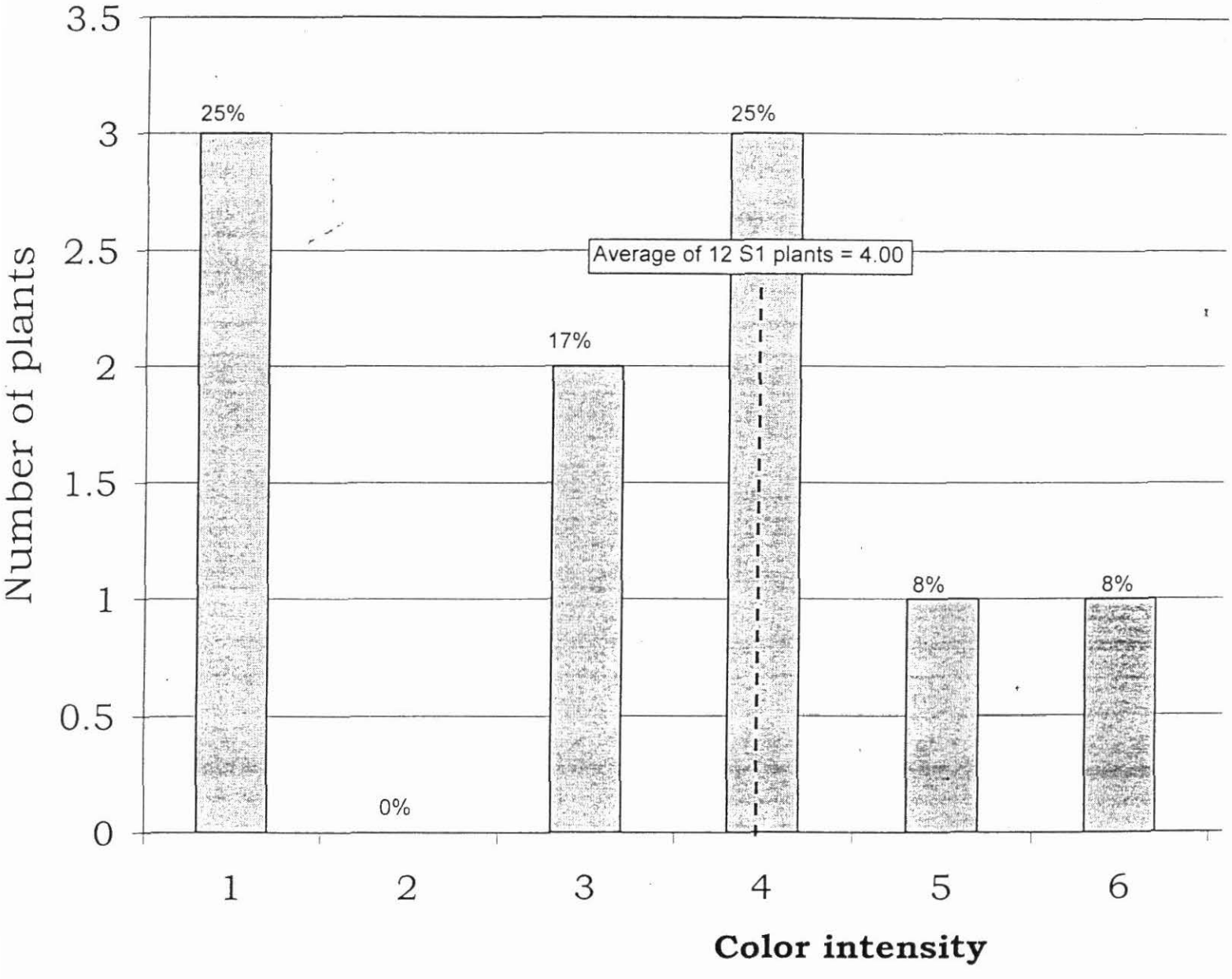

Figure 4. Segregations for color intensity in 12 S_1 plants from CM 4919-9 (Scale 1= white; 5= deep yellow; 8= pinkish roots).

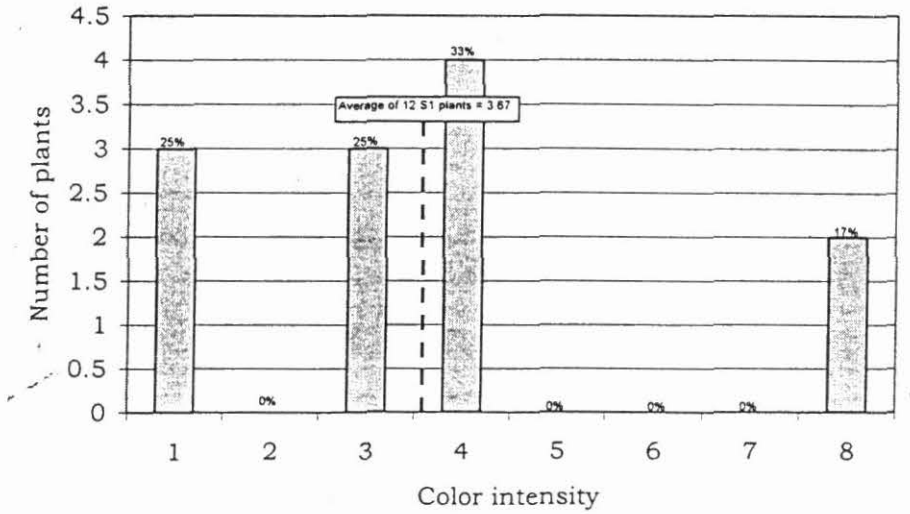

Figure 5. Segregations for color intensity in 12 S_1 plants from CM 2772-3 (Scale 1= white; 5= deep yellow; 8= pinkish roots).

1.3.21 Evaluation of segregation of traits related to Post-Harvest Physiological Deterioration

Introduction

Post-harvest physiological deterioration (PPD) remains one major constraint for cassava development . As such the problem is dully addressed by CIAT scientists. A manuscript of a scientific article in this regard was reproduced in Output 1. This activity reports data of recently harvested clones developed to analyze the inheritance and factors affecting PPD.

Specific Objectives

a) Produce crosses between two clones contrasting for different variables.
b) Germinate the botanical seed produced and from the resulting clones obtain stakes.
c) Plant the clones genotypes in an evaluation trial.
d) Analize the cloned genotypes for the relevant variables.

Results

Two clones were selected for this activity. CM 523-7 (ICA-Catumare) has white roots, with high dry matter content and high susceptibility to PPD. Clon MBRA 337 has yellow roots, low dry matter content, intense yellow roots and tolerant to PPD.

A total of 38 hybrids from the cross between CM 523-7 x MBRA 337 could be cloned and planted in two replications at Santander de Quilichao (Cauca Department). Each plot had 8 plants that were harvested. The following variables will be analyzed for each plant individually: PPD, dry matter content, total starch, total sugar, reducing sugars, amylase/amylopectin ratio, cyanogenic potential. Carotenes will also be measured in pooled samples from 2-3 plants. These results will be used to learn about the individual segregations for each trait, as well as for determining the relationship among them.

1.3.22 Development of CSSLs and associated marker systems in two interspecific *O. sativa* x *O. glaberrima* populations

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Partners: IRD
Project funded by CIAT, IRD and the Generation Challenge Program

Introduction

The future of crop improvement depends on the availability of genetic variation. Most modern crop varieties have undergone a genetic bottleneck associated with the process of domestication resulting in a restriction of the genetic options that are available to plant breeders. There is a larger pool of genetic variation available in landraces and wild relatives of crops. These resources are known to contain many interesting traits for breeding, including good to strong tolerance to abiotic and biotic stresses and various nutritional traits of interest (Sun et al 2001). However, it is often difficult to utilize these natural sources of genetic diversity because of fertility barriers, linkage drag, the time and resources required to recover useful recombinants.

To take advantage of the unexploited reservoir that exists in the wild relatives of cultivated rice (*Oryza sativa* L.), we started to develop interspecific introgression lines that will be of immediate use to breeders and will simultaneously serve to enhance our understanding of the "wild alleles" that contribute favorably to plant performance under drought stress. These lines are called Chromosome Segment Substitution Lines (CSSLs).

CSSLs are particularly valuable when complex, quantitatively inherited phenotypes are the breeding target. Because they represent permanent (inbred) genetic resources that can be easily replicated by seed and distributed to collaborators working in different environments. Each set of CSSLs consists of a relatively small number of lines that can be evaluated in replicated trials. They are constructed to provide maximum power of statistical analysis because each line can be compared to all others or may simply be compared to the recurrent parent, making it possible to extract a great deal of valuable information from a relatively small number of lines crops. For phenotypes that are difficult to measure, or require repeated evaluation over years and environments, the ability to focus quickly on a small number of lines is a critical component of success (Ghesquière et al, 1997).

In addition to the targeted introgression of traits that can be identified phenotypically in the wild material, such as biotic or abiotic stress tolerance, it has been demonstrated that alleles hidden in low yielding, agronomically undesirable ancestors can enhance the productivity of many of the world's most important crop varieties. These yield-enhancing alleles are the basis of 'transgressive variation' and may confer an advantage in both favorable (irrigated) and unfavorable conditions (drought and weed competition) (Moncada et al., 2000; Gur and Zamir, 2004). Thus, the use of wild and exotic germplasm for CSSLs construction carries with it the possibility that favorable transgressive segregants will be identified, providing the basis for studies aimed at understanding the genetic basis of transgressive variation associated with the trait of interest.

Wide spread utilization of *O. sativa* relatives remains limited due to the fact that: (1) no extensive study has been carried out to explore the range of allelic diversity in any of the *Oryza* AA genome relatives, (2) the genetic basis of heterosis or transgressive variation in interspecific crosses remains largely unknown, (3) interspecific crossing barriers have hampered full utilization of rice relatives for breeding and genetic studies, (4) very few genomic resources have yet been developed to facilitate breeding efforts using *O. sativa* relatives. In particular, the lack of a cost effective, high throughput marker system that targets gene-based polymorphisms impedes efforts to efficiently and systematically select the best introgression lines and to evaluate the gene content of those lines in the context of comparative cereal genomics.

Materials and Methods

Genetic material

Two populations derived from *O. sativa* x *O. glaberrima* crosses were chosen for searching CSSLs:
a BC3DH population produced by C. P. Martinez and Z. Lentini at CIAT. This population was derived from the cross Caiapo (tropical japonica) x IRGC103544 and was made of more than 600 lines.

A BC3F4/5 population produced by M. Lorieux and A. Ghesquière at IRD. The parents of this population are IR64 (indica) and TOG5681.
Preliminary data análisis

For the Caiapo x IRGC103544 population, we started for data generated by C.P. Martinez and constituted by genotyping of 312 lines using 99 SSRs. The lines were searched for contiguous chromosomal segments that would cover the all-*O. glaberrima* genome. To this aim, a computer program was specially designed (CSSL Finder). This program allows searching for a subset of lines (starting from the entire set) that have contiguous segments. Several parameters values can be chosen, like desired segment size, smaller and larger allowed segment size, possibility of retaining lines with segments tagged by only one markers, minimum tiling path, etc. Then, graphical genotypes of the selected lines were obtained using another software that we developed (GraphGenot).
For the IR64 x TOG5681 cross, BC3F4/5 lines were generated at IRD using a marker-assisted selection of genotypes at BC1, BC2 and BC3 stages. The GraphGenot software was then used to draw graphical genotypes of the selected lines.
Marker analyses
The 312 genotyped lines of the Caiapo x IRGC103544 population were screened for additional markers, in order to fill the gaps in the genetic map made of the 99 SSRs. Standard conditions for PCR and gel migration (PAGE/silver staining) were used.
The IR64 x TOG5681 population was grown at CIAT HQs during the first semester of 2004. DNA was isolated from 354 lines using a modified version of the CTAB protocol (Lorieux et al, 2003). The population is currently screened using a set of evenly dispersed (each 15-20 cM) SSR markers. When possible (i.e. when polymorphism was > 5bp), PCR products were separated on 2.5-3% agarose gels or 3% Metaphor gels and stained with ethidium bromide. We choose to use that technique instead of PAGE as it is more flexible, quicker and gels are easier to stain.
We have initiated a standardization of electrophoretic conditions on agarose or Metaphor gels for polymorphism detection, using 12 rice accessions that are the parents of various segregating populations developed at CIAT and IRD or accessions of general interest. The *O. sativa* accessions are: Nipponbare, IR36, Azucena, IR64, Caiapo, Bg90-2, and Orizica 3; the other accessions were: one *O. barthii,* one *O. longistaminata*, two *O. glaberrima* (Tog 5681 and IRGC103544), and one *O. rufipogon*. This survey will let us have a universal core set of easy-to-screen SSR markers useful for numerous genetic analyses using diverse rice segregating populations.
Final data analysis
After completion of the genotyping of the Caiapo x IRGC103544, CSSL Finder was ran again to redefine the best CSSL set within the 312 lines set. Several parameters values were tried to optimize the choice of the final set of lines. Graphical Genotyping of the final set was obtained using GraphGenot.

Results

Caiapo x IRGC103544 population

The preliminary data analysis consisted in running CSSL Finder on the first data set of 99 markers. This analysis permitted to identify a subset of 97 lines that maximized the *O. glaberrima* genome covering. As an immediate output, this subset of lines constituted a core set for drought resistance screening trials carried out at three locations (La Libertad Station – Colombia; CNPAF – Brazil – WARDA – Mali) in the framework of the Generation Challenge Program (SP2 C4).

The marker analysis allowed us to add 51 new SSRs to the data matrix and to improve significantly the genome coverage.

CSSL searching was run on the new data set using several parameter values. We finally retained the lines set obtained with a desired segment size of 25 cM and allowing for one-marker segments. An example of Graphical Genotyping of a set of lines is shown in Fig. 1.
IR64 x Azucena population
We have completed the evaluation of twenty-one SSR markers on the 354 lines (Fig. 3).
CSSL searching will be carried out while the complete data set of chosen markers will be available.

SSR polymorphisms on agarose gels

We select 181 SSRs to be evaluated, known as polymorphic between Caiapo and IRGC103544 and/or between IR 64 and Tog 5681.
For the IR64 x TOG5681 cross, polymorphism for 65 of these markers could be detected in separating the PCR products on 3% agarose gels, 13 using 2.5% agarose gels, and 22 using the 3% high resolution Metaphor agarose (Fig. 2). Fifty-six SSRs were found to show a polymorphism corresponding to a size difference of 5 bp or less and only could be resolved by PAGE. The PCR and gel separation conditions standardization for the remaining SSRs is on going.

Discussion

CSSLs were proven as very a powerful tool for gene discovery in different crops. They are of particular value for studies involving wild progenitors as they 1) often permit to overcome interspecific sterility barriers as a large part of the cultivated species is recovered in advanced generations, 2) allow a direct comparison of the introgressed lines to the cultivated parent, permitting to display the effect of the wild progenitor on the phenotype.
The Caiapo x IRGC103544 population was already successfully used to identify chromosomal segments linked to quality traits (Aluko et al 2004).

The IR64 x TOG5681 lines also already permit to physically map a major gene for Nematode resistance (Lorieux et al 2003).

We hope that the development of full-genome coverage CSSL populations will contribute significantly to the set of genetic and genomic tools available for breeding and gene discovery in rice.

References

Aluko G., C. Martinez, J. Tohme, C. Castano, C. Bergman, J.H. Oard 2004. QTL Mapping of Grain Quality Traits From the Interspecific Cross *Oryza sativa* x *O. glaberrima*. Theor. Appl. Genet. 109:630-639

Ghesquière, A., J. Séquier, et al. (1997). "First steps towards a rational use of African rice, *Oryza glaberrima*, in rice breeding through a 'contig line' concept." Euphytica **96**: 31-39.

Gur A and D Zamir (2004) Unused natural variation can lift yield barriers in plant breeding. Public Library of Science (in press)

Moncada, M.P., C.P. Martínez, J. Tohme, E. Guimaraes, M. Chatel, J. Borrero, H. Gauch, Jr. and S.R. McCouch, 2001. Quantitative trait loci for yield and yield components in an *Oryza sativa* x *Oryza rufipogon* BC_2F_2 population evaluated in an upland environment. Theor Appl Genet 102:41–52

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*. Theo. Appl. Genet 107:691-696

Sun, CQ, X. K. Wang, Z. C. Li, A. Yoshimura, N. Iwata. 2001. Comparison of the genetic diversity of common wild rice (Oryza rufipogon Griff.) and cultivated rice (O. sativa L.) using RFLP markers. Theo. Appl. Genet 102:157-162

Figure 1. An example of graphical genotyping of a set of lines chosen using CSSL Finder. Using a combination of parameters, the total number of lines able to cover about 95 % of the *O. glaberrima* genome could be reduced to 91.

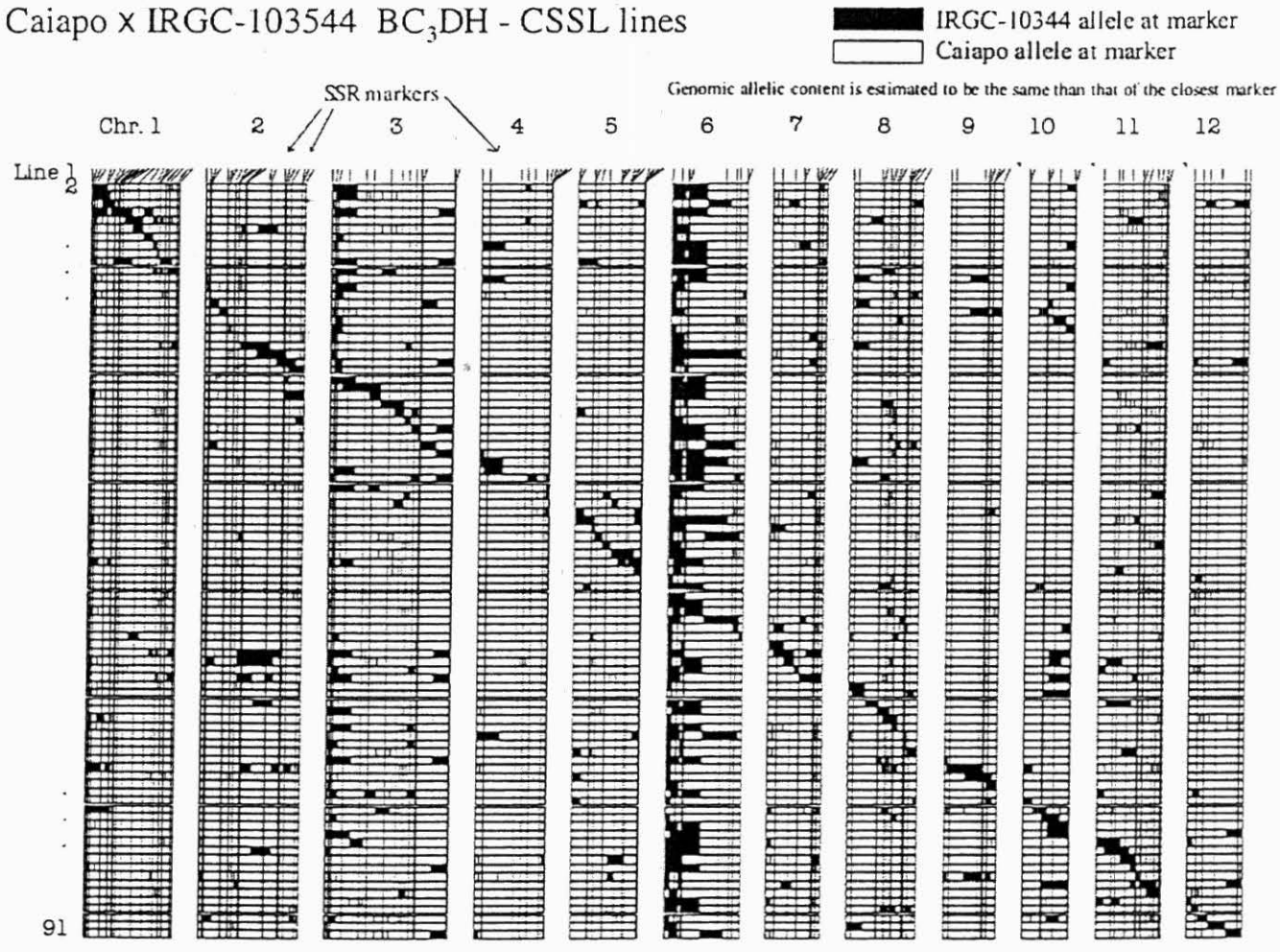
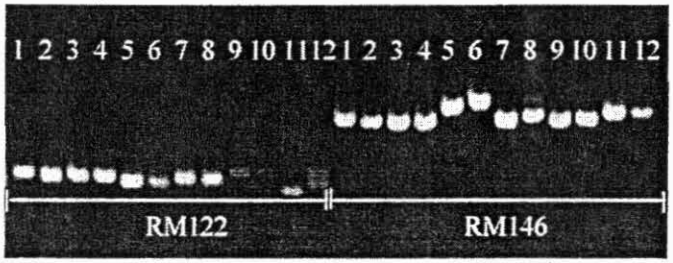


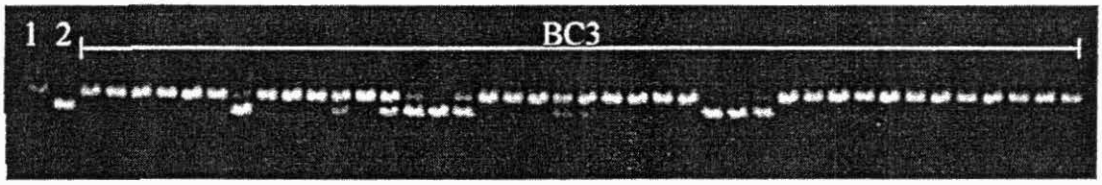
Figure 2. Polymorphism for two SSRs markers. Differences of size between the allele from IR64 (column 4) and TOG5681 (column 5) is big enough to be scored in Metaphor agarose (RM 122) and in 3% Agarose (RM 146).

1. Nipponbare, 2. IR36, 3. Azucena, 4. IR64, 5. TOG5681, 6. IRGC103544, 7. Caiapo, 8. *O. rufipogon*. 9.



Bg90-2, 10. Oryzica 3, 11. *O. barthii*, 12. *O. longistaminata*.

Figure 3. Evaluation for SSR RM7 of the population derived from the IR64 x TOG5681 cross. 3% agarose gel.



1. IR64, 2.TOG5681.

1.3.23 Development of a quantitative genetics estimate of the standard error for test for epistasis.

Introduction

Epistasis remains one of the less understood genetic effects in plant breeding and quantitative genetics. One of the problems that make epistasis so difficult to analyze is that genetic designs for its analysis require the isolation of the within-family genetic variation, which is difficult to do. Epistasis, therefore, is frequently considered to be negligible in most quantitative genetics models. The literature seldom reports on quantitative genetics estimates of epistasis, and only occasionally the tools for determining its statistical significance is provided and used.

Specific Objectives

a) Develop a formula for estimating the standard error of epistasis test.

Results

In previous reports the results of diallel studies were reported. Three different diallel mating designs were used to generate F1 crosses among 9 (sub-humid and mid-altitude valleys) or 10 (acid-soil savannas) parents. Inbreeding level of parental lines was considered zero because no self-pollination has been involved in cassava breeding and crosses among related clones are generally avoided. Controlled pollinations were performed following the standard procedures at CIAT (Output 3). Many parental clones were initially involved but the parents ultimately used (as well as the number of parents involved) were those that allowed for as a balanced set of crosses as possible. Botanical seed were germinated and grown in a screen house until the seedlings were two-months old, when they were transplanted to the field at CIAT experimental station in Palmira. F_1 plants were grown in the field for ten months. Among the many genotypes (> 30) from a given F_1 cross, 30 were randomly chosen for this study based solely on their capacity to produce at least six vegetative cuttings. Each of these stakes was planted in one of three replications at one of two locations in the respective target environments of each diallel. Table.1 provides the expectations for each mean square in the analysis of variance.

The analysis of variance was conducted following the expectations for each mean square described in Table 1. The total genetic variance has been partitioned into the between-family variation (σ^2_{F1}) and the within-family variation ($\sigma^2_{c/F1}$). The between-family variation, in turn, was partitioned into the well-known variances related to general (σ^2_{GCA}) and specific (σ^2_{SCA}) combining ability, which in turn allow the estimation of σ^2_A and σ^2_D (Griffing 1956; Hallauer and Miranda 1988):

$$\sigma^2_{GCA} = (\text{Cov.HS}) = 1/4\sigma^2_A + 1/16\sigma^2_{AA} + 1/64\ \sigma^2_{AAA} + \ldots \text{ etc.} \quad [1a]$$

$$\sigma^2_{SCA} = (\text{Cov.FS} - 2\ \text{Cov.HS}) = 1/4\ \sigma^2_D + 1/8\ \sigma^2_{AA} + 1/8\ \sigma^2_{AD} + 1/16\ \sigma^2_{DD} \ldots \text{ etc.} \quad [1b]$$

Genetic parameters were estimated using the following mean squares from Table 1:

$$\sigma^2_{GCA} = [\ MS_{31} - MS_{32} - MS_{41} + MS_{42}\ ] / \text{rak}\ (p-2) \quad [2a]$$

$$\sigma^2_{SCA} = [\ MS_{32} - MS_{42}\ ] / \text{rak} \quad [2b]$$

Variance for these estimates were calculated as follows:

$$\text{Var}(\sigma^2_{GCA}) = \{2/[rak(p-2)]^2\}$$

$$[(MS^2_{31}/df_{31}+2)+(MS^2_{32}/df_{32}+2)+(MS^2_{41}/df_{41}+2)+(MS^2_{42}/df_{42}+2)] \quad [3a]$$

$$\text{Var}(\sigma^2_{SCA}) = [2/(rak)^2] \quad [(MS^2_{32} / df_{32}+2) + (MS^2_{42} / df_{42}+2)]$$

[3b]

In this evaluation, in addition to the usual between-family variation, the vegetative propagation of cassava allowed the analysis of the within-family variation. By cloning individual genotypes, they could be planted in two locations with three replications in each location. Therefore it was possible to partition the within-family variation into its genetic ($\sigma^2_{c/F1}$), genotype by environment ($\sigma^2_{c/F1*E}$) and the environmental (σ^2_e) components, as illustrated in Table 1.

The within-family analysis allows obtaining information on the relative importance of epistatic effects. In the absence of epistasis the equation:

$$\sigma^2_{c/F1} - 3 \text{ Cov FS} + 4 \text{ Cov HS} \approx 0$$

[4]

The variance for this test is expected to be large (Hallauer and Miranda, 1988) because of the complexity of this linear function. The variance was estimated following the principles established in Lynch and Walsh (1998) and Isk et al. (2003), as follows:

$$\begin{aligned}\text{Var (Test)} &= \text{Var}[\sigma^2_{c/F1} - 3(\sigma^2_{SCA} + 2\sigma^2_{GCA}) + 4\sigma^2_{GCA}] \\ &= \text{Var}[\sigma^2_{c/F1} - 3\sigma^2_{SCA} - 6\sigma^2_{GCA} + 4\sigma^2_{GCA}] \\ &= \text{Var}[\sigma^2_{c/F1} - 3\sigma^2_{SCA} - 2\sigma^2_{GCA}] \\ &= \text{Var}(\sigma^2_{c/F1}) + \text{Var}(3\sigma^2_{SCA}) + \text{Var}(2\sigma^2_{GCA}) - 6\,\text{Cov}(\sigma^2_{c/F1}, \sigma^2_{SCA}) - \\ &\quad - 4\,\text{Cov}(\sigma^2_{c/F1}, \sigma^2_{GCA}) + 12\,\text{Cov.}(\sigma^2_{SCA}, \sigma^2_{GCA})\end{aligned}$$

[5]

However, since Cov ($\sigma^2_{c/F1}$, σ^2_{SCA}) =0 and 4 Cov ($\sigma^2_{c/F1}$, σ^2_{GCA}) = 0, the formula can be simplified:

$$\text{Var (Test)} = \text{Var}(\sigma^2_{c/F1}) + 9\,\text{Var}(\sigma^2_{SCA}) + 4\,\text{Var}(\sigma^2_{GCA}) + 12\,\text{Cov}(\sigma^2_{SCA}, \sigma^2_{GCA})$$

[6]

The last term in the equation can be estimated as:

$$\begin{aligned}\text{Cov}(\sigma^2_{SCA}, \sigma^2_{GCA}) = [(1/rak) * (1/rak(p-2)] * [&\text{Cov}(MS_{32}, MS_{31}) - \text{Cov}(MS_{32}, MS_{32}) - \\ &\text{Cov}(MS_{32}, MS_{41}) + \text{Cov}(MS_{32}, MS_{42}) - \text{Cov}(MS_{42}, MS_{31}) + \text{Cov}(MS_{42}, \\ &MS_{32}) + \text{Cov}(MS_{42}, MS_{41}) - \text{Cov}(MS_{42}, MS_{42})]\end{aligned}$$

in the above equation:

$Cov(MS_{32}, MS_{31}) = Cov(MS_{32}, MS_{41}) = Cov(MS_{42}, MS_{31}) = Cov(MS_{42}, MS_{41}) = 0$
$Cov(MS_{32}, MS_{32}) = Var(MS_{32})$
$Cov(MS_{42}, MS_{42}) = Var(MS_{42})$

Therefore,
$Cov(\sigma^2_{SCA}, \sigma^2_{GCA}) =$
$= [(1/rak) * (1/rak(p-2)] * [- Var(MS_{32}) - Var(MS_{42}) + 2 Cov(MS_{32}, MS_{42})] =$
$= -[2/(r^2a^2k^2(p-2)] * [(MS_{32})^2/(df+2) + MS_{42})^2/(df+2)]$

Equation 6 can now be written as follows:

Var (Test) =
$Var(\sigma^2_{c/F1}) + 9\ Var(\sigma^2_{SCA}) + 4\ Var(\sigma^2_{GCA}) - 12\ [2/(r^2a^2k^2(p-2)]*[(MS_{32})^2/(df+2) + MS_{42})^2/(df+2)]$

The estimates of additive and dominance variances are overestimated because they contain portions of epistatic variances (Equations 1a and 1b).

Table 1. Analysis of variance and expected mean squares for a 9-parents diallel design in which the 30 cassava genotypes representing each F_1 cross were clonally propagated.

Source of variation	Degrees freedom[¶]	MS	Expected mean squares
Environment (E)	a-1	MS_1	
Rep/E	a(r-1)	MS_2	
F1	[p(p-1)/2]-1	MS_3	$\sigma^2_e + k\sigma^2_\varepsilon + rk\sigma^2_{F1*E} + rka\sigma^2_{F1}$
GCA	p-1	MS_{31}	$\sigma^2_e + k\sigma^2_\varepsilon + rk\sigma^2_{SCA*E} + rk(p-2)\sigma^2_{GCA*E} + rka + \sigma^2_{SCA} + + rka(p-2)\sigma^2_{GCA}$
SCA	p(p-3)/2	MS_{32}	$\sigma^2_e + k\sigma^2_\varepsilon + rk\sigma^2_{SCA*E} + rka\sigma^2_{SCA}$
F1*E	(a-1)([p(p-1)/2]-1)	MS_4	$\sigma^2_e + k\sigma^2_\varepsilon + rk\sigma^2_{F1*E}$
GCA*E	(a-1)(p-1)	MS_{41}	$\sigma^2_e + k\sigma^2_\varepsilon + rk\sigma^2_{SCA*E} + rk(p-2)\sigma^2_{GCA*E}$
SCA*E	(a-1)(p(p-3)/2)	MS_{42}	$\sigma^2_e + k\sigma^2_\varepsilon + rk\sigma^2_{SCA*E}$
Error (a)	a([p(p-1)/2]-1)(r-1)	MS_5	$\sigma^2_e + k\sigma^2_\varepsilon$
Clones/F1	(p(p-1)/2)(k-1)	MS_6	$\sigma^2_e + r\sigma^2_{c/F1*E} + ra\sigma^2_{c/F1}$
Clones/F1*E	(p(p-1)/2)(k-1)(a-1)	MS_7	$\sigma^2_e + r\sigma^2_{c/F1*E}$
Error (b)	a(p(p-1)/2)(k-1)(r-1)	MS_8	σ^2_e

[¶] a= number of environments evaluated (2); r= number of replications within each environment (3); p= number of parents involved in the diallel crosses (9); k= number of cloned genotypes representing each F1 cross (30).

CONTENT

OUTPUT 2. Genes and genes combinations use to braden thec genetic base

Activity 2.1 Transfer of gene and gene combinations using cellular and molecular techniques

2.1.1 Coculture temperature and light positively affect *Agrobacteirum*-mediated transformation efficiency of cassava friable embryogenic callus

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Introduction

The most common method for the foreign DNA transfer to cassava is *Agrobacterium*-mediated transformation. This method however is not very efficient when it comes to producing independent transgenic lines. Besides, it depends very much on the performance of the genotype. The major bottleneck resides on the conversion of transgenic embryos to whole plants. A typical transformation experiment can produce thousands of transgenic, gus-expressing cell lines of friable embryogenic callus (FEC), but the conversion of embryos to plants usually halves the number of regenerated plants. There are two ways to approach the problem and find solutions: 1) improving the conversion of embryos to plants or 2) substantially increasing the number of transgenic cells lines before regenerating plants. This report summarizes the findings of experiments done following the second approach.

Materials and Methods

Two protocols of transformation were used. The first one, known as *treatment*, consisted on inoculating the cells without applying vacuum, cocultured them with *Agrobacterium* at 28°C, under light for 48 hours. In the *control* protocol the cells were subjected to 25-psi vacuum pressure for 1 minute to allow better contact with bacteria. The coculture temperature was 21°C, in the darkness for 48 hours.

The experiment required the use of friable embryogenic callus (FEC) of clones TMS 60444 (line 1 and line 2), SM1219-9 and MCOL 2215, with an average age of 3 years. Similarly, the experiment also required the use of *Agrobacterium tumefaciens*, strain AGL1 with plasmid pCAMBIA 1305.2, containing the GUS plus gene as reporter. After coculture, bacteria were eliminated from FEC by washing them off for one week in liquid medium. Then, plants were regenerated. An indicator of the success of transformation was the analysis of stable gus expression, which was performed on embryos and plants.

Results and Discusions

A preliminary assay was carried out to establish the viability of the *treatment*, and to know the response of each clone. All clones did generate independent cell lines with stable gus expression, indicating that the treatment had a positive influence in the transformation of cells (Table 1). The presence of the gene *hpt* (hygromycin phospho-transferase) in two lines (52 and 53) of clone 60444-Line1 was verified by means of Real Time PCR (see Beltran et. al. in this issue).

A second assay corroborated the positive effect of the *treatment* compared with *control*, with the former producing more independent transgenic lines since all clones tested did produce more transgenic lines, some of which were gus positive. Another advantage of the *treatment* was that the initial amount of tissue required to achieving a good transformation was lower. Table 2 shows that with 5 g of FEC is enough to get hundreds of cell lines and 12 transgenic plants with clone 60444. Since transformation is genotype dependant, Mcol2215 had a much lower response and plants, at the moment of writing this report, had not been regenerated. Meanwhile, clone SM1219-9 did produce two plants in the first round of experiments, being the first transgenics ever produced with this cultivar.

Table 1. Testing the effect of coculturing FEC with *Agrobacterium* at 21°C under light. The response, transformation efficiency, seems to be genotype- and line-dependant. These are results of a preliminary assay where controls were ommited.

Clone	Initial FEC (grams)	FEC independent lines isolated	GUS+ independent lines (Embryos)	GUS+ independent plants	Transformation Efficiency * (Percentage)
SM1219-9	20	391	6	2	10
TMS 60444 Line 1 (L1)	20	440	25	12	60
TMS 60444 Line (L2)	16	1360	13	3	18,75

*Transformation efficiency = Gus (+) Plant / initial CEF x 100.

Table 2. Comparative assay of two transformation protocols for cassava FEC using *Agrobacterium*: *control* at 28°C, in the dark, and *treatment* at 21°C under light. The efficiency

Clone	Inicial FEC (grams)	FEC independent lines isolated	No. of GUS+ independent lines (Embryos)	No. of GUS+ independent plants	Transformation Efficiency * (Percentage)
60444 *control*	5	49	12	7	140
60444 *treatment*	5	125	22	12	240
MCOL 2215 *control*	5	148	-	-	-
MCOL 2215 *treatment*	5	435	-	-	-

*Transformation efficiency = Gus (+) Plant / initial CEF x 100.

Summarizing, the results indicated an appreciable increase in the transformation efficiency when using the *treatment*, which produced 240 plants (gus positive) per 100 grams of transformed CEF, while in the *control* it was 140 plant for 100 grams of CEF, almost half that of the *treatment*.

Baron et al. (2001), found that the process of gene transfer from bacteria to plant cells is sensible to temperature, mainly because virulence of bacteria is diminished at temperatures around 28°C. This occurs mainly by the degradation of proteins that are part of the denominated secretion machinery type IV, which play an important role in the formation of pili and of the complex of transference of the T-DNA. The light by it self may not be a factor that affects bacteria, but it may play a role in plant cells, although more experiments are needed to determine if FEC is more efficiently transformed under light that in the dark. The effect of the vaccum during the infection would be purely mechanic facilitating the contact of the bacteria with the embryogenics units of the FEC. Another interesting aspect is that the clonal origin of the FEC affects efficiency. Clone 600444 stands out for being the one with highest transformation frequency, and ability to produce plants (vigor?) between the three evaluated clones.

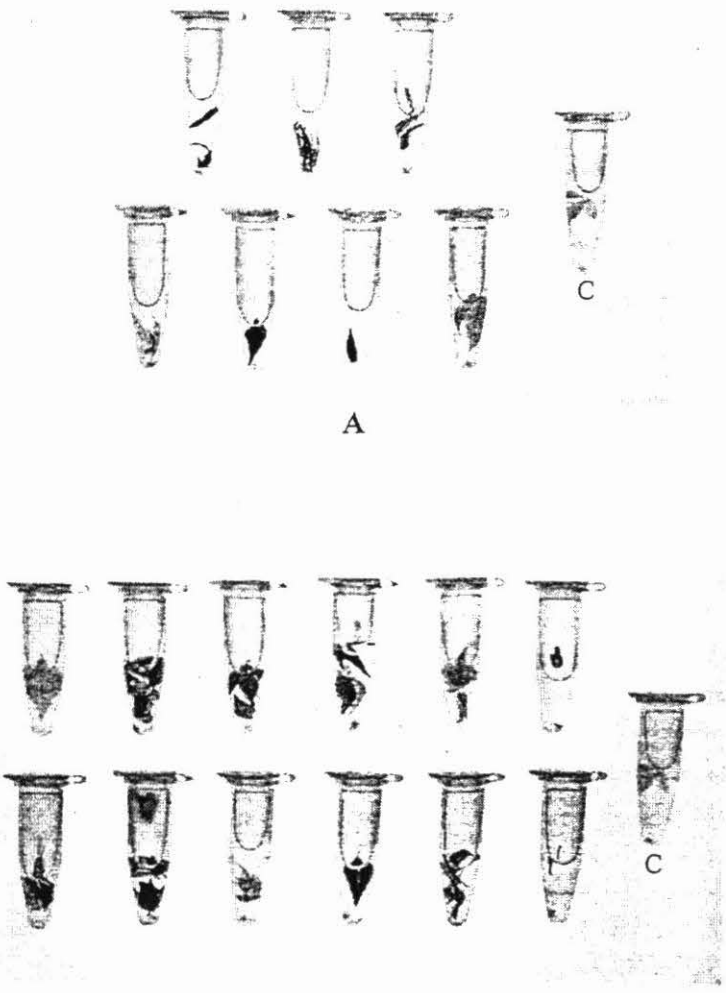

Figure 1. Stable expression of the GUS plus gene in young leaves from all plants regenerated from independent transformation events mentioned in Table

2. A. Standard protocol (*control*). B. New protocol (*treatment*). (C = Not transformed control plant).

Future Activities

- *Agrobacterium*-mediated transformation, in addition to being affected by light, temperature, and vacuum, may also be affected by the clonal origin of plant tissues. Therefore, to increase the efficiency for particular clones it will be necessary to test and adjust new conditions for those clones.

- Conversion of embryos to plants is also critical to increase transformation efficiency. More experiments have to be designed to test the ability of different cassava clones, under different treatments, to produce plants from somatic embryos.

Acknowledgments

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References

Baron, C., N.Domke., M. Beinhofer.& S. Hapfelmejer. 2001. Elevated temperature differentially afects virulence, vir B protein accumulation, and T-pilus formation in different Agrobacterium tumefaciens and Agrobacterium vitis strains. Journal of Bacteriology 23:6852-6861.

2.1.2 Molecular Characterization of A Putative Waxy Cassava Starch GMO Obtained by Anti-sense Mediated Silencing of the Granule Bound Starch Synthase I (GBSSI) gene

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1.CIAT; 2.IAEA, Austria

Funding: Ministerio de Agricultura y Desarrollo Rural (MADR), Colombia, CIAT.

Important Outputs

1) PCR and Southern hybridization analysis of transgenic cassava plants expressing a full-length GBSSI gene in the sense orientation revealed stable expression of the gene.
2) Application for permission to transfer biochemical evaluation of the waxy trait.
3) Preparation of 6 additional transgenic events expressing the full-length GBSSI gene in the anti-sense sense orientation for transfer to the screen house

Introduction

With funds from the Colombian Ministry of Agriculture and Rural Development, a project was initiated in 2002 to genetically engineer industrial cassava varieties to produce waxy starch via the sense- and anti-sense down-regulation of the GBSSI gene, the predominant starch synthase gene that catalyses the conversion of ADP-glucose to amylose. Preliminary transformation work was carried out with the model transformation cultivar, MNig11 (60444). One independent transgenic event was produced last year for the sense construct and plantlets were transferred to the screen house (CIAT 2003). Six independent events were produced for the anti-sense constructs and about 5 plants per event have been transferred to the screen house. We describe here molecular characterization via PCR and Southern hybridization of the sense transgenic line, Permission to transfer these plants to the field has also been requested from the CIAT biosafety committee for biochemical evaluation of the waxy phenotype.

Methodology

The isolation of a full length GBSSI cDNA clone, construction of sense- and anti-sense transformation cassettes and their insertion into the genome of the variety TMS60444 via *Agrobacterium tumefaciens* have been described earlier (CIAT 2003). Molecular characterization of the sense transgenic event was using PCR primers designed from the 3'and 5'ends of the GBSSI gene and primers for the *GUS* reporter gene, the PCR primers and conditions for PCR have also been described earlier (CIAT 2003). Southern analysis was using ten micrograms of total genomic DNA from 4 transgenic plants of the single event and 2 non-transgenic cassava genotypes using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI* . The RFLP probe was a full length GBSSI cDNA clone (clone-3) from a root cDNA library from TMS30572. Preparation and Southern hybridization of the filters were as described by Fregene et al. (1997).

Friable embryogenic callus (FECs) of the cassava genotypes 60444 and MCol2215 were transformed via *Agrobacterium tumefaciens* with the GBSSI gene in anti-sense orientation as described earlier (CIAT 2003). Plants could be regenerated for a single transgenic event and 5 events for the genotypes 60444 and MCol22 respectively.

Results

PCR amplification with the GBSSI and *GUS* gene primers yielded positive amplification in the transgenic plants of the expected size of a 2.1 kb and 700 bp fragment respectively (Fig 1). Southern hybridization of the GBSSI to genomic DNA digested with *PstI* also gave the expected size of 2.1kb in the transgenic plants but not in the control non-transgenic plants suggesting stable integration in the genome (Fig 2). Large molecular weight bands in the non-transgenic plants are indigenous GBSSI genes found in cassava containing introns.

Following the confirmation of successful and stable incorporation of the sense GBSSI construct into the genome of the cassava genotype 60444 as revealed by molecular characterization of transgenic plants, the stage is set for biochemical evaluation of roots in field experiments. Application for field trials has been prepared for submission to the Colombian plant quarantine authorities through the CIAT Biosafety committee. Transgenic plants are at the moment maintained in the CIAT biosafety green house. At the same time, regenerated plants representing 6 transgenic events of the anti-sense constructs from the genotypes 60444 and MCol22 have been transferred to the screen house in preparation for molecular characterization.

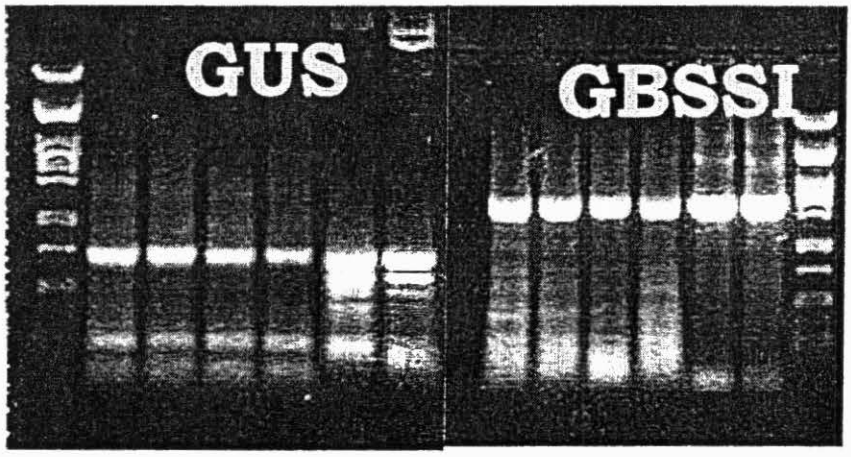

Figure 1. (A) PCR amplification of the *gus* using primers specific for the gene. The first and last lanes is molecular weight marker Lambda DNA, digested with *PstI*, the next four lanes from the left (section GUS) are PCR amplification of the *GUS* gene in regenerated transgenic plants transformed with the GBSSI sense construct, the next two lanes are control PCR amplification of the construct in the plasmid pCAMBIA 1305.

(B) PCR amplification using primers specific for the GBSSI gene. The first four lanes in the section on the right (labeled GBSSI) are PCR amplification of the gene in regenerated transgenic plants transformed with the GBSSI sense and the last two lanes are control PCR staring of a cDNA GBSS clone (clone 3).

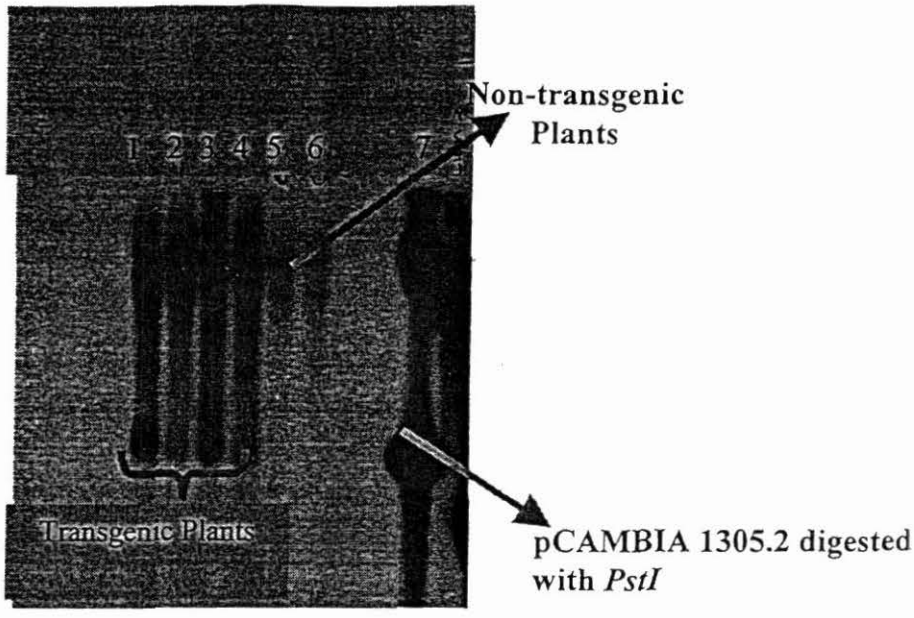

Figure 2. Southern hybridization of a GBSS full-length clone hybridized to genomic DNA digested with *Pst I* of transgenic, non-trangenic and the transformation construct. Lane 1-4 are transgenic plants transformed with a sense GBSSI construct, lanes 5-6 are non-transgenic control DNA, lane 7 is the construct in pCAMBIA1305.2. Large molecular weight bands in the non-transgenic plants are indigenous GBSSI genes in cassava containing introns.

Conclusion and Perspective

We have successfully transformed a full-length sense construct of the GBSSI gene into the model cassava transformation variety 60444. Molecular characterization by PCR and Southern hybridization revealed stable incorporation of the gene, biochemical tests to confirm the waxy phenotype will be conducted on field grown plants once a permit has been obtained to transfer these plants to the field. Additional transgenic events using an anti-sense construct have also been obtained and transferred to the biosafety green house in preparation for molecular characterization.

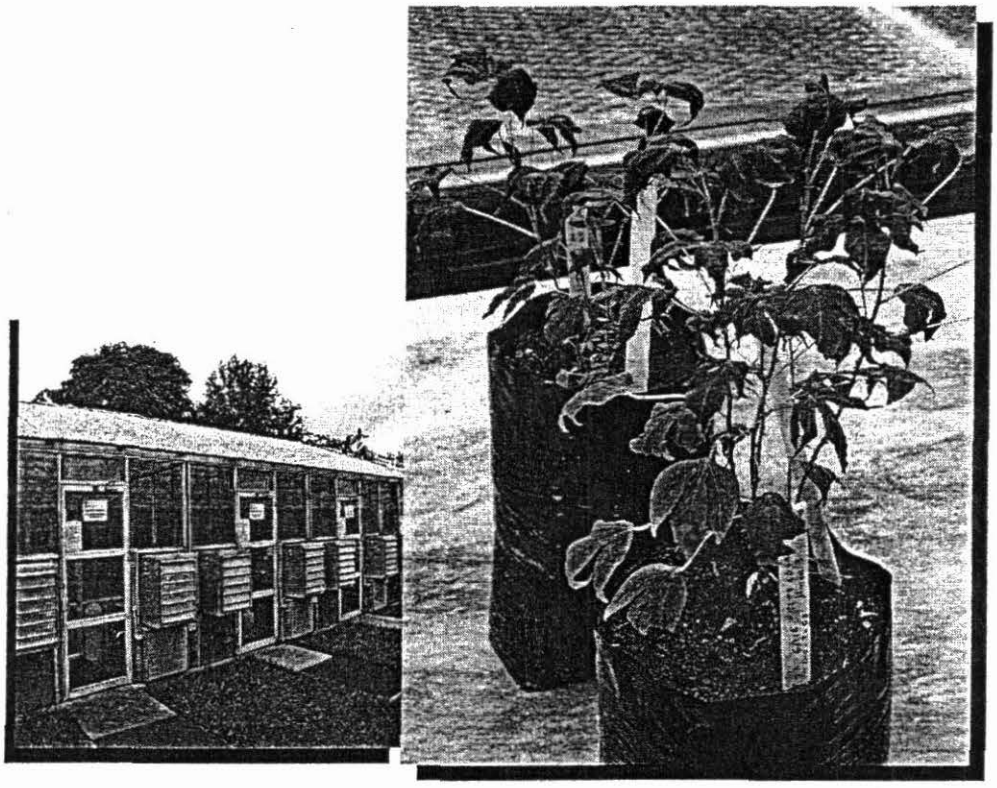

Figure 3. Transgenic plant in biosafety green house

References

CIAT(Centro Internacional de Agricultura Tropical). 2003. Annual Report Project SB2, Assessing and Utilizing Agrobiodiversity through Biotecnology. Cali, CO. p.202-205

FREGENE, M.; Angel F.; Gomez, R.; Rodriguez, F.; Chavarriaga, P,; Roca, W.; Tohme, J., Bonierbale, M. 1997. A molecular genetics map of cassava (Manihot esculenta Crantz) Theor Appl Genet 95:431-441.

2.1.3 Modification of Flowering in Cassava

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Funding: Rockefeller Foundation

Important outputs

1) Acquisition of the APETALA and ethanol inducible promoter ***AlcR*** genes identified in other species for modification to control flowering for use in Cassava
2) Preparation of friable embryogenic callus (FECs) from the model cassava transformation variety 60444 (MNig 11)

Introduction

Flowering and its control is one of the most important challenges of cassava breeding. Shy and non-synchronized flowering can result in an inability to use a very valuable genotype in breeding. On the other hand, profuse early flowering is normally an undesired characteristic as it is associated with heavy branching and tends to lead to low harvest index and yield (Cock and El Sharkaway 1988). Conversely high yielding genotypes either do not branch or branch late and the first branches formed often do not produce fertile flowers. This leads to a dilemma for breeders who must produce shy flowering types for high yield, but require profuse early flowering types for making genetic crosses. In cassava an approach that will increase flower number and specifically target the timing of flowering would be a great asset to the field breeder.

There have been major advances in the understanding and control of flowering. For example the discovery of the LEAFY gene and its action has allowed researchers in the United States of America to induce flowering in three month old aspen trees, which normally flower earliest when they are eight years old (Salk Plant Biology). It is probable that homologous genes control flowering in cassava and much of the knowledge gained in other temperate species could be used to rapidly control flowering in cassava. Furthemore, the amount and timing of flowering can be manipulated through controlled expression of flowering-time genes under the control of an ethanol-inducible promoter (AlcR), ethanol is a common and readily available/storable compound that could be used to regulate the expression of a transgene in the field. This AlcR gene-expression system functions in many plants and works in controlled breeding environments (Sweetman et al. 2002), suggesting it will be useful in cassava.

With funding from the Rockefeller foundation and in collaboration with Dr Seth Davies group at the Max Planck Institute for Plant Breeding Research, Cologne, Germany, a project has been initiated this year to modify the control of flowering in cassava via genetic transformation using the flowering gene APETALA and LEAFY driven by an ethanol inducible promoter (AlcR). Immediate benefits of the project include making accessible to conventional breeding the many excellent cassava genotypes that are recalcitrant to flowering, and easing the difficulties of synchronizing flowering between cassava genotypes which currently flower at different times in the breeding cycle.

Methodology

The first step in the project is the acquisition of the flowering genes and ethanol inducible promoters (*AlcR*) via negotiated agreements with the intellectual owners of the LEAFY, APETALA genes and the AlcR promoter for their use in cassava. The ethanol-inducible promoter AlcR, is owned by Syngenta, the APETALA gene (*AP1*) is owned by the University of California San Diego, and the LEAFY (gene) *LFY* is owned by the Salk Institute. The LFY gene is still being negotiated with the Salk Institute. The ethanol-inducible promoter will be separately fused to the two flowering-time genes and then cloned into a non-antibiotic selection marker; in this project we will be using a binary vector containing LUC PLUS and the NPT II gene. These constructs will be fully selectable and/or screenable in Arabidopsis, cassava and GUS PLUS will be used for cassava. These constructs will be generated at the Max Planck Institute in Cologne and tested in Arabidopsis genotypes that mimic "shy" flowering. *LFY* and *AP1* are genetically both necessary and sufficient for conversion of vegetative meristems to reproductive meristems (Mouradov *et al.* 2002). The choice of these two genes lies in the one that allows the bypass of the genetic blocks of multiple pathways.

The above constructs will be then be transferred to CIAT and inserted into the cassava varieties CM3306-4 and 60444 that flower moderately. Constructs with the flowering gene will also be made with other inducible promoter systems, for example promoter systems based on sterol or tet, provided agreements for their use can be negotiated with the owners. The transformation protocol to be followed is that described by Schopke et al. (1996) with some modifications made at CIAT and has been used with success to produce several independent transgenic events (CIAT 2002). At least seven independent transformation events will be generated for each of the flowering gene constructs. The genetic pathway blocks of flowering are unknown in cassava therefore the choice of 2 genes that lie far downstream of floral initiation will facilitate the production of flowering in widely differing genotypes of cassava, irrespective of the molecular mechanisms that inhibit flowering in this plant. The effects of the flowering genes will be assessed by spraying ethanol on the transgenic plants at 4 months after planting to induce flowering.

Results

Syngenta and the University of San Diego California have made available the *AlcR* and *AP1* genes to CIAT under a limited 'for research only' MTA, another MTA will be negotiated for permission to use these genes in new cassava varieties. Over 300 apical meristem and nodal cuttings of the model cassava transformation plants 60444 and another variety CM3306-4 have been cultured in preparation for induction of Friable Embryogenic Callus (FECs).

Conclusions and perspectives

A project to modulate the control of flowering in cassava using the flowering genes LEAFY and APETALA under the ethanol inducible promoter *Alcr* have been initiated at CIAT in collaboration with Max Planck Institute for Plant Breeding Research, Cologne, Germany, and funding from the Rockefeller foundation. Constructs for the 2 flowering genes are being carried out at the moment and will be ready by the end of the year for introduction into cassava by agrobacterium transformation.

References

CIAT, (2002). Annual Report Project SB2, Assessing and Utilizing Agrobiodiversity through Biotechnology, CIAT, Cali, Colombia, pp 203-207.

CIAT (2002). Annual Report Project SB2, Assessing and Utilizing Agrobiodiversity through Biotechnology, CIAT, Cali, Colombia, pp 212-214.

Schöpke, C., N. Taylor, R. Carcamo, N.K. Konan, P. Marmey, G.G. Henshaw, R.N. Beachy and C. Fauquet. 1996. Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) from microbombarded embryogenic suspension cultures. Nature Biotechnol. 14: 731-735.

Cock, J. H., and M.A. El-Sharkawy. 1988. Physiological characteristics for cassava selection. Expl. Agric. 24: 443 - 448.

Sweetman J.P., Chu C., Qu N., Greenland A.J., Sonnewald U., Jepson I. (2002) Ethanol vapor is an efficient inducer of the alc gene expression system in model and crop plant species. Plant Physiol. 129: 943-948.

2.1.4 Assessment of transgenic status in cassava using Real Time PCR

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Introduction

Real Time PCR appeared recently as a more accurate option for molecular analysis of transgenic plants (Ingham et al 2001, Mason et al 2002). The technique, which uses a fluorescent dye, makes possible the quantification of nucleic acids during the PCR reaction, in real time, without the need of post-PCR analyses. The specificity of the DNA product being amplified in the PCR reaction is determined by product's melting curves and emission of fluorescence, which are determined by the base sequence or composition of the melting product. Thus, it permits the quantification and statistical analyses of transgen copy number, as well as transgene expression with more precision than conventional PCR, Southern and Northern blots. We describe the implementation of this technique for the analyses of putative transgenic cassava plants.

Materials and Methods

Transgenic cassava plants of cultivars 60444, SM1219-9, CM3306-4, Tapicina and Bujá Preta were analyzed with Real Time PCR, Opticon 2 from MJ Research. These plants contained at least one of the following genes: *uidA, npt* II *Y hpt*. DNA obtained from plants and plasmids, or eluted from agarosa gels, was quantified with Hoescht® using a fluorometer (Hoefer, DyNA quant 200 flurometer).

Standard amplification curves were obtained for genes *uidA, npt*II and *hpt*, using a DyNamo® kit, with SYBR® green as fluorochrome. PCR Amplifications were analyzed through melting curves that allowed the differentiation of transgenic plants from non transgenic ones. Positive controls were *npt*II and *uid*A amplicons, eluted from agarose gels, and the plasmid pCAMBIA1305.2 for the *hpt* gene. Negative controls were non-transgenic plants, and samples without DNA.

Three individuals from clone 604444, lines 92, 80 and 270400, were evaluated with the method for absolute copy number quantification to determine the number of transgenes inserted. The *npt* II and *uid*A amplicons, eluted from agarose gels, were used as external standards. Each sample was amplified three times to obtain an average and standard deviation. These values were compared with previous results from Southern blot analyses.

Results and Discussion

The distinction between transgenic and non-transgenic individuals is summarized in Table 1. Transgenic lines showed overlapping melting curves with the positive controls. Negative

controls, as well as non-transgenic samples showed non-overlapping melting curves (Figure 1).

Cassava clone	# Of lines evaluated	Plasmid	Genes evaluated with Real Time PCR			Gus expression	Transgénica
			UidA	npt II	hpt II		
CM 3306-4	8 lines	pBigCry	(-)	(-)	*	No	No
CM 3306-4	1 line	pSGMan	(-)	(-)	*	No	No
SM1219-9	2 lines	pBigCry	(-)	(-)	*	No	No
Buja Preta BMG1467	1 line	pCAMBIA 1305.2	*	*	(-)	No	No
Tapicina 1063	1 line	pCAMBIA 1305.2	(-)	*	*	No	No
TMS60444	L52	pCAMBIA 1305.2	*	*	(+)	Yes	Yes
	L54	pCAMBIA 1305.2	*	*	(+)	Yes	Yes
TMS60444	L92	pBigCry	(+)	(+)	*	Yes	Yes
	L80	pBigCry	(-)	(+)	*	No	Yes
	L270400	pBigCry	(-)	(+)	*	No	Yes

Table 1. Identification of cassava transgenic lines using Real Time PCR. The (+) indicates an amplification for the corresponding gen, while (-) is a negative amplification that did not overlap the melting curve (peaks in graphs below) with positive controls. The (*) means no evaluation of the gene in this individual. *uidA*: gus gene; *npt II*: neomycin phosphotransferase gene for resistance to aminoglicoside antibiotics; *hpt II*: hygromycin phosphotransferase gene for resistance to antibiotics.

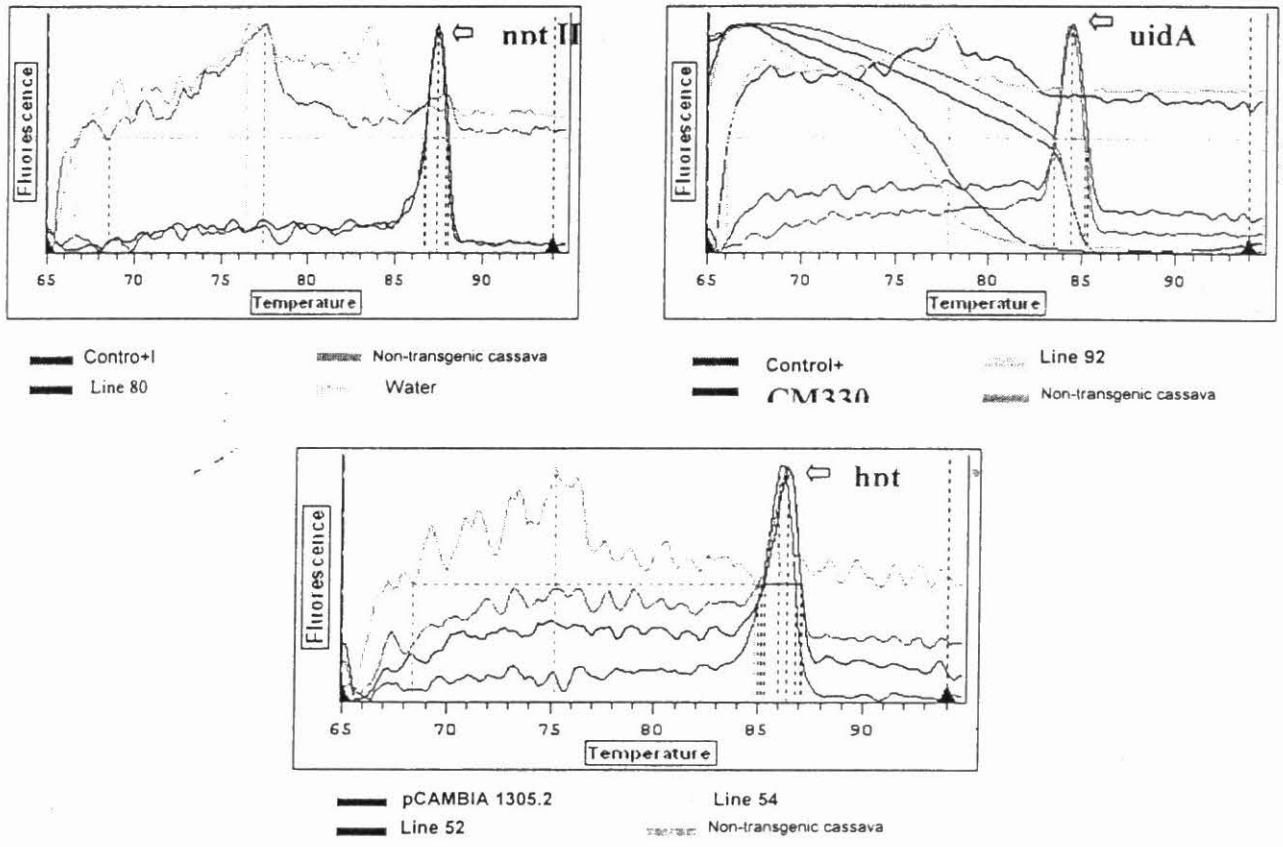


Figure 1. Identification of transgenic and non-transgenic lines through melting curves. In each curve, the peaks that overlap, indicated by arrows, confirm the presence of either of the transgenes nptII, uidA or hpt.

Lines 80 and 270400 showed specific amplifications for gene npt II, but not with uidA. Similar results were observed with line 80 when it was evaluated with Southern blot (not shown). This probably means that at least line 80 suffered a rearrangement that caused the partial or total lost of the uidA gene (Ladino et al 2002). It's worth noting that this line does not express the uidA gene, as neither does line 270400.

Absolute quantification of copy number

Standard curves for the uidA and nptII genes are shown in 2a and 2b. They were used to statistically determine the most likely number of copies, of each transgene analyzed, inserted in each transgenic line. The results are presented in the Table 2, which compares with previous results obtained with Southern blot.

The results show differences between Real Time PCR and Southern blot estimates (Figure3; Ladino et al 2002). Similar discrepancies have been reported by Bubner et al (2004), although in their study they used the method of relative quantification of copy number. Absolute quantification of transgene copy number, as reported by Roche Technical Note N. LC 11/2000, is probably less precise, which stresses the need of implementing the relative method.

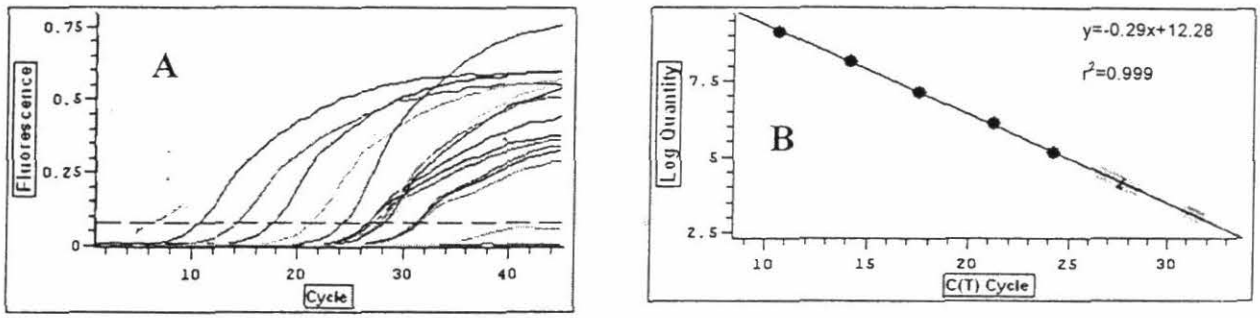


Figure 2. A. Real Time PCR for absolute quantification of copy number of nptII. The curves represent amplicons of a DNA standard from nptII (between 1 and 10^{-6} ng). B. Standard lineal regression curve generated from curves in 2A to determine copy number

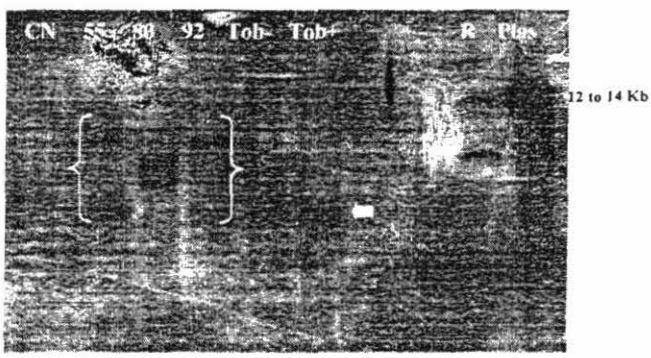


Figure 3. Southern blots of genomic DNA from lines 55, 80 and 92, restricted with BamHI -or EcoRI-, to determine the number of insertions (brackets and arrowhead). Southern probed with a 0,7 kb PCR-derived nptII probe (CN= negative control; Tob- = tobacco genomic DNA; Tob+ = transgenic tobacco transformed with pBIGCry; R = Rice genomic DNA; Plas = plasmid PBIGCry cut with same enzymes –12 to 14 Kb)

Trangenic lines	Transgen	Medium value of transgen copy	Standard Desviation (SD) of copy	Copies in Southern blot
L.92	*uidA*	2.6	0.35	*
L.92	*nptII*	0.006	0.325x10-5	1
L.270400	*nptII*	0.006	0.1x10-2	*
L.80	*nptII*	0.14	0.3x10-3	2

Table 2. Absolute quantification of transgene copy number and comparison with Southern blot data for lines 92 and 80, using the nptII gene as target. (*) Not determined.

When comparing the data obtained with Real time PCR and Souhtern, although the numbers in the former are not integers, they show that line 92 has less copies than line 80, when tested for the gene nptII. It does agree with the Southern where line 92 shows one copy and line 80 shows at least two. It is necessary to polish the technology to detect more precisely the number of transgenes inserted. It probably requires using the relative method and more plants for which Souhern blots have been clearly established.

Conclusions and Perspectives

Real Time PCR may be a tool useful for the rapid detection of transgenic plants, in the sense that it allows to determine if a plant contains or not a gene. However, more work needs to be done to use Real Time PCR for quantifying the number of copies inserted in a genome and to do expression analysis of genes inserted.

References

Bubner, B., Gase, K & Baldwin T. 2004. Two fold differences are the detection limit for determinig transgene copy number by real-time PCR. BMC Biotecnhology 4:14

Ingham, D.J., Beer, S., Money, S & Genevieve H. 2001. Quantitative Real-Time PCR assay for determining transgene copy number in transformed plants. Biotechniques 31: 132-140.

Ladino JL. et al (2002) Genetic transformation of cassava: Confirmation of transgenesis in clone 60444 and analysis of CRY1Ab protein in transgenic lines. Preliminary data on transformation of farmer-preferred cultivars SM1219-9 and CM3306-4. SB-2 Annual Report, October 2002, CIAT, pp 203-207.

Mason, G., Prover, P., Vaira, A. M &Acotto G. 2002. Estimating the number of integrations in transformed plants by quantitative real-time PCR. BMC Biotechnology 2: 20 .

Roche Molecular Biochemicals. Technical Note No. LC 11/2000.
Ladino et al 2002 Annual Report

2.1.5 Evaluation and Selection in the Field in 2004 of Advanced Breeding Generations from crosses with Transgenic Rice Resistant to RHBV. Part I.

L. Fory[1], T. Agrono, E. Bolaños[2], E. Tabares[1] C. Martínez [1,2], J. Borreo[2], and Z. Lentini[1,2].
[1]SB2, [2]IP4

Introduction

The Biotechnology Research Unit at the International Center for Tropical Agriculture (CIAT) had generated transgenic rice lines resistant to the Rice Hoja Blanca Virus (RHBV) disease (Lentini et al., 2003). The main goal of this project is to confer protection to RHBV to plants younger than 25 days-old, age at which the standard resistance breeding source does not protect the plants. The variety Cica 8 was originally selected as the ideal parental line since it has broad adaptation to less favorable conditions has good yield potential and quality traits making it an interesting parent to deploy the transgenic resistance into other varieties. Transgenic plants outperforming commercial varieties for the RHBV resistance and with good agronomic traits were identified. Advanced generations (F_7) of the original transgenic events had been selected. Best transgenic events were backcrossed to Cica 8 or crossed to Iniap 12, Fedearroz 50 or Oryzica 1, to further improve agronomic characteristics. The selection process has been conducted in the field including stepwise evaluations for RHBV resistance in the greenhouse or field. Last year we reported the selection of the 11 of the 82 derived crosses, and 202 transgenic plants derived from 6 of the original transgenic events. These plants were selected based on their resistance to RHBV, high fertility, vigor, and yield potential. Many of the crossed lines are still segregating since they are in F_3 or F_4 generations. This year we report selection of advanced lines with stable RHBV resistance and good yield performance in the field.

Materials and Methods

Evaluation of RHBV under greenhouse conditions in 2003. The evaluations were conducted using progeny plants from 38 crosses between resistant transgenic Cica 8 plants and the commercial varieties Fedearroz 50, Oryzica 1, and Iniap 12. Plants were selected by the agronomic performance in the field in 2002. Fifteen progeny plants per each selected line were inoculated with four proven viruliferous insects per plant at 15 days old in the greenhouse. Disease evaluations were conducted at 15, 30, 45 and 60 days after inoculation using a 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. Agronomic traits were evaluated according the IRRI scale (1996).

Molecular analysis of the transgenic rice plants. DNA was extracted from 1.0 g fresh leaves (McCouch et al., 1998). Southern and PCR analyses were conducted to detect the presence of the N-protein and hygromicin gene. Fifteen µg of DNA was digested with Bam HI and KPN I. The gels were denatured and neutralized by standard procedures. The filters

were hybridized at 60°C. The presence of 35S promoter sequence was display mediated PCR. The electrophoresis separation was carried out in 1% agarose (Sambrook et al., 1987).

Results and Discussion

Eighty individual plants resistant to RHBV from 11 crosses of the total 38 evaluated were selected based on their agronomic performance in the field (Table 1). The crosses with Iniap-12 were eliminated due to high susceptibility to RHBV. Crosses with Fedearroz 50 and Oryzica 1 were selected. The transgenic crosses showed an increased resistance to RHBV (score ≤ 4) than the corresponding non-transgenic cross Cica 8/ Fedearroz 50 (score >7) or Cica 8/ Oryzica 1 (score >5). Transgenic crosses with the Fedearroz 50 showed the characteristic dark green leaves as Fedearroz 50 and erect plants, while the crosses with Oryzica 1 showed broad leaves. In general, the 11 crosses selected were taller, with more tillers, and higher yield potential respect to Cica 8 (Table 1).

Plants derived from the transgenic event A3-49-60-12-3 were highly resistant to RHBV (score <3) compared to control non transgenic Cica 8 plants (score 7-9), but some sibling plants showed susceptibility to the virus (Figure 1A). Resistant plants derived from the A3-49-60-12-3 line, show a hypersensitivity disease resistance reaction upon challenge with the virus (Lentini et al., 2003). Resistant plants contain multiple rearranged copies of the RHBV N- protein gene, as well as the hpt gene encoding for hygromycin resistance and the CaMV 35S promoter sequence (Figure 1B to 1E). In contrast, susceptible transgenic plants appeared to have lost the transgenic genes and sequences in advanced generations through the self-cross process (Figure 1B to 1E). Small size RNA molecules have been found in susceptible and resistant transgenic plants (Garavito, 2003). The presence of these molecules has been associated with the process of post-transcriptional gene silencing (Hamilton and Baulcome, 1999; Hamilton et al., 2002; Sijen and Kooter, 2000). This mechanism depends more on the characteristics of the transcript than on the gene dosage (Voinnet et al., 1998). It is possible that the transgene is transcribed using other plant promoters and that in both lines the process of post-transcriptional silencing is active, but the RNA small sequence may be actively degraded only in the resistant transgenic line as suggested by Garavito (2003).

Future Activity

Subsequent generations obtained from self-cross of the selected advanced breeding and transgenic lines were evaluated in the field for agronomic performance in 2004 (Quintero et al., 2004; herein SB2 Annual Report). Seeds of selected plants will be multiplied to conduct replicated yield trials and evaluations for other disease resistance and grain quality traits in 2005.

References

Garavito, A.2003. Asociación del silenciamiento post-transcripcional de genes y la reacción de hipersensibilidad, con la resistencia transgénica al virus de la hoja blanca del arroz (*Oryza sativa* L.). Tesis de maestría. Universidad de los Andes. Bogotá.

Hamilton. J. and Baulcombed. C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. En: Science 286:950-2

Hamilton, A., Voinnet, O., C happell L., Baulcombed. D. 2002. Two classes of short interfering RNA in RNA silencing. Embo. J 21:467-14679.

McCouch S.R.; Chen, X.; Panaud, O.;Temnykh, S.; Xu, Y.; Cho, Y.G.; Huang, N.; Ishii, T. Blair, M.. 1998. Microsatellite marker development, mapping and applications in rice genetics and breeding. Plant Molecular Biology 35: 89-99.

Lentini Z., Lozano I, Tabares E., Fory L., Domínguez J., Cuervo M., Calvert L. 2003. 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.

Sijent T. and Kooter J. M. 2000 Post-transcriptional gene silencing: RNA's on the attack or on the defense?. Bioassays 22: 520-531

Voinnet, O., Vain, P., Angell, S., Baulcombe, D. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell. 16: 177-187.

Table 1. Agronomic performance in the field in 2003 of RHBV transgenic resistant F4 lines derived from backcross with Cica 8, Oryzica 1, Fedearroz 50, or Iniap 12.

Transgenic line	P[1]	DF[2]	RHBV		PLANT HEIGHT	TILLERS	F[5]	WEIGHT (GR) PER PLANT
			2000[3]	2003[4]				
A-3-49-60-4-13/Oryzica 1-15A-M-M	10	110	4.3	3.5	77	7	91	12.4
A-3-49-101-18-19/Oryzica 1-14-M-M	12	105	5.0	3.6	76	7	82	12.9
A-3-49-60-4-5/Oryzica 1-15-M-M	8	109	6.3	3.9	90	9	82	18.8
A-3-49-60-4-5/Fedearroz 50-12 A-M-M	9	115	NE	4.1	76	8	84	17.1
A-3-49-60-4-13/ Fedearroz 50-18-M-M	10	115	5.0	4.2	80	7	70	17.4
A-3-49-60-4-13/Oryzica 1-13III-M-M	7	118	5.0	4.5	89	8	89	20.8
A-3-49-60-4-5/ Fedearroz 50-68-M-M	11	111	5.0	4.5	79	5	86	8.7
A-3-49-60-4-5/Oryzica 1-232-M-M	7	118	4.3	4.6	75	7	89	15.6
A-3-49-60-4-5/ Fedearroz 50-16 A-M-M	7	112	NE	4.6	82	11	69	17.7
A-3-49-60-4-5/ Fedearroz 50-66-M-M	3	110	5.0	4.7	80	10	60	14.5
A-3-49-60-4-5/ Fedearroz 50-19-M-M	6	111	5.0	4.8	83	8	66	15.2
Cica 8	NA	112	8.0	8.3	54	6	93	10.7
Cica 8/Iniap-12	NA	111	7.0	8.6	64	5	72	7.2
Cica 8/Fedearroz 50	NA	114	8.0	7.6	74	5	76	7.8
Cica 8/Oryzica 1	NA	113	7.0	6.1	69	7	79	13.3
Fedearroz 2000	NA	110	2.7	2.3	74	7	96	15.5

[1] Number of individual plants selected; [2] Days to 50% plants flowering; [3] and [4]RHBV evaluations in 2000 in the field and in the greenhouse in 2003; [5]F, Mean percentage of fertility per line.

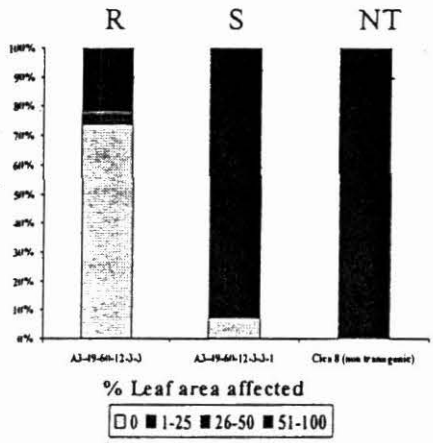


Figure 1 A

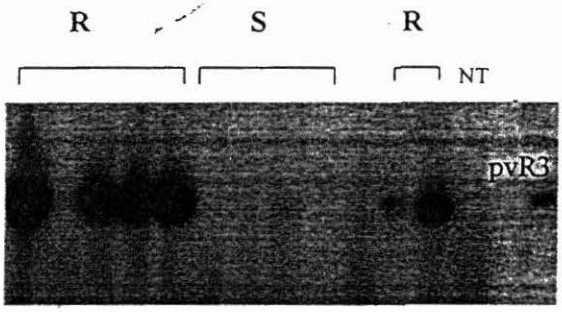


Figure 1 B

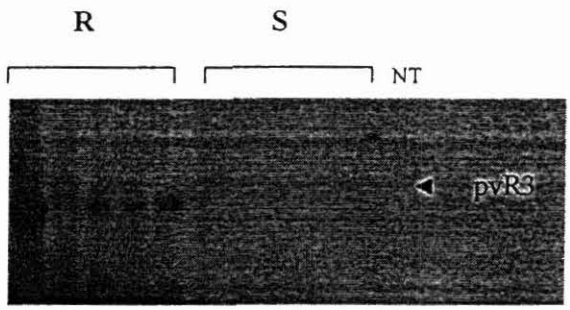


Figure 1 C

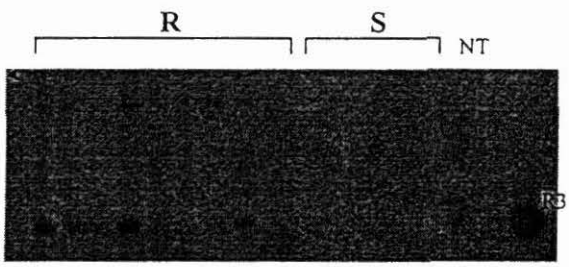


Figure 1 D

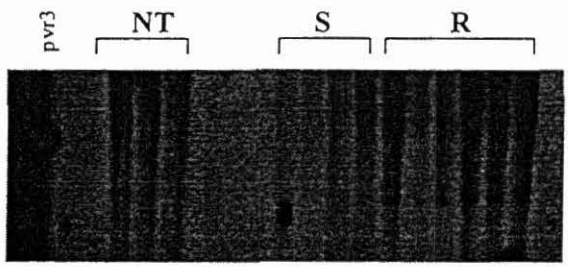


Figure 1 E

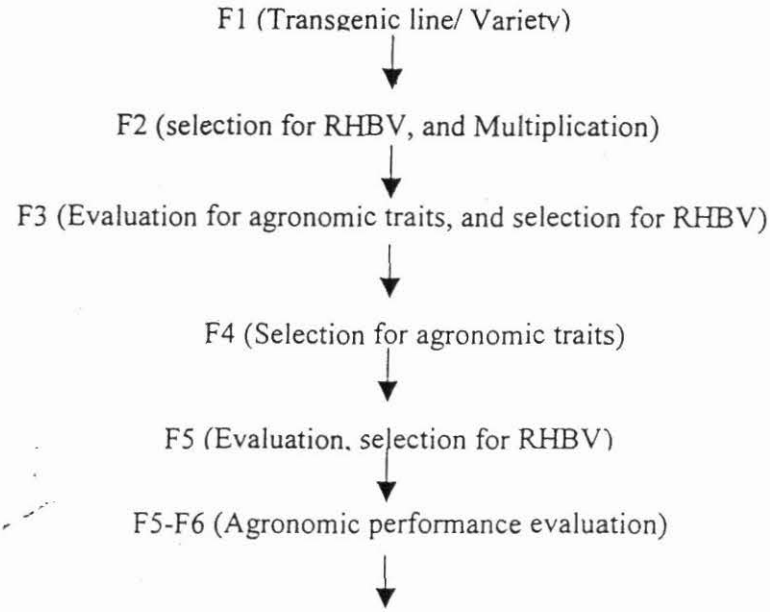
Figure 1. A) Disease resistance on T4 transgenic plants inoculated at 14 day-old, B) Southern blot of PCR amplified sequence of CaMV 35S Promoter. C) Southern blot of PCR amplified sequence of RHBV- N protein gene. D) Southern blot of genomic DNA for hygromycin resistance gene. E) Southern blot of genomic DNA for RHBV N- protein gene. R resistant line, S susceptible line, and NT non-transgenic control Cica 8.

2.1.6 Evaluation and Selection in the Field in 2004 of Advanced Breeding Generations from crosses with Transgenic Rice Resistant to RHBV. Part II.

M. Quintero[2], J. Borrero[2], T. Agrono, E. Bolaños[2], E. Tabares[1] C. Martínez [1,2], and Z. Lentini[1,2]
[1]SB2, [2]IP4

Introduction

The main goal of this project is to provide new source(s) of resistance to complement the single breeding resistance source present in most of the commercial varieties grown in Latin America nowadays. Highly RHBV resistant transgenic rice encoded by the RHBV-nucleoprotein gene was generated by particle bombardment (Lentini et al., 2003). The resistance in these plants appears to be mediated by post-transcriptional gene silencing inducing a hypersensitive reaction upon challenge with the virus. Transgenic lines outperform the level of resistance of commercial varieties released so far even when inoculated at 10-day-old seedling stage. Previous analyses indicated that the transgenic resistance is inherited stably through several generations of self-cross and complements the single resistance source used so far in breeding. Since the approval of the first transgenic field tests, our group has been sequentially scaling up the evaluation and selection of lines to be incorporated into breeding. Crosses were generated with selected popular commercial varieties (Fedearroz 50, Oryzica 1 and Iniap 12) to transfer the transgenic resistance into improved genetic backgrounds. Lines from the original transgenic events and those derived from the different crosses had been submitted to a process of multiplication and selection for RHBV resistance and agronomic traits following the pedigree method, which involves the advancement of six generations of evaluations alternative in the greenhouse and the field as shown below.



Evaluation and selection of advanced generations of transgenic events and derived progeny plants from crosses. Field evaluations were conducted using 300 progeny plants (from T_6 up) of 8 transgenic events RHBV resistant, and 141 F_5 progeny plants derived from crosses between selected transgenic events and Fedearroz 50, Oryzica 1 or Iniap 12. Plants were transplanted in the field using an experimental complete randomized block design with two replications, of 63 plants per each replicate. Agronomic traits were evaluated through the cycle up to maturity. Days to flowering, plant vigor, plant height, number of tiller and fertility were recorded. Individual plants were selected and harvested.

Results and discussion

The experimental plot was of about 1 ha, the largest transgenic rice plot planted so far at CIAT experimental station for evaluation of agromonic traits (Figure 1). Twenty one individual plants derived either from crosses with Fedearroz 50 (48%) or Oryzica 1 (52%) were selected. The crosses from Oryzica 1 showed a higher vigor than those from Fedearroz 50 (Table 1). No significant differences were noted between the selected plants and the commercial varieties respect to flowering cycle, vigor, plant height, tillering capacity and fertility. Fertility ranged from 79% to 89% likewise the commercial varieties (Table 1).

Plants derived from crosses with Fedearroz 50 were characterized by a good tillering capacity, fewer slender leaves and with delayed senescence, which is associated with increased grain yield. Panicles showed good exertion. Plants started flowering between 101 and 118 days after planting and reaching 50% anthesis at 125-150 days (Table 1). Selected

plants from the crosses with Oryzica 1 showed good tillering capacity, profuse vegetative growth. Panicles were long of about 24 cm in length. Plants with good panicle exertion were selected; however most plants derived from crosses with Oryzica 1 did not show good panicle exertion. Flowering started at about 95 to 110 days after planting reaching 50% anthesis at 102 to 118 days. The total cycle ranged from 132 to 150 days. Some plants showed delayed senescence.

Plants derived from the transgenic events were submitted to a strong selection process, identifying about the 10% best top materials from the 300 progeny plants evaluated in the field in 2004. These plants are highly fertile (80% to 96%, Table 2). Low fertility (male sterility) had been a major bottleneck for selecting materials from the original transgenic events, which complicated the selection of non-segregating materials in advance generations (T_6 to T_{11}) because the sterility made more prone the lines to out-cross with neighbor plants. No significant differences were noted between the selected plants and the commercial varieties for the various agronomic traits evaluated (Table 2). Plants with early senescence were discarded. The selected plants show good tillering capacity, slender-to-broad leaves, and intermediate plant height and good vigor likewise the commercial varieties. Short flag leaves, panicles of about 24 cm in length and good exertion were taken into account. Flowering initiates at 89 to 107 days reaching 50% anthesis at 105 to 116 days, and a complete cycle at 135 to 146 days (Table 2).

Future Activities

Selected plants will be evaluated for RHBV resistance in the field, and multiplied seed sources will be used to conduct replicated yield trials and evaluation for other disease resistances and quality traits in 2005.

References

Lentini Z., Lozano I, Tabares E., Fory L., Domínguez J., Cuervo M., Calvert L. 2003. 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.

Table 1. Agronomic performance of F_5-F_6 lines derived from backcross to Cica 8 or crosses to Oryzica 1, Fedearroz 50, and Iniap 12.

CROSSES	P[1]	VIGOR[2]	DF[3]	DF(50%)[4]	PLANT HEIGHT (CM)	TILLER	F[5]
A3-49-60-4-5/Fedearroz 50-12[a]-M-M-M	2	3	113	118	110	3	86
A3-49-60-4-5/Fedearroz 50-16[a]-M-M-M	2	3	94	101	120	3	81
A3-49-60-4-5/Fedearroz 50-19M--M-M	3	3	112	117	115	3	84
A3-49-60-4-13/Fedearroz 50-18-M-M-M	3	3	102	108	118	3	82
A3-49-60-4-5/Oryzica 1- 15- M-M-M	7	3	95	102	120	3	79
A3-49-60-4-5/Oryzica 1- 232- M-M-M	4	1	108	115	120	3	83
A3-49-60-4-13/Oryzica 1-13III-M-M-M	3	1	110	118	110	3	89
A3-49-60-4-13/Oryzica 1-15A-M-M-M	5	1	98	105	120	3	83
A3-49-101-18-19/Oryzica 1-14--M-M-M	6	3	97	104	110	3	83
Total	**21**						
Cica 8/Fedearroz 50		3	92	102	124	1	80
Cica 8/Oryzica 1		1	96	111	120	1	81
Fedearroz 50		1	104	111	115	3	80
Cica 8		1	99	106	105	3	82
Oryzica 1		3	97	106	105	1	81

[1] Number of individual plants selected. [2] Plant vigor: (1) highest vigor and (5) lowest vigor. [3]DF days to flowering initiation. [4] Days to 50% anthesis. [5]F= mean fertility % per line.

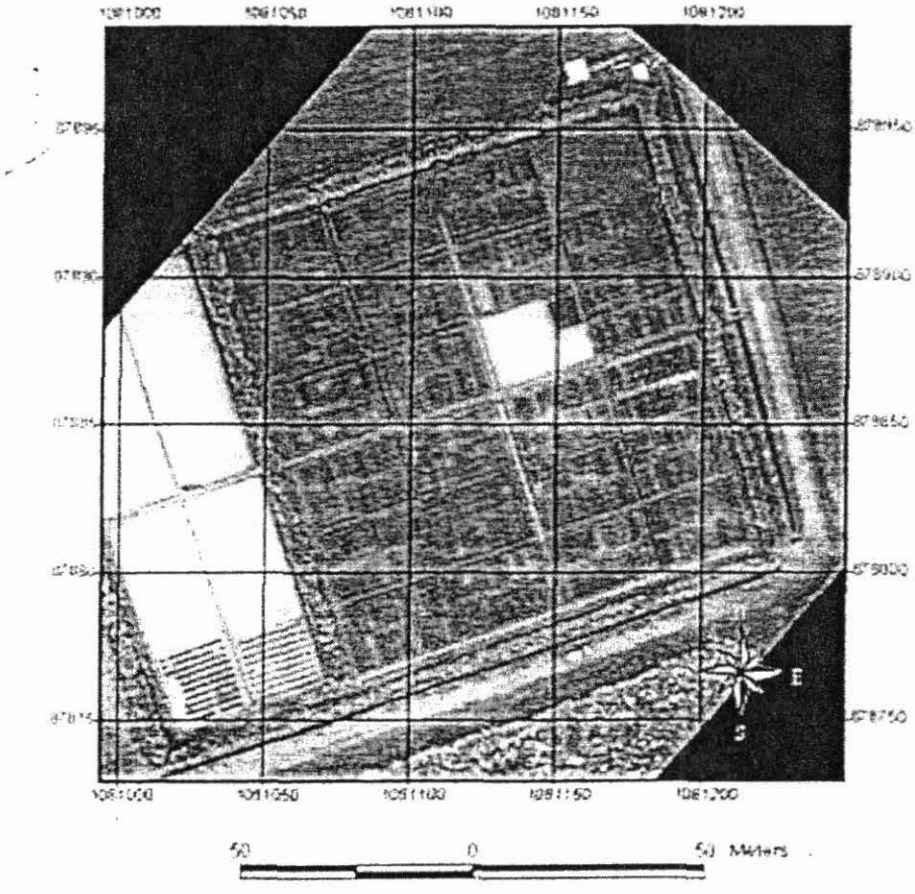


Figure 2. Aerial view of field plot (about 1 ha) for agronomic performance *evaluation of transgenic* rice RHBV resistant in 2004.

Table 2. Agronomic performance of RHBV resistant transgenic plants in the field 2004.

LINES	P[1]	VIGOR[2]	DF[3]	DF(50%)[4]	PLANT HEIGHT (CM)	TILLERS	F[5] (%)
A3-49-56-10-37-M-M	1	3	107	114	90	3	80
A3-49-60-13-69-M-1	1	3	101	108	105	3	85
A3-49-60-19-27-M-1	2	5	100	106	90	3	90
A3-49-60-4-13-16-M	1	5	104	111	95	5	80
A3-49-56-15-24-M-M-10	3	3	99	107	120	3	80
A3-49-60-13-40-M-M-1	3	3	107	116	95	3	91
A3-49-60-4-13-6-M-2	1	3	89	107	120	5	92
A3-49-60-12-3-3-75-M-3	1	3	95	105	115	3	88
A3-49-60-4-13-18-M-M-10	2	3	94	107	110	3	94
A3-49-60-12-3-20-M-M-M-3	8	3	98	108	110	1	90
A3-49-60-12-3-3-20-M-M-1	1	3	95	106	115	3	97
A3-49-60-12-3-3-58-M-M-14	1	3	110	116	105	3	91
A3-49-60-12-3-3-59-M-M-3	2	3	104	109	115	3	88
A3-49-60-12-3-3-65-M-M-4	1	3	92	106	120	1	80
A3-49-60-12-3-3-31-M-M-M-3	2	3	102	110	95	3	85
Total	**30**						
Fedearroz 50	-	3	107	113	115	3	82
Fedearroz 2000	-	3	110	119	110	3	85
Cica 8	-	3	100	109	105	3	80
Oryzica 1		3	97	106	105	3	80

[1] Number of individual plants selected. [2] Plant vigor: (1) highest vigor and (5) lowest vigor. [3]DF days to flowering initiation. [4] Days to 50% anthesis. [5]F= mean fertility per line.

2.1.7 Foreign genes as novel sources for increased efficiency of water use in rice

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[1]SB2 project, [2] IP4 project

Introduction

Rice yield potential (irrigated and upland rice) is highly dependent of adequate availability of water throughout the plant growth cycle. Irrigated rice currently accounts for most productivity worldwide. Irrigated rice requires large amount of water competing with water usage for other human activities. Flooded paddy rice fields significantly contribute with methane emission associated with the earth greenhouse effect and consequently climate change. In addition, water is becoming a scarce resource, also in Latin America which currently accounts for the largest ratio of water availability per person, thus breeding

rice for increased efficiency in water use must be a priority. Knowledge on molecular metabolism associated with drought tolerance in rice is limited. The physiological response to water stress is driven by changes in gene expression at the cellular level. Several genes had been demonstrated to be associated with tolerance to drought, salinity and cold, and the proteins encoded by these genes are thought to protect cells from these stresses (Shinozaki and Yamaguchi- Shinozaki, 1997). Some genes respond to water stress very rapidly, whereas others are induced after the accumulation of abcisic acid (ABA). Analyses in *Arabidopsis thaliana* of gene promoters induced by dehydration and cold have identified several *cis*-acting elements that are involved in ABA dependent and ABA independent responses to water stress. The DRE element (Dehydration Responsive Element) has been implicated in the regulation of dehydration responsive gene expression and found in promoter regions of dehydration and cold stress inducible genes (Kasuga et al., 1999). The main gene controlling the expression of these stress inducible genes is DREB (Dehydration Responsive Element Binding protein), which has been characterized as an early response transcription factor controlling the expression of multiple genes under drought stress. DREB (also known as CBF) genes have been isolated and characterized from *Arabidopsis thaliana* (Liu et al., 1998), and rice (*Oryza sativa*, Dubouzet et al., 2003). The *Arabidospis* DREB have been used in heterologous system to test its transgenic expression in tomato (Hsieh et al., 2002). Other transgenic approaches have been tested to improve stress tolerance in plants using genes encoding for enzymes involved in the biosynthesis of different osmo-protectants or encoding for modified membrane lipids, such as LEA protein and detoxification enzymes (cited by Kasuga et al., 1999).

The main objective of this work is to test DREB genes, and other sequences associated with tolerance to drought stress, in order to confer increased water use efficiency in commercial rice lines adapted to Latin American tropical conditions, and to understand the molecular mechanisms underlining the stress tolerance in these plants. The DREB genes (from rice and *Arabidospis*) were kindly provided by Dr. Shinozaki (from the Japan International Research Center for Agricultural Sciences, JIRCAS). In addition, a group from the Biotechnology Research Unit (Manabu Ishitani's group) has isolated several DREB genes from common bean and is testing the expression of these genes under stress. Here we report the progress made in rice during the past six months.

Materials and Methods

Plasmid constructions. Two gene cassette-constructs *Lip9::AtDREB1A* and *Lip9::OsDREB1B* were used for *Agrobacterium* mediated transformation of rice. The *Lip9::AtDREB1A* contains the *Arabidopsis thaliana DREB1A* transcription factor conferring tolerance to drought and low temperatures (Lui et al., 1998), while the *Lip9::OsDREB1B* contains the rice (*O. sativa*) *DREB1B* (Dubouzet, et al., 2003). Both constructs are driven by *Lip 9* stress inducible promoter and spliced into the plasmid *pBIG*, carrying the hygromycin resistance gene (JIRCAS). *Lip 9* corresponds to a rice promoter whose expression is not affected by ABA (Fig.1).

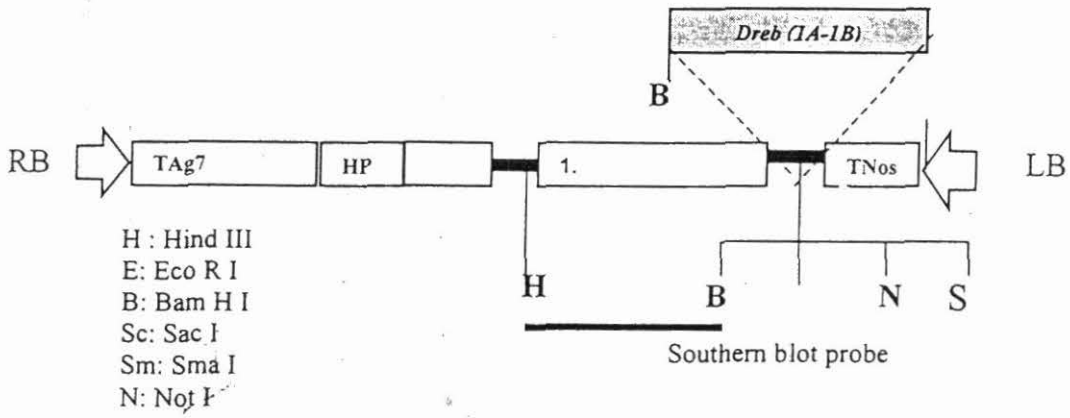

Figure 1. Gene cassette construct maps. Some restriction sites are showed. *HPT* **higromycin resistance gene,** *Pnos* **nos promotor,** *Tag7* **Tag7 terminator,** *Tnos* **nos terminator,** *RB* **right border,** *LB* **left border.**

Sequence confirmation. Rice *Lip 9::OsDREB1B* sequence was previously confirmed at JIRCAS. *Lip9::AtDREB1A* was confirmed using 7 primers (Table 1) specifically designed for *Lip9* (sequence provided by JIRCAS) and *DREB1A* (Genebank accession AB007787).

Table 1. Primer's sequences.

Name	Sequence	Bases	GC content	Tm
Lip9 fw1	5'-aagctttcatcagctatcatcaaa-3'	24	33.3%	54.7°C
Lip9 fw2	5'-tgtcaccgtccaggtctctg-3'	20	60%	59.4°C
Lip9 fw3	5'-taaatggatcgctccgctca-3'	20	50%	57.4°C
Lip9 fw4	5'-ccccatctcgtcgccatttc-3'	20	60%	59.2°C
At DREB rv1	5'-ggatccttaataactccataacga-3'	24	37.5%	53.5°C
At DREB rv2	5'-cacatctcatcctgaaacgc-3'	20	50%	54.7°C
At DREB rv3	5'-attgggtgacgagtctcacg-3'	20	55%	57.8°C

Rice genetic transformation. Mature embryo-derived callus of rice Cica 8, Palmar (*indica* type) and CT6241-1-15-1 (*japonica* upland type) were transformed using *Agrobacterium tumefaciens* strain Agl1 (Wang et al., 1997) which contained the plasmid carrying the DREB1A or DREB1B genes.

Molecular analyses of the transgenic rice plants. Genomic DNA was extracted from 1g of rice leaves according to McCouch (1988). Because hundreds of plants were regenerated, a first preliminary screening was conducted bulking DNA of plants regenerated from the same transformation event. A primary Southern blot was performed digesting DNA with BamHI and HindIII. Gels were denatured and neutralized by standard procedures and membranes were hybridized using a labeled promoter specific probe at 60°C also

generated by digesting the plasmids with HindIII and BamHI (Sambrook *et al.*, 1989). Confirmation by PCR for DREB1A was performed using Lip 9 fw 1 and At DREB rv1 primers, which are located in the promoter region and inside of the transcription factor (DREB). An additional Southern blot of the PCR amplified fragment was performed by hybridizing the probe to the confirmation PCR fragments. The *DREB1B*-PCR is under standardization process as well as the Real Time PCR tests.

Results and Discussion

About 700 plants from 400 events were regenerated from 1760 callus originally agro-infected (Table 2). The transformation efficiency varied from 18% to 28 % (mean of 24%) depending of the rice variety.

Table 2. Transformation efficiency of two *Indica* and one *Japonica* rice genotypes using *Lip9: :AtDREB1A* and *Lip 9::OsDREB1B* constructs.

Genotype	Gene	Callus agro-infected	Plants regenerated	Events	Transformation efficiency (events/ callus) %
Cica 8	*DREB1A*	200	72	43	21.5
CT6241		200	50	40	20.0
Palmar		380	179	105	27.6
Total		780	301	188	24.1
Cica-8	*DREB1B*	180	45	38	21.1
CT6241		280	56	51	18.2
Palmar		520	296	139	26.7
Total		980	397	228	23.3
	Total	1,760	698	416	23.6

Molecular analyses of the putative transgenic rice plants are in progress. Preliminary molecular analyses when using *Lip9fw1* and *At DREB rv1* primers amplified an 1,100 pb fragment (Figure 2A). The sequence specificity of this amplified fragment was confirmed by Southern blot- analysis, using as a probe the promoter region excised by HindIII/ BamHI digestion of the original plasmid (Figure 2B). Preliminary results using Real Time PCR amplification of bulked DNA of putative transgenic plants showed a similar peak between the positive control (plasmid construct) and the transgenic plants, and a clear-cut difference with the non-transgenic control, but there are additional background peaks that requires further analyses (Figure 2C). Southern blot of genomic DNA of the putative transgenic plants is in progress.

Future Activities

Corresponding molecular analyses of T0 transgenic plants are currently in progress to identify plants containing and expressing the *DREB1A* and *DREB1B* genes, and to determine the gene inheritance pattern.

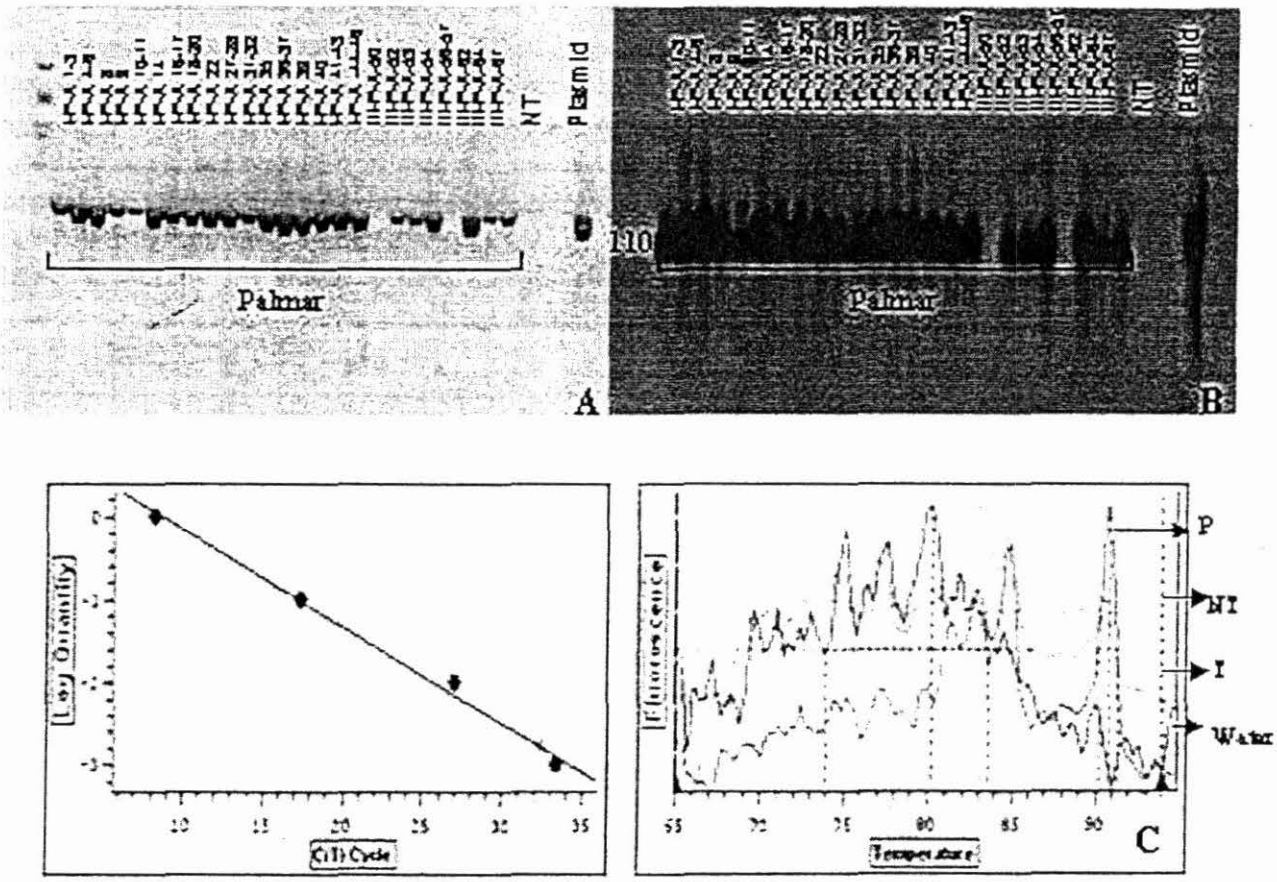

Figure 2. Molecular analysis of bulked DNA from putative transgenic plants. (A) *Lip9::DREB1A* PCR analysis. (B) *Lip9::DREB1A* southern-blot of PCR-amplified fragment. (C) Real Time PCR of regenerated plants using *Lip9-DREB1A*. P= amplification of *Lip9::DREB1A* plasmid. T = putative transgenic plant. NT = non-transgenic control plant.

References

Dubouzet, J., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E., Miura, S., Seki, M., Shinozaki, K. and Yamaguchi- Shinozaki, K. 2003. OsDREB genes in rice, *Oryza sativa* L.,encode transcription activators that function in drought, high-salt-and cold –responsive gene expression. The Plant Journal 33: 751-763.

Tsai-Hung Hsieh, Jent-turn Lee, Yee-yung Charng, and Ming-Tsair Chan. 2002. Tomato Plants Ectopically Expressing Arabidopsis CBF1 Show Enhanced Resistance to Water Deficit Stress Plant Physiol. 130: 618–626.

Kasuga, M., Liu Q., Miura, S., Yamaguchi- Shinozaki, K and Shinozaki, K.1999 Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology 17:1999.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi -Shinozaki, K. and Shinozaki, K. 1999. Two transcription factors DREB1 and DREB2, with an EREBP/AP2 DNA-binding domain separate two cellular signal transduction pathways in drought and low temperature responsive gene expression respectively in Arabidosis. Plant Cell 10: 1391-1406.

Shinozaki, K. and Yamaguchi- Shinozaki, K. 1997. Gene expression and signal transduction in water stress response. Plant Physiol. 115: 327-334.

2.1.8 Use of weedy rice as potential trait source for 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. Hybridization between crops and their wild relatives sometimes brings genes into wild populations, occasionally resulting in the evolution of aggressive weeds and/ or endangerment of rare species. Latin American wild *Oryza* species are new sources of genetic diversity not found in the cultigens (Buso *et al.*, 1998). However many 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). The weedy rice complex, commonly known as red rice, is composed by *Oryza sativa f. spontanea* and other annual *Oryza* species with feral traits in tropical America (Holm et al., 1997). Weedy rice has similar morphological traits as cultivated rice at vegetative phase but generally it has profuse tillering, vigorous growth and re-growth after pruning making this weed highly competitive respect to rice. According to Langevin *et al.* (1990), the weedy rice can be grouped in ecotypes with characters alike-cultivated rice or wild rice (Oka and Chang, 1961). Our work using microssateliites markers had indicated that weedy 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. glaberrima* analyzed (Gonzalez *et al.*, 2003) and used so far in breeding at CIAT, including added value traits such as aromatic and high anthocyanin content in the grain. Thus weedy rice could be a potential source to broaden the genetic base of rice varieties. The main objective of this study was the characterization of agronomic traits of weedy rice accessions pre-selected based on previous phenotypic, phenological and molecular

characterization (Gonzalez *et al.*, 2002 and Fory et al., 2003, SB2 Annual Report 2002 and 2003, respectively).

Materials and Methods

Weedy rice materials were pre-selected based on previous phenotypic, phenological and molecular characterization. Priority selection was given to accessions with resistance to RHBV, higher tiller number, yield potential and high re-growth capacity upon harvest and pruning respect to commercial varieties. Selection criteria also included materials genetically diverse identified using the principal coordinate and component analyses, and microsatellite molecular characterization from previous studies conducted last year.

Third generation of self-progeny seeds derived from the original weedy plants collected in the farmer's fields were planted in a randomized plot design with 3 replications with 51 plants for replication. The evaluations were conducted on a total of 65 accessions of weedy rice. Six commercial rice varieties and twelve advanced breeding lines of FLAR and CIAT were used as control. The evaluated traits included: flowering cycle, tillering capacity, effective tillering capacity (referring to those producing panicles), re-growth capacity and re-growth height after 12 days of harvest and pruning, plant height, plant type, total grain weight per plant, seed shattering rate, and grain quality traits.

Results and Discussion

Accessions were selected based on a Principal Three Component Analysis. The cluster analysis arranged the accessions in seven groups (Figure 1). Groups 3 and 6 were mainly composed by weedy rice accessions, the other groups contained accessions of weedy rice, advanced FLAR and CIAT breeding lines and commercial varieties. Selection of weedy rice accessions was conducted in order to include highly diverse materials as well as those clustering closer to either commercial varieties and/or advanced breeding lines but distinguishing from them in at least one superior characteristic. Of the 65 weedy rice accessions evaluated, ten accessions were selected by its agronomic performance. Four accessions selected were clustered in group 1, two accessions in cluster 4, two accessions in cluster 6 and two accessions in cluster 7. Accessions from 1 to 4 (Table 1) were the most distant from the commercial varieties (Figure 1). Accessions from clusters 5 to 8 were the most similar to the commercial varieties, and accession 9 and 10 the closest to *O. rufipogon* (Figure 1).

The most distant accessions from the commercial varieties were characterized by open plant type, fewer short and slender leaves, and exerted panicles. They start flowering at 84 to 91days, and end flowering at 97-100 days. Plant height ranged from 72 to 115 cm. Number of tillers and effective tillers (tillers with panicles) were higher than the commercial varieties and breeding lines. Accession 1-5-3 showed an extremely high number of tiller re-growth and re-growth height upon harvest and pruning (Table 1).

Accessions No. 2 to 4 showed the largest number of panicles per squared meter and with high percent of effective tillers.

The accessions closer to the commercial varieties have semi-open to compact plant type, broader leaves, large flag leaf and erect or decumbent. Flowering initiated between 88 to 100 days and ended between 98 and 118 days. Plant height was intermediate (IRRI standard evaluation scale, 1996). Plants showed the effective number of tillers similar to Fedearroz 50. The accession 5-66-2 was characterized by a high number of tiller re-growth and re-growth height, and all the accessions showed good grain weight per plant (Table 1).

The accessions closest to *O. rufipogon* have open plant type, intermediate width leaves, short flag leaf and erect or decumbent. Similar to *O. rufipogon,* these accessions show a very early flowering initiation at 84 at 87 days which ended between 91 at 93 days, and intermediate to tall plant height (IRRI standard evaluation scale, 1996). In contrast to *O. rufipogon*, they have improved number of effective tillers and a significant higher number of tillers re-growth, re-growth height, and potential grain weight per plant (Table 1).

The grain quality of all accessions in relation to white center oscillated between 1 and 3 (translucent to chalkiness), and the amylose content varied from 25% to 27%. Grains were of medium size and thick (IRRI standard evaluation scale, 1996). Not significant difference in grain weight per plant was found between the weedy rice accessions and the commercial varieties, except for accession 1-5-3 and 6-3-2 which showed a low value due to grain shattering. Shattering ranged from 60% at 70% introducing a large variation in the data collection for yield potential. Shattering is a major bottleneck to be overcome, however MAS to eliminate this dominant trait is possible in few generation since molecular markers tightly linked to this trait had already been identified.

Future activities

Selected materials will be planted for further chacaraterization for disease resistance (*Rhyzocthonia sp*, *Pyricularia sp*, Helminthosporium *sp* and grain discoloration, among others). Selected plants will be crossed with Fedearroz 50, Fedearroz Victoria 1, CIAT and FLAR advanced lines, and segregating populations will be evaluated and selected at the F_2 generation and processed through anther culture to develop doubled haploids fix lines to initiate yield potential and regional evaluations earlier.

Reference

Buso, G., Rangel, P. and Ferreira, M. 1998. Analysis of genetic variability of South American wild rice populations Oryza *glumaepatula*) with isozyme and RAPD markers. Molecular Ecology 7. 107-117.

Burke, J. and Arnold, M. 2001. Genetics and the fitness of hybrids. Annu. Rev. Genet. 35: 31-52.

González, E., Fory, L., Vásquez, J., Ruiz, P., Mora, A., Silva, J., Duque, M., Corredor, E. and Z, Lentini. 2002. Molecular characterization of rice and wild/ weedy relatives by microsatellites and their use to assess gene flow in the Neo-Tropics. Annual Report Project SB-2.

Fory, L., Corredor, E., Ruiz, P., Vásquez, J., Mora A., González, E., Agrono, T., Ordoñez, C., Bolaños, E., Silva, J., Duque, M., and Lentini, Z. 2003. Use of weedy rice as potential trait source for commercial rice breeding. Annual Report Project SB-2.

Holm , L., J. Doll, E. Holm, J. Pancho and J. Herbeger.1997. The wild rices. In World Weeds: Natutal history and distribution. New York. J. Wiley. pp: 531-547.

Langevin, S., Clay, K. and Grace, J. 1990. The incidence and effects of hybridization between cultivated rice and its related weed weedy rice (*Oryza sativa L.*). Evolution 44: 1000-1008.

Oka, H. 1998. Origin of cultivated rice. In Developments of crop science. JSSP/Elsevier. Tokyo. 254 p.

Oka , H. and Chang, W. 1961. Hybrid swarms between wild and cultivated rice species *Oryza perennis* and *Oryza sativa*. Evolution 15: 418-430.

Table 1. Agronomic performance of weedy rice accessions, commercial varieties and advanced breeding CIAT and FLAR lines.

No.	Line	FI	T	ET	%ET	PH	TRg	HRg	GP	Cluster (Fig. 1)
1	1-11-2	91	45	31	67.8	92	13	42	2,072	1
2	1-9-6	91	62	40	63.9	98	11	46	2,522	1
3	1-3-4	87	63	47	74.6	115	0	0	2,120	6
4	1-5-3	84	59	42	71.2	72	59	49	1,636	6
5	6-2-4	93	50	22	66.9	101	11	50	3,222	1
6	5-66-2	99	48	37	76.7	107	37	58	2,738	1
7	3-12-6	100	42	34	81.3	103	9	41	4,832	4
8	1-19-1	88	44	35	79.5	94	0	0	6,856	4
9	1-13-2	87	27	22	81.5	134	8	55	3,018	7
10	6-3-2	84	41	33	80.5	94	23	49	1,244	7
13	Cimarron	89	37	28	75.8	87	21	51	4,342	4
14	F. Victoria I	98	46	38	82.6	101	16	51	3,080	1
15	Fedearroz 2000	95	39	29	74.9	103	30	59	6,540	4
16	Fedearroz 50	102	39	35	89.9	102	29	54	2,826	1
17	CT11014-10-1-2	98	37	32	86.5	97	22	53	1,416	1
18	CT7201-16-5P	85	36	32	88.9	118	15	52	4,278	7
19	FLAR LINES	105	43	35	81.4	93	9	53	2,469	1
20	FLAR LINES	103	42	35	83.3	94	6	47	5,664	4
21	*O. rufipogon*	80	42	19	44.0	101	0	0	842	7

FI = flowering initiation (days); T = number of tillers; ET = number of tillers with panicles; %ET = percentage of tillers with panicles; PH = plant height; TRg = number of tiller with re-growth; HRg = re-growth tiller height; GP =grain per plant; Cluster = cluster according to Figure 1.

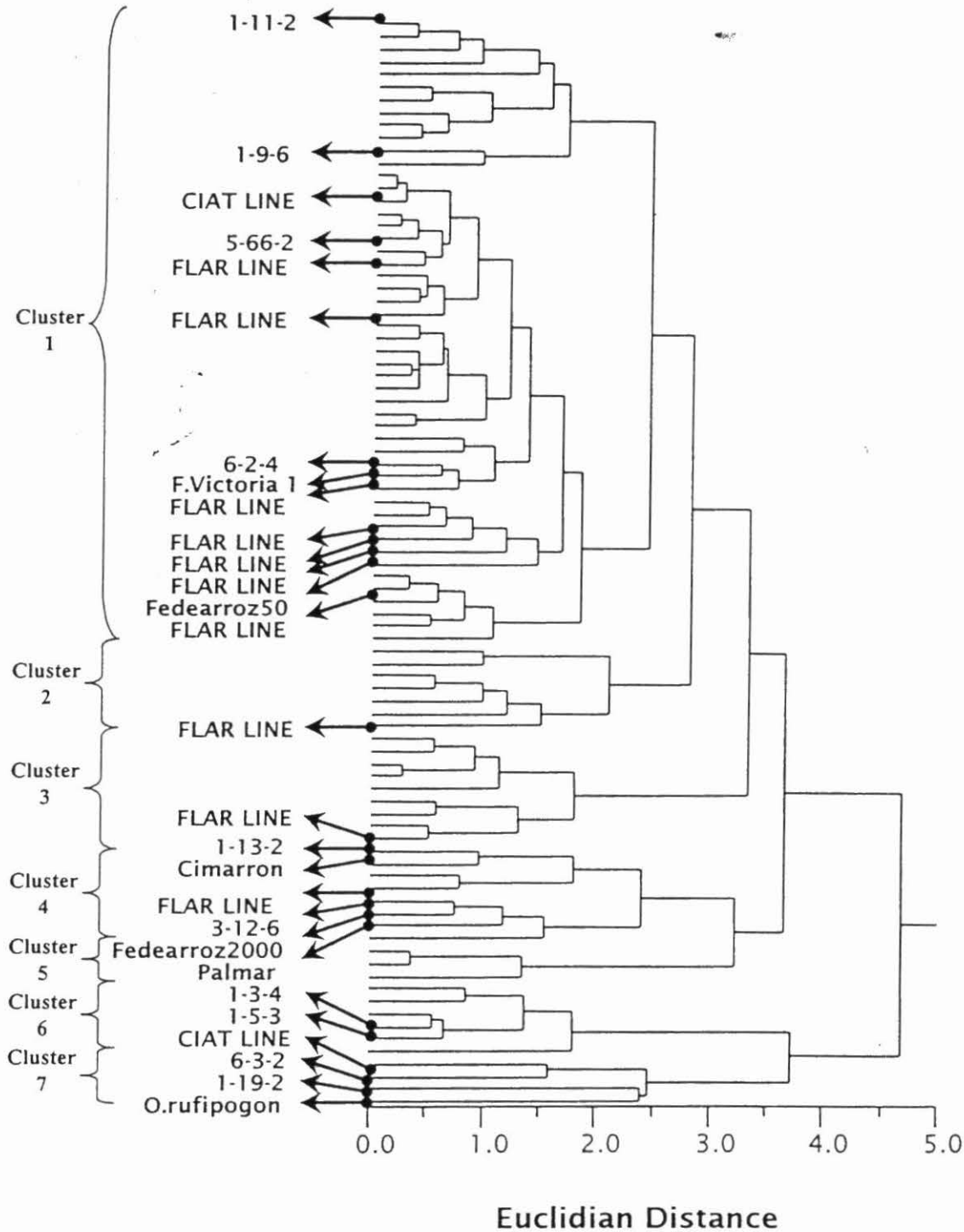

Figure 1. Dendogram using three principal components (flowering, plant height, tillers and yield) from cluster analysis. Selected weedy rice lines, advanced FLAR and CIAT breeding materials and *O. rufipogon*.

2.1.9 Field trials of transgenic cassava plants at CIAT: Pioneering steps towards biosafety standards

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SB-2 Project, CIAT

Introduction

Testing the ability of transgenic crops to perform under cultivated conditions is the ultimate proof of their real value to be included in a breeding program, or to be released as a variety for commercial purposes. This year CIAT received permission from the Colombian authorities, the National Biosafety Technical Committee (NBTC), to plant in the field few transgenic cassava lines that carry a gene for insect resistance (*cry*1Ab). The plants were transplanted on the 9 of August, 2004, following strict biosafety regulations, becoming the first ones to be planted in the open field in continental America, Asia or Africa. The main purpose of growing them in the field is to let them mature and produce vigorous stems that can be used as planting material to perform experiments with statistical significance in the next growing cycle. Therefore, this report does not detail the analysis of behavior of transgenic plants in the field, but rather it focuses on the biosafety implications of testing transgenic cassava in CIAT, in the middle of the CG-Center that preserves the world largest collection of cassava germplasm.

Implementing biosafety standards for transgenic cassava in the field

Location of the experimental plot and isolation from other cassava crops

For field trials, a plot of 2072 m^2 is available at CIAT's experiment station in Palmira. It is separated by more than 500 m from the nearest cassava crops. Transgenic plants are not permitted to flower in the field when flowering is not needed for studying the heredity of transgenes or for crossings. Flowering in the field, for this particular trial, requires approval from the NBTC of Colombia. The plot is surrounded by a natural barrier of elephant grass to minimize pollen movement (i.e., gene flow). Sugarcane plantations that also serve as barrier to pollen movement by wind also surround the plot.

The cassava germplasm bank held at CIAT carries more than 6000 accessions, which are currently kept *in vitro* and in greenhouses. In terms of biosafety and public perception, having no germplasm bank planted in the field is an advantage for conducting trials with transgenic plants.

Surveillance, water treatment, and residue management:

Because the plot is inside CIAT's installations, access is restricted to authorized CIAT personnel. The plot can be reached by road at any hour of the day or night, which

guarantees constant surveillance of the crop. The plot was irrigated manually, immediately after planting, for 2 or 3 weeks or until the plants develop deeper roots to capture moisture below the soil surface. Although planting is usually done in the rainy season, the rains are not intense and plot drainage guarantees that water runs towards collector canals without dragging with it planted stakes or plantlets.

An industrial incinerator is available for plant residues, as is fumigation equipment. Personnel are trained to maintain the crops free of weeds, diseases, and pests, and to irrigate appropriately.

Detecting movements of transgenes

The molecular biology laboratories can rapidly detect movements of transgenes where necessary. CIAT has PCR equipment for carrying out rapid tests for gene detection by amplifying specific sequences (primers are available for all three genes of the T-DNA: *npt*II, *gus*A and *cry*1Ab). A diagnosis of a gene's presence takes 3 to 5 days. There is also RealTime® PCR, which is a much more specific and safer application of PCR for detecting genes through fluorescence using also specific primers of the same genes. It is also possible to carry out analyses with Southern, Northern, and Western blots for detecting genes, their transcripts, and the proteins they produce. Finally, CIAT has the equipment for sequencing DNA, making it possible to decipher the sequence of bases of any gene.

All applications made to CIAT's internal biosafety committee and the NBTC provide the complete sequences of the genes that were inserted into the plants being studied. This guarantees that highly specific primers can be designed that would permit following up genes through either PCR or RealTime® PCR. Many of the transgenic plants produced at CIAT carry marker genes with resistance to antibiotics. These genes may also be detected in histological tests, either through *in vitro* tests or using very simple, cheap, and fast histological stains.

Potential for genetic exchange between cassava and other organisms

The largest center of diversity for cassava (*Manihot esculenta*) is in the Amazon Basin and the Orinoco. The *Manihot* genus contains about 90 wild species, of which *M. esculenta* subspp. *flabellifolia* and *peruviana* are regarded as the subspecies closest to cassava (Olsen and Schaal, 1999). The natural habitat of these subspecies is found mainly in the Brazilian states of Acre, Goiás, Rondônia, Mato Grosso, and Tocantins. These subspecies are not found at CIAT in their wild form, although some of them are indeed part of the *in vitro* and greenhouse germplasm collections kept at the Center.

Careful examination of the gene flow between cultivated cassava and wild *Manihot* species shows that interspecific gene flow occurs in nature, for example, between cassava and *M. glaziovii*. The latter is a species that originates in Brazil and was introduced into Côte

d'Ivoire and Nigeria. Beeching et al. (1993) reported the existence of natural hybrids in Côte d'Ivoire, a finding that was later confirmed by using DNA molecular markers and isozymes (Wanyera et al, 1994). *M. glaziovii* is sympatric with cultivated cassava in northeast Brazil, but has not been reported in Colombia. The apparent absence of this species in Colombia may, in fact, be a result of a lack of information on its presence in this side of the continent. Accordingly, we always need to consider the possibility that this and other species can mate with cassava in Colombia, even though no reports to that effect have yet been made.

The most recent data based on DNA molecular markers have shown the existence of wild cassava species (i.e., uncultivated) in Brazil (Olsen and Schaal 1999; 2001), corroborating previous findings by Allem in 1994. Gene flow has not been demonstrated under natural conditions between cultivated cassava and its closest wild relatives *M. esculenta* subspp. *flabellifolia* and *peruviana*. Although little information is available and, hence, little understanding of the distribution of either subspecies in relation to cassava in Colombia, neither has the presence of either of these subspecies in the country been demonstrated. However, preliminary studies carried out by Roa et al. (1997) show that seeds can be obtained from artificial crosses between cultivated cassava and the subspecies *flabellifolia*.

The species *M. carthaginensis* is found along Colombia's North Coast (collections were made in El Rodadero and Neguanje Cove in the Tayrona National Natural Park, Santa Marta; Roa, 1996). Even so, no evidence exists of natural crossing between this species and cassava. Likewise, *M. brachyloba* has been collected in the Municipalities of Barrancabermeja (village district El Centro) and Girón (Puente Sogamoso) in Santander, and in Bahía Solano (Playa Mecana) in Chocó (Roa, 1996). Again, no evidence exists to suggest that natural crosses occur between this species and cassava.

In Colombia, the possible unplanned or undesired transmission of genes between wild *Manihot* species and transgenic cassava is less important than that between the numerous traditional cultivars (landraces), and transgenic cassava that are likely to be cohabiting future field plantings. We can ensure that, under CIAT conditions, non-transgenic cassava will not be planted within 500 m of trials with transgenic cassava, thus minimizing gene flow. Moreover, the natural barriers of sugarcane and elephant grass, and elimination of immature flowers practically eliminate it. However, under conditions found outside the research center, gene flow cannot be impeded if transgenic cassava is planted close to traditional cultivars.

Alternatives for containing transgenes in plants and preventing their undesirable distribution (Daniell 2002) include restricting the insertion of genes of interest to the chloroplast genome. This would be possible in cassava if we knew the sequence of its chloroplast genome. CIAT still does not have the technology, but efforts are being made to seek financing so we may apply this technology to those of CIAT's crops involved in transgenesis, such as cassava, beans, and rice.

In the Americas, one barrier to crossing occurring between cultivated cassava and related wild species seems to be the low levels of flowering (although this depends on the clone), low seed fertility (usually one in three in each fruit of cultivated cassava crosses), asynchrony of reproductive organs in reaching maturity (e.g., anthesis), the nature of fruit dehiscence (seeds usually fall very close to the parent plant, thus limiting expansion of the species), the short-term viability of pollen (1 to <6 h) and, above all, the loss of the wild species' natural habitats.

Adaptation and survival of cassava:
Cassava in nature is adapted to a hot climate (temperatures of 18°C to >24°C), poor acid soils, and little or minimal precipitation (<500 to 1000 mm). But cassava can also adapt to humid environments (in the exceptional cases of certain varieties, up to 8000 mm). It has been observed growing in the Colombian Pacific coastal region, and in the departments of Valle del Cauca and Chocó, among others. However, cultivation is usually carried out under more favorable conditions, such as along the Colombian North Coast, particularly if the crop is destined for the starch or animal feed industries.

Cultivated cassava depends entirely on the farmer for its spread and survival in the environment. Although cassava is cross-pollinated by insects or wind and can produce fertile seed, propagation is mostly through asexual seed known as "cangres", that is, stem cuttings, better known as stakes. Hence, the genotype of the variety is conserved. Cassava is highly heterozygous, which means that propagating it by sexual seed would mean losing the original genotype. Sexual seed production also depends on the variety, as some varieties do not flower, or typically flower very little, and others produce abundant flowers and sexual seed.

Sexual seed is not normally used for commercial reproduction of the species. Plants obtained from sexual seed are usually less vigorous, take more time to establish, and are smaller than those obtained from stakes. The fruit is dehiscent, opening explosively once mature and throwing seed close to the parent plant. Seed fertility varies with the clone. Normally, one fertile seed is obtained from the three seeds found in each fruit. Fresh seed is dormant and needs 3 to 6 months at room temperature to germinate (Jennings and Iglesias 2002).

Although the importance of sexual seeds for cassava propagation may be regarded as not critical in the maintenance of the crop for medium-to-large, even some small-scale farmers, one can not ignore that traditional cassava cultivators in the Amazon recruit seedlings germinated in plantations to replant their fields after what they call a "bad" year, possibly meaning a year in which planting material left after the harvest is not enough for the next cycle of cultivation (Emperaire et al, 1998). In some cases, plants derived from sexual seeds account for 30% or more of the total plantation, highlighting thus the importance of the sexual seed in enhancing the variability of the crop and helping farmers to maintain the crop as part of their diets in the most extreme conditions.

In their natural habitat, cassava's closest relatives probably reproduce sexually (Allem 1994); at least they produce seed, although the possibility that they may do so asexually cannot be ruled out. Wild cassava (subspp. *flabellifolia* and *peruviana*) is usually found growing in patches of virgin jungle in the State of Mato Grosso, Brazil, as groups of individuals that grow like lianas over the vegetation, as Allem (1994) says: ". . . the habitat was that of subscandent, woody, vine-like shrubs, climbing over other vegetation with branches to 7 m long" (p. 139). This morphology permits the branches to fracture easily through the action of, mostly, birds and monkeys, thus falling to the ground where they generate new plants.

Conclusion and Perspectives

There is not doubt that the implementation of the first open-field trials of transgenic cassava in CIAT has implications for biosafety and public concern. Especially if the trials are carried out in the CG center responsible for preserving the world's cassava germplasm. However, as far as we could foresee, all measures have been taken to minimize or eliminate risks. For instance, eliminating flowers from plants in the field will eliminate gene flow through pollen movement. This is of course a utopia in larger plantations with thousands of transgenic plants. Then, the two major concerns that come out of these transgenic trials are:

Are there wild relatives that crossbreed with cultivated cassava in Colombia?

How will cross-pollination be avoided between transgenic and conventional cassava growing side by side in the field?

Two concerns that need basic studies on the actual distribution of cassava and its relatives in Colombia, and on methods to avoid transmission of genes through pollen.

References

Allem A 1994. The origin of *Manihot esculenta* Crantz. Genet Res Crop Evol 41:133–150.

Beeching JR; Marmey P; Gavalda MC; Noirot M; Haysom HR; Hughes MA; Charrier A 1993. An assessment of genetic diversity within a collection of cassava germplasm using molecular markers. Ann Bot 72:515–520.

Daniell H 2002. Molecular strategies for gene containment in transgenic crops. Nat. Biotechnology 20:581-586

Emperaire L; Pinto F; Second G 1998. Une gestion dynamiqué de la diversité varietale du Manioc in Amazonie du Nord-Ouest. 6:27-42.

Jennings DL; Iglesias C. 2002. Breeding for crop improvement. In Hillocks RJ; Thresh JM; Bellotti A, eds. Cassava: biology, production and utilization. CABI Publishing, Oxon, UK. pp 149–166.

Olsen K; Schaal B. 1999. Evidence on the origin of cassava: phylogeography of *Manihot esculenta*. PNAS 96:5586–5591.

Olsen K; Schaal B. 2001. Microsatellite variation in cassava and its wild relatives: further evidence for a southern Amazonian origin of domestication. Am J Bot 88(1):131–142.

Roa AC. 1996. Estimación de la diversidad genética en *Manihot* spp. mediante morfología y marcadores moleculares. MSc thesis. Department of Biology, Universidad del Valle, Cali, Colombia. 221 pp.

Roa AC; Maya MM, Duque MC, Tohme J, Allem AC & Bonierbale MW, 1997. AFLP analysis of relationships among cassava and other *Manihot* species. TAG 95:741–750.

Wanyera NMW, Hahn SK and Aken'Ova ME, 1994. Introgression of ceara rubber (*Manihot glaziovii* Muell-Arg) into cassava (*M. esculenta* Crantz): a morphological and electrophoretic evidence. Proc. 5th Symp. ISTRC-AB, 1994, pp 125-130.

2.1.10 Temporary Immersion System (RITA) for Rice Anther Culture

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Introduction

Automation is an attractive alternative for practical mass production of somatic embryos. The establishment of automated culture protocols could offer substantial labor cost reductions and be useful to study somatic embryogenesis, in addition to improve embryo production efficiency. Some key parameters to optimize include the volumes of the container and nutrient medium, especially for shoot proliferation. Several reports demonstrates increased efficacy from temporary immersion when using liquid medium for micropropagation. Current trends involve scaling up the use of temporary immersion for embryogenesis and shoots proliferation procedures at commercial level (Berthouly and Etienne, 2002).

For several plant species including rice, microspore can readily be isolated in very large numbers. Standardized system for generating haploid/doubled-haploid plants from isolated microspores offers considerable opportunities for various applications, including the rapid detection of homozygous mutations, production of recombinant pure lines and homozygous transgenic plants, and fundament studies of embryogenesis including its molecular basis. In the past 6-8 years, significant progress has been made in the development of isolated microspore culture systems even in the comparatively recalcitrant cereal species (Jahne and Lorz, 1995; Raina 1997). Microspore –derived plants without prior anther culture were recovered in barley (Hunter, 1987), maize (Coumans et al., 1989; Pescitellie et al., 1989) and wheat (Mejza et al., 1993; Konzak et al., 2001). In rice, plant regeneration from microspores has been reproducible attained from pre-cultured anthers (Jia et al., 1987; Cho and Zapata, 1988 and 1990; Xie et al., 1995; Raina et al., 1998). Several plant growth regulators have been used successfully in cereals anther culture.

Phenyl acetic acid (PAA) is a naturally occurring auxin with much weaker activity than indole –3-acetic acid (AIA) or naphthalene acetic acid (ANA). Leuba and Le Tourneau (1990) reported that PAA enabled the initiation and growth of callus suspension cultures of several dicot species. Ziauddin et al. (1992) found improved plantlet regeneration in wheat anther culture and barley microspore culture using PAA.

Last year we reported a comparative analysis including various *indic*a and *japonica* rice genotypes using RITA system for the induction of embryogenic anther culture-derived callus (Quintero et al., 2003 in SB2 annual report 2003). This year, the work focused in three main aspects to improve embryogenesis and plant regeneration from recalcitrant *indica* genotypes. First, the effect of temporary immersion system and continuous immersion cultures with agitation treatments on callus induction, embryogenesis and plant regeneration. Second, the effect of PAA on rice anther culture, and third the study of various parameters and protocols for an efficient isolation and culture of rice isolated microspores.

Materials and methods

Anther Culture. Anther donor plants were grown in the field, and the rice panicles were harvested, and anthers cultured according to Lentini et al. (1995). Anthers were cultured without agitation (stationary) or with agitation (shaker) in liquid medium contained in baby food jars closed with perforated plastic caps with a foam plug in a hole for aeration (Treatment 1, stationary; Treatment 3, shaker); or tightly closed without perforated plastic caps (Treatment 2, stationary control; Treatment 4, shaker); or in RITA vessels (Treatment 5).

Plant regeneration. Induced callus from each culture treatment was transferred onto solid plant regeneration medium according to Lentini et al. (1995). Six treatments were tested to increase plant regeneration from anther derived callus. Treatment 1 (control) consisted on MS medium as in Lentini et al. (1995) supplemented with 3% sucrose. In treatments 2, 3, and 4, auxins were replaced by PAA at 0.45 mg/l, 2.25 mg/l, or 4.5 mg/l, respectively. Treatment 5 consisted of adding PAA 0.45mg/l without kinetin.

Effect of PAA on embryogenesis and plant regeneration. Callus was induced from *indica* rice Cica 8 and CT 11275, and Japonica rice CT6241 by culturing anthers on media containing PAA at 0, 10, 20, 30 and 40 mg/l. Anthers were cultured in liquid medium contained in baby food jars sealed with solid plastic caps and kept on a shelf (control) or on a shaker (with agitation), or closed with perforated plastic caps with a foam plugs in a hole for aeration and maintained stationary without agitation.

Microspore isolation and culture. Donors plants were grown in the field, panicles harvest and anthers cultured according to Lentini et al. (1995). Anthers were isolated and pre-cultured in 5 ml of 0.3 M mannitol and placed in the dark for 5 days at 26 C. after pre-treatment, microspores were released from the anthers either by blending, stirring,

vortexing or macerating them. Clean isolated microspore suspensions were cultured in liquid medium. The effects of anther pre-treatment prior microspore isolation, microspore density and viability were evaluated.

Microspore isolation by blending method. Fifty flower buds were cut from their bases and allowed to drop into the open blender cup. Mannitol 0.3 M (40 ml) was added to the blender cup and sealed. Buds were blended for 20 s at low speed to release most microspores. The blended slurry was poured into a sterile 100µm stainless-steel mesh and the blender top rinsed twice with 5 ml of 0.3 M mannitol. The filtrate was placed into 15 ml sterile centrifuge tubes and centrifuged at 800 rpm x 3 min. The supernatant was discarded from the tubes, and the pellet was re-suspended with 5 ml of 0.3 M mannitol followed by filtration through a 50µm mesh sieve. The filtrate was centrifuged again at 800 rpm x 3 min. The total number of microspores was counted using a haemocytometer (Nebauer) and the percentage of viable microspores was determined by staining with fluorescein diacetate (Widholm, 1972). The final microspore solution was centrifuged again and the pellet washed and re-suspended twice in liquid medium prior final culture in 1 ml of liquid medium and plated in smaller (60x15mm) Petri dishes.
Microspore isolation by macerating method. Anthers were excised directly from the immature panicles or first pre-cultured after excision, and then macerated in liquid medium with a glass pestle. The macerated tissue was filter through a 100µm-mesh followed by a 50µm-mesh. The isolated microspores were sieved, washed and incubated as described above.

Microspore isolation by vortexing or stirring technique. Anthers were pre-cultured for 5 days prior isolation in 0.3 M mannitol at 26 C. The anthers and medium were transferred to a sterile 50 ml Erlenmeyer flask with a stir bar. The volume was adjusted to about 20 ml by adding 0.3 M mannitol and the anthers stirred for fifteen minutes on a stir plate. Stirring was replaced by vortexing under the same conditions. Microspores were sieve, washed, and incubated as described above.

Results and Discussion

A two fold increased in callus induction was obtained when PAA 4.5 m/l was added to the induction medium (Figure 1). PAA 0, 0.45, 2.25 mg/l were sub-optimal (Figure 1). Similar results were obtained on embryogenesis (data not shown). In relation to the different aeration treatments (with/without shaker, with/without foam plugs, and RITA system), results showed that embryogenesis was significantly higher (90%) with the RITA system.

Further analysis to evaluate the interaction between PAA concentration and aeration indicated that while more responsive genotypes such CT6241 and CT11275 the optimal PAA concentration for callus induction and embryogenesis was at PAA 10 mg/l (Figure 2A and 2B), recalcitrant genotypes such as Cica 8 requires higher concentrations showing an optimal callus induction and embryogenesis at PAA 30 mg/l (Figure 2C and 2D).

Response significantly increased in Cica 8 when culture in a shaker allowing a better aeration of the culture vessels (Figure 2C and 2D).

Preliminary results testing different methods for isolating microspores indicate that with the blender it is possible to yield about 10 fold higher concentration of microspores from either *japonica* or *indica* rice respect to the other treatments (Table 1). Additionally, microspores released by blending the anthers showed 60-70% viability after isolation in contrast to those released by maceration (20-40%), steering (8-30%), or vortexing (10-40%).

Since there is a minimum density to ensure embryogenesis from microspore culture, further work will include the analysis of microspore culture density on *in vitro* response. The study will be undertaken with different genotypes to evaluate the effect of genotype on microspore culture response. Additional studies on the conditions of the donor plants prior microspore isolation will be evaluated as well as various pre-treatment to the panicles/ anthers prior culture.

Future activities

- To study systematically different factors affecting the emission and action of ethylene on *in vitro* culture
- To study the effects of PAA embryogenesis and plant regeneration
- To evaluate various modifications of the culture vessel allowing aeration and/or temporary immersion for reducing current cost for the implementation of the RITA system
- To evaluate the effect of physiological condition of anthers/ microspores donor plants and various pre-treatments on microspore culture response

References

Alvarad, D.; Cote, F.; Teisson, C. (1993). Comparison of methods of liquid medium culture for banana micropropagation. Plant Cell Tiss. Org. Cult. 32: 55-60

Berthouly, M.; Dufour, M.; Alvarad, D.; Carasco, C.; Alemanno, L.; Teisson, C. (1995). Coffee micropropagation in liquid medium using temporary *immersion technique*. ASIC, Kyoto, Vol II: 514-519C.

Cabasson, D.; Alvarad, D.; Dambier, D.; Ollitrault, P.; Teisson, C. 1997. Improvement of Citrus somatic embryo development by temporary immersion. Plant Cell Tiss. Org. Cult. 1-5

Coumans MP, Sukhinder S. Swanson SB (1999) Plant Cell Rep 7 :618-621

Cho MS, Zapata FJ (1988) Plant Science 58: 239-244

Escalant, J.V.; Teisson, C.; Cote, F. (1994). Amplified somatic embryogenesis from male flowers triploid banana and plantain (Musa sp.). In Vitro Cell. Dev. Biol. 30: 181-186

Escobar, R. H.; Roca, W. M. (1999). Cassava micropropagation for rapid "seed" production using temporary immersion bioreactors. CIAT Annual Report Project SB-02 p 84-86

Etiene, H.; Lartaud, M.; Michaux-Ferriére, N.; Carron, M.P.; Berthouly, M.; Teisson, C. (1997). Improvement of somatic embryogenesis in Hevea brasiliensis (Müll. Arg) using the temporary immersion technique. In Vitro Cell Dev. Biol. 33:81-87

Hunter CP (1987) Australian patent application number AU-A-7271. In : Plant regeneratiom method , Shell international Research .

Jhane A, Lorz H (1995) Plant Science 109 : 1-12

Jia Y, Abe T, Futsuhara Y (1987 Rice Genet Newsl 4: 109-110

Khanna, H. y Raina S. (1998). Genotype x culture media interaction effects on regeneration response of the *indica* rice cultivars. Plant Cell Tissue and Organ Culture 52: 143-145

Konzak CF, Polhé EA, Liu W,Zheng Y (2001) Cultured of freshly isolated wheat (Triticum aestivum L.) microspores treated with inducer chemicals (2001) Plant Cell Rep 20: 685-690

Lentini, Z.; Reyes, P.; Martínez, C.; y Roca, W. (1995). Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Science 110: 127-138

Leuba V, Le Tourneau D (1990) J Plan t Growth Regul 8 : 163-165

Mejza SJ , Morgant V, DiBona DE . Wong JR (1993) Plant Cell Rep 12 : 149-153

Murashige T.; Y Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473 – 497

Pescitelli SM Mitchell JC, Jones AM . Pareddy DR. Petolino JF (1989) Plant Cell Rep 7 : 673-676

Raina SK, Irfan ST (1998) Plant Cell Rep 17 : 957-962

Teisson, C. & Alvarad, D. (1998). In vitro propagation of potato micro tubers in liquid medium using temporary immersion. Conf. Potato Seed Production by Tissue Culture, Brussels, Cost 822, European Commission.

Tabares, E.; Delgado, G.; Escobar, R.; Lentini, Z. (2001). Temporary immersion system (RITA) for anther culture of rice. Annual Report, 2001. CIAT, Cali-Colombia. pp 19 – 20.

Tabares, E.; Delgado, G.; Escobar, R.; Lentini, Z. (2002). Osmotic stress as a tool to enhance plant regeneration in rice. Annual Report, 2002. CIAT, Cali-Colombia, pp. 55 – 57

Widholm, J.M., 1972 The use of fluorescein diacetate and phenosafranine for determining the viability of cultured plant cells . Stain Technol 47 : 189-194.

Xie J, Gao M, CaI Q,Cheng X, Shen Y, Liang Z (1995) Plant Cell Tissue Organ Cult 42 : 245-250

Ziauddin, A.; Marsolais, A.; Simion, E.; and Kasha, K.J. (1992). Improved plant regeneration from wheat anther and barley microspore culture using phenyl acetic acid (PAA). Plant Cell Report Vol. 11, pp. 489-498

Table 1.- Yield of isolated microspore suing different isolation treatments on *japonica* (CT6241-17-1-5-1) and *indica* (Fedearroz 2000) rice genotypes.

Genotype	Treatment	Density
CT6241	Vortex	7.4×10^4
		7.4×10^4
		1.5×10^4
	Stirring	1.3×10^4
		1.3×10^4
		1.9×10^4
	Macerating	6.5×10^4
		4.0×10^5
		2.0×10^5
	Blender	2.3×10^5
		1.5×10^5
Fedearroz 2000	Blender	2.3×10^5
		1.7×10^5

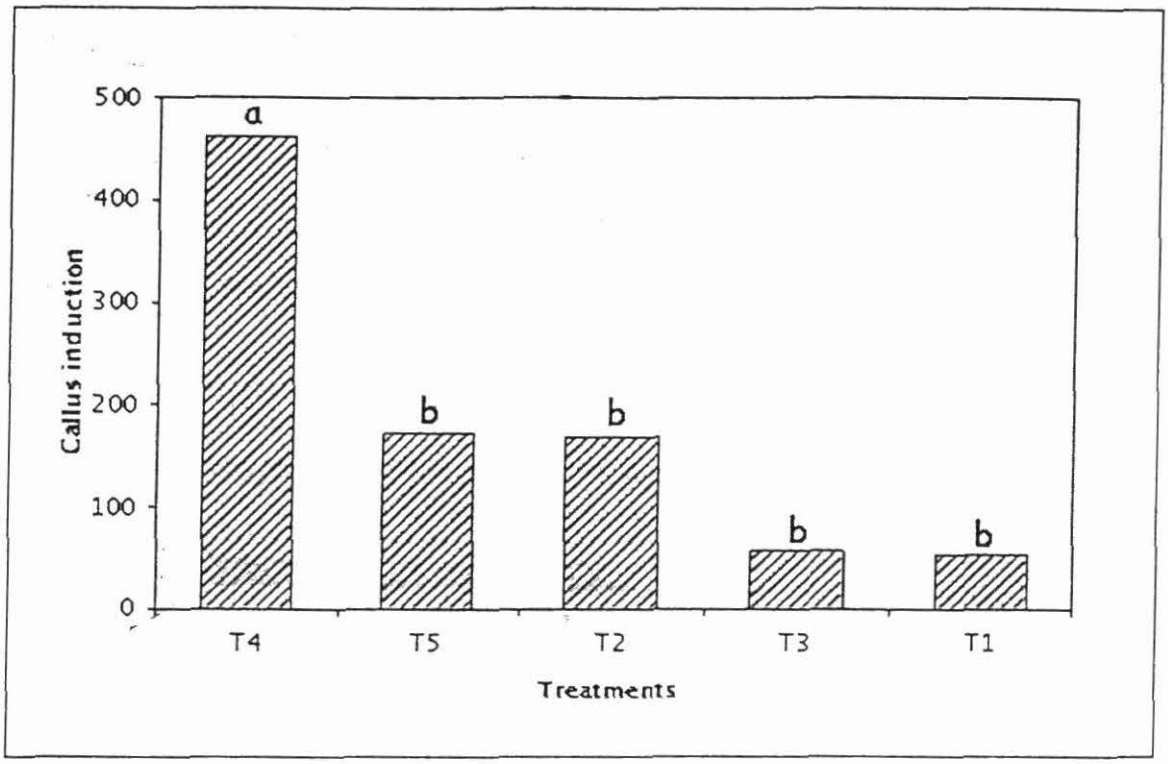

Figure 1.- Callus induction index using different treatments. Anthers were cultured without agitation (stationary) or with agitation (shaker) in liquid medium contained in baby food jars closed with perforated plastic caps with a foam plug in a hole for aeration (Treatment 1, stationary; Treatment 3, shaker); or tightly closed without perforated plastic caps (Treatment 2, stationary control; Treatment 4, shaker); or in RITA vessels (Treatment 5). Mean values followed by the same letter are not significant different (Multiple test Ryan-Einot-Gabriel-Welsch).

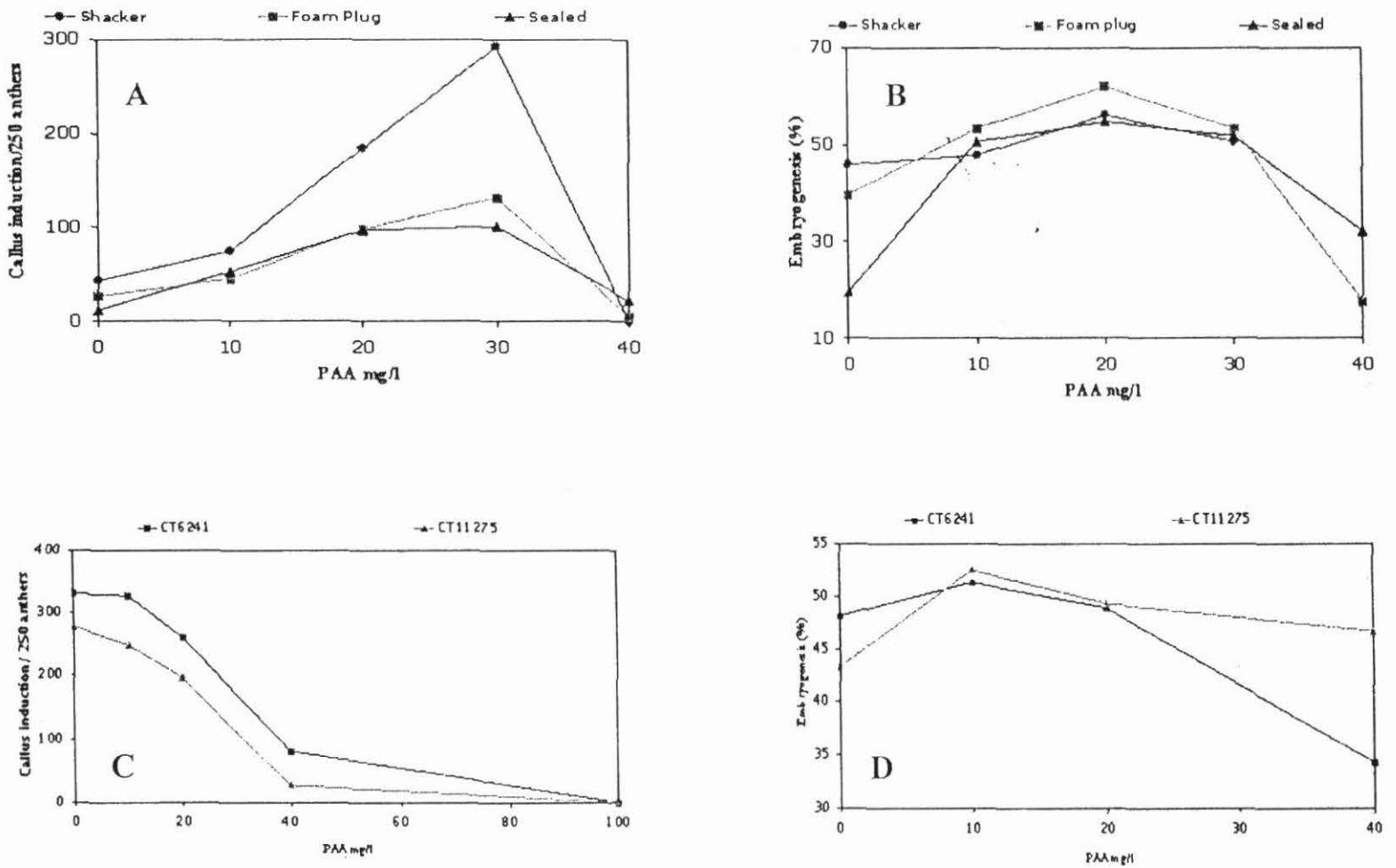

Figure 2.- Effect of PAA on callus induction and embryogenesis of recalcitrant *indica* genotype Cica 8 (A and B) and more responsive genotypes (C and D).

2.1.11 Development of an *In Vitro* Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding

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Introduction

Cassava is an important crop for subsistence farming in the tropical and subtropical regions of Africa, Latin America and Asia. The globalization of world economy offers cassava new opportunities for becoming an even more important source of raw materials for different industrial uses. To achieve this, cassava productivity must increase steadily and reliably based on an efficient breeding approach to maintain its competitiveness respect to other commodities. Cassava breeding is cumbersome and inefficient compared to other crops. This project proposes the development of an *in vitro* protocol for the generation of doubled haploids from cultured anthers or microspores via androgenesis, establishing a suitable model system for different ecotypes of cassava. This work will serve as a baseline for the development of populations allowing the identification of high-value recessive traits in early generations, and facilitating the application of molecular tools in breeding. Last year we presented a preliminary study directed to the identification of the different microspore developmental stages across different genotypes and environments, including cytogenetics studies, as well as the optimization of environmental conditions for shipment of flower buds to establish collaboration with Jan Custer at ARI, University of Wageningen (The Netherlands) as part of a Rockefeller Foundation funded project. This year, the work focused on analyzing various protocols and treatments for isolating and culturing cassava microspores of various genotypes. Usually anther culture has been preferred to isolated microspore. But isolated microspore culture could offer the opportunity of producing large amounts of green plants with less effort and reduced cost. Detail analysis is needed for the optimization of the different phases of this methodology in order to be applicable to different breeding lines.

Materials and Methods

Analyses of developmental stages of flower buds and microspore of cassava genotypes MCOL 1505, HMC-1, SM998-3 and CM523-7. Clones were grown at ICA experimental Station (Palmira, Valle del Cauca). Genotypes of contrasting flowering cycle (early, intermediate and late) were used: MCol-1505, HMC-1, CM7190 and SM1495-22. 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) and confirmed by this group last year, flower buds were collected when they were between 0.8 mm and 2.5 mm in diameter and fixed in a solution of 3:1 ethanol: glacial acetic acid with 0.5% ferric chloride for 24 hr

prior determining the stage of microspore development according to flower bud size. Microspore size was determined according to the stage of development.

Viability analysis of microspores under simulated shipment conditions. Microspore/ pollen viability was determined with FDA staining at different intervals between zero and 1 week, the maximum number of days after shipment from CIAT and arrival at Wageningen. Surface sterilized flower buds were kept under the same conditions as shipment (contained in moisture 50 ml Falcon tubes enclosed in a box with refrigerant gel) or either at 4 C in the refrigerator or at room temperature.

Isolation and culture of cassava microspores. Donors plants were grown in the field, and immature flower buds were collected when plants were about 8-12 month-old. Anthers were isolated and pre-cultured in 5 ml of 0.3 M mannitol and placed in the dark for 5 days at 26 C. After pre-treatment, microspores were released from the anthers either by blending, stirring, vortexing or macerating them. Clean isolated microspore suspensions were cultured in liquid medium. The effects of anther pre-treatment prior microspore isolation, microspore density and viability were evaluated.

Isolation by blending method. Fifty flower buds were cut from their bases and allowed to drop into the open blender cup. Mannitol 0.3 M (40 ml) was added to the blender cup and sealed. Buds were blended for 20 s at low speed to release most microspores. The blended slurry was poured into a sterile 100µm stainless-steel mesh and the blender top rinsed twice with 5 ml of 0.3 M mannitol. The filtrate was placed into 15 ml sterile centrifuge tubes and centrifuged at 800 rpm x 3 min. The supernatant was discarded from the tubes, and the pellet was re-suspended with 5 ml of 0.3 M mannitol followed by filtration through a 50µm mesh sieve. The filtrate was centrifuged again at 800 rpm x 3 min. The total number of microspores was counted using a haemocytometer (Nebauer) and the percentage of viable microspores was determined by staining with fluorescein diacetate (Widholm, 1972). The final microspore solution was centrifuged again and the pellet washed and re-suspended twice in liquid medium prior final culture in 1 ml of liquid medium and plated in smaller (60x15mm) Petri dishes. Isolated microspores were cultured in liquid medium NL (Lentini et al., 1995), B5 or MS4 medium at a density of approximately 1.2 X 10^4 micropores/ ml. Petri dishes were sealed with *Parafilm* and incubated in the dark at 26-27oC.

Isolation by macerating method. Anthers excised directly from the immature flower buds or previously pre-cultured after excision in 5 ml of 0.3 M mannitol and placed in the dark for 5 days at 26 C. Anthers were then macerated in liquid medium with a glass pestle. The macerated tissue was filter through a 100µm-mesh followed by a 50µm-mesh. The isolated microspores were sieved, washed and incubated as described above.

Results and discussion

Cassava pollen is generally 65 μm to 123 μm in size, which is large compared to other flowering plants. Preliminary work suggests that most cassava clones show microspore in the tetrad stage of development when flower buds are between 0.8 to 1 mm in diameter. Flower buds of 1.2-1.5 mm in diameter contain microspores at the uninucleate stage, whereas those of 1.6-1.8 mm in diameter are at the binucleate. Most flower buds contain mature pollen grains when they reach 2.0-2.8 mm in diameter. This association between flower bud size and microspore developmental stages appears to change with each peak of flowering (Table 1).

For the 4 clones tested, no clear differences in micropospore viability were noted during the first three days after flower bud excision when maintained either under room temperature or refrigerated conditions. After this period, a drastic reduction of viability is observed at room temperature respect to those under refrigerated conditions. Results suggest that flower buds should arrive at final destination within 3 days upon shipment to warranty high viability of microspore and preserve response to *in vitro* culture.

Preliminary results testing different methods for isolating microspores indicate that with the blender it is possible to yield about 2 fold higher concentrations of microspores from cassava clones respect to maceration (Table 2). Additionally, microspores released by blending the anthers showed about 60% viability after isolation in contrast to those released by maceration about 40%.

Since there is a minimum density to ensure embryogenesis from microspore culture, further work will include the analysis of microspore culture density on *in vitro* response. The study will be undertaken with different genotypes to evaluate the effect of genotype on microspore culture response. Additional studies on the conditions of the donor plants prior microspore isolation will be evaluated as well as various pre-treatment to the panicles/ anthers prior culture.

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 will be used as experimental genotypes to develop a protocol for efficient induction of androgenesis *in vitro* from isolated microspores. Additional studies on the conditions of the donor plants prior microspore isolation will be evaluated as well as various pre-treatment to the flower buds/ anthers prior culture.

References

Andersen S.B., I. Christiansen, B. Farestveit. 1990. Carrot (*Daucus carota* L.): In vitro production of haploids and field trials. *In:* Y.P.S. Bajaj (Ed.). Biotechnology in Agriculture and Forestry. Vol 12. Springer-Verlag. Berlin. P: 393-402

CIAT (Centro Internacional de agricultura Tropical) .2003 Annual Report Proyect SB2 .

Cistue L, Ziauddin, Simion E. Kasha KJ (1995) Plant Cell Tissue Organ Cult 42:163-169

Carvahlo R., M. Guerra, P.C. Carvahlo. 1999. Occurrence of spontaneous triploidy in Manihot esculenta Crantz. Jap Mendel Soc 64: 137-140

Carvahlo R., M. Guerra. 2002. Cytogenetics of Manihot esculenta Crantz (cassava) and eight related species. Hereditas 136: 159-168

Custers , J.B.M., J.H.G.Cordewener , Y. Nollen , H. J.M. Dons and M.M Van Lookeren Campagne, 1994, Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep .13 :267-271

Jhane A ,Lorz H (1995) Cereal microspore culture. Plant Sci 109 : 1-12

Konzak, C. F., E.A. Polle , W .Liu, and M.Y. Zheng. 2000 . Methods for generating Doubled –haploid plants . Published international patent application .PCT/US99/ 19498.

Konzak, C. F., E.A. Polle , W .Liu, and M.Y. Zheng. 2000 . Methods for generating Doubled –haploid plants . U. S. patent 6, 362,393.

Mordhorst AP, Lorz H (1993) Embryogenesis and development of isolated barley (*Hodeum vulgare L)* microspores are influenced by the amount and composition of nitrogen sources in cu8lture medium. J Plant Physiol 142: 485-492

Paillard M., P. Lashermes, and V. Pétiard. 1996. Construction of a molecular linkage map in coffee. TAG 93: 41-47

Raina SK, Irfan ST (1998) Plant Cell Rep17: 957-962

Roca, W.M. and K.Morman; R. Chavez and M.L.Marin. 1989. Cassava. In Working document. Biotechnology Research Unit. Annual Report 1988. No Wd 48. CIAT. Pp 8-22.

Roca W.M., C. Iglesias. 1993. Isolation and in vitro culture of cassava immature pollen and zygotic embryos. CIAT Annual Report. Biotechnology Research Unit. P: 175-179

Zhang J., W. Guo, T. Zhang. 2002. Molecular linkage map of allotetraploidy cotton (*Gossypium hirsutum L. X Gossypium babadense* L.) with a haploid population. TAG 105: 1166-1174

Table 1.- Developmental stages of cassava microspores according to flower bud and microspore sizes.

Clone	Flower bud (mm)	Developmental stage	Number cells analyzed	Microspore sizes min-max (µm)
HMC-1	<0.8	PMC	25	76-79
	≤ 1.0	Tetrad	25	91-104*
	≤ 1.5	Uninucleate	25	65-69
	≥ 2.0	Pollen	25	114-123
MCOL1505	<0.8	PMC	25	75-80
	≤ 1.0	Tetrad	25	85-96*
	≤ 1.5	Uninucleate	25	68-70
	≥ 2.0	Pollen	25	98-123
SM998-3	<0.8	PMC	ND	ND
	≤ 1.0	Tetrad	25	75-96*
	≤ 1.5	Uninucleate	25	64-70
	≥ 2.0	Pollen	25	82-105
CM523-7	<0.8	PMC	ND	ND
	≤ 1.0	Tetrad	25	75-96*
	≤ 1.5	Uninucleate	25	54-71
	≥ 2.0	Pollen	25	83-104

* Size includes the four microspore cells

Table 2. Yield of isolated microspores and viability with different isolation protocols

Genotype	Treatments	Density	FDA (Viability) %
HMC-1	Blender	1.7×10^4	60
HMC-1		1.2×10^4	80
HMC-1		1.7×10^4	62
SM998-3		2.3×10^4	80
CM523-7		0.7×10^4	60
CM523-7		0.5×10^4	50
Fedearroz 2000		9.3×10^4	60
Fedearroz 2000		7.0×10^4	60
MPER-183		1.2×10^4	50
Mean		2.8×10^4	62
HMC-1	Macerating	1.8×10^4	50
HMC-1		1.1×10^4	50
SM998-3		1.0×10^4	40
Mean		1.3×10^4	47

2.1.12 *In vitro* propagation of elite clones of Solanum quitoense (Lulo) selected by farmers in the field

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Introduction

Solanum quitoense, also known as lulo in Colombia and naranjilla in other countries, has great potential to become a premium product for local and export markets. Recently, in Colombia, lulo evolved from being a fruit just for local fresh consumption to become an important ingredient of commercial juices, yogurt, flavoring and processed food products, increasing its market value. Various diseases and pests affect its production, and plant breeding is at a young stage. A major constraint for the rapid adoption of lulo by the local farmers is the limited availability of elite clonally propagated germplasm free of pathogens. Rapid clonal multiplication of high-quality planting materials is of paramount importance to obtain uniform elite plants. Genetic transformation could also facilitate splicing in genes for traits of interest. Plant regeneration is usually a bottleneck for the development of efficient genetic transformation protocols. Last year we reported optimized conditions for propagation and plant regeneration in vitro. *In vitro* propagation under aerated conditions was optimized and uniform plants with normally and healthy developed roots and leaves are obtained on medium A. In addition, an efficient plant regeneration protocol was optimized using petioles as starting explants. Regenerated plants developed likewise control clones in the field, showing a similar fruit yield and quality. This year report summarizes the progress attained in selecting elite clones with experienced farmers in two commercial regions of Colombia using participatory research approaches; with the aim of propagating *in vitro* the selected material and testing the performance of these plants respect to conventionally propagated materials through seeds in the field. If successful, *in vitro* propagated plants could be a potential source of healthy and clean material for farmers.

Materials and Methods

Selection of elite clones by farmers in the field. In June 2003, CIAT and Corpoica held a meeting at CIAT with farmers selected from two of the most important zones for commercial production of lulo (Cauca and Huila). The farmers participating all have several years of experience growing the crop commercially. At the meeting, the main crop constraints were identified with the farmers and new alternatives were discussed including the use of *in vitro* propagated plants as a potential source of healthy-clean starting material. Farmers agreed in conducting a comparative evaluation of the *in vitro* plants with their corresponding seed derived plants representing elite clones selected by them under their field conditions. The most outstanding five clones of each region were selected based on their high productivity, premium fruit quality, plant type, resistance/tolerance to abiotic/

biotic stresses. In each region, two field plots were selected to represent the diverse environmental condition of each site.

Collection and establishment of selected elite clones in the greenhouse and in vitro. After selection of elite clones following the criteria described above, farmers collected botanical seed and stakes from each clone for vegetative propagation at the farmer's field. Two-old-month plants were brought to CIAT and divided into 2 or 3 stakes of 20 centimeters of length each, with 2 or 3 buds. The stakes were disinfected in a fungicide solution (Pervicur 3 cm^3/L or Inex-A 2 cm^3/L for 5 minutes) and planted in plastic bags with soil from the original farm. To induce profuse root development, at least two buds were cover with soil. After two months of weekly application of Benlate 2 g/L, Vertimec 2 cm^3/L, Confidor 2.5 cm^3/L and Inex-A 2 cm^3/L, to clean the material from pests and diseases, plants were transferred to the greenhouse at CIAT. *In vitro* multiplication of each clone was carried out by using greenhouse grown material as described in last year report with some modifications to enhance roots and foliar development. Induction medium was supplemented with MS (Murashige and Skoog, 1962) salts and vitamins MS, sucrose 3 %, gelrite 7 g/L, pH 5.7 and supplemented with BAP 0.02 mg/L and ANA 0.02 mg/L (Hendrix *et al.* 1987).

Experimental design for the comparative evaluation of clones in the field. The *in vitro* propagated clones will be evaluated and compared with their corresponding botanical seed derived plants in the farmer's fields. Several criteria were considered to select the experimental field plots:

a) Field sites may represent the soil and topographical conditions, allowing the elite clones to express all its potential and reflect standard conditions for commercial production.
b) Sites selected may facilitate the development of farmers' organizations in collaborative projects for crop production, germplasm evaluation and commercialization.
c) Sites may offer ease transportation, accessibility, and a port of entry for potential dissemination of new clones in the near future.

The *in vitro* propagated plants were established from greenhouse grown materials. *In vitro* plants were sub-cultured in order to sufficient material to conduct replicated trials. Conditions were standardized in order to have *in vitro* plants in a similar stage of development for the field trials. Thus, *in vitro* plants were grown for 30 to 45 days prior transferring them to the greenhouse for a stepwise process of acclimatizing for about 15 to 20 days, after which will be taken to the field sites for a final acclimatization period of about a week before planting them in the field. On each experimental field site, 26 *in vitro* propagated plants and 15 seed-derived plants of each originally collected clone will be planted. Materials will be planted following a randomized split-plot design.

Results and Discussion

A total of twenty farmers from two commercial production regions of lulo in Colombia (Pescador, Cauca, and Tierradentro, Huila), were selected and organized as with the collaboration of IPRA-CIAT and CORPOICA-Popayán (Table 1). The experimental sites, clones and methods of evaluations were selected following participatory means with the farmers. In Pescador, half of the farmers have commercially grown lulo for 9 to15 years, and the rest at least 1 year. Half of them cultivate between 1200- 2000 plants, the rest of the farmers usually grow 50 plants per cycle but have the potential to reach up to 2000 plants. Most farmers at Tierradentro have in average about 3 years of experience cultivating lulo, and some up to 7 years of experience. About half of them cultivate between 1200 and 5200 plants per cycle. Tierradentro is a remote area, with more difficult. All the farmers sell the fruits at markets distant from the production area through inter-mediators.

Based on the set of criteria established with the farmers, a total of 11 elite clones were selected in the two sites (Pescador and Tierradentro) (Figures 1 and 2). After the selection of the clones by the farmers, 10 stakes from each clone were used for clonally propagation in the field and became the starting material (donor plants) for the generation of the *in vitro* plants in the laboratory. The established clonally propagated plants were transferred to CIAT, and after phytosanitary treatments, clean materials were transplanted in the greenhouse in 10 kg pots with a mixture of 2 cachaza: 1 soil : 1 san) (Table 2). NAA 0.02 mg/l was added to medium A (composed of MS salts, thiamine HCL 0.5 mg/L, calcium panthotenate 2.5 mg/L, pyridoxine HCL 1 mg/L, nicotinic acid 5 mg/L, sucrose 30 g/L, gelrite 3.5g/L and pH 5.9) in order to accelerate a profuse root development of the initial cuttings *in vitro*. Plants initiate an active growth approximately 15 to 25 days upon culture, and then transferred to medium without growth regulators to allow a complete development. *In vitro* plants were sub-cultured every 30 to 45 days, to increase the numbers per clone. The initial phytosanitary condition of the field clonally propagated donor plants significantly affected the efficiency for establishing the material *in vitro* (Table 2). The efficiency for *in vitro* propagation ranged from 61% to 100%, with a mean of 82% yielding a total of 1,313 *in vitro* propagated plants in 9 months from the 62 original clonally propagated plants in the field by the farmers for the 11 clones (Table 2).

In Pescador (Cauca), two sites were selected to conduct the study. On site is located in the corregimiento of Siberia at about 1900 meters over sea level, in the property of the farmer Pedro Nel Herrera (Table 1). The other site is at the Municipio Pescador minicipio at 1550 meters over sea level in the property of the farmer Hermes Vitelio Menza (Table 1). In Tierradentro (Huila), two experiemntal sites were selected. One site is located at the corregimiento Pedregal at 1750 meters over sea level in the property of the farmer Saúl Salazar, and the other site is in the carregimiento Ramo at 1850 meters over sea level in the property of the farmers Orlando and Jose Valverde (Table 1).

Future activities

Of the total 11 clones originally selected in field, at present *in vitro plants* from 10 of them are ready for distribution to the farmers at Pescador and corresponding evaluation for this growing season starting in November 2004. Meanwhile, efforts continue in order to be able to distribute *in vitro* plants from the 11 original clones to farmers at Tierradentro, whose growing season starts early in 2005.

In vitro propagated plants will be planted in field and evaluated jointly with plants derived from botanical seeds of each original clone (the farmer's standard procedure). Plants derived from the different propagation systems (botanical seeds and *in vitro*) will be evaluated by the farmers following participatory approaches. Plants will be assessed by their capacity for survival in the field upon transplant, growth and development including flowering cycle, productivity, fruit yield and quality, tolerance/ resistance to pests and diseases, as well as adaptation to the standard abiotic stresses during the growing season.

If *in vitro* plants prove to be superior materials with potential for increasing revenues to the farmers, a low cost *in vitro* mass propagation system will be adapted and transferred to the farmers by building their own capacity to multiply themselves the *in vitro* materials according to their needs.

References

Atkinson, R.. and Gardner R. 1993. Regeneration of transgenic tamarillo plants. Plant Cell Rep. 12: 347-351.

Hendrix, R., Litz R. and Kirchoff B. 1987. *In vitro* organogenesis and plant regeneration from leaves of *Solanum candidum*, *S. quitoense* (naranjilla) and *S. sessiliflorum*. Plant Cell Tissue Organ Cult. 11: 67-73.

Hussey, G and Stacey N. J., 1981. *In vitro* propagation of potato (*Solanum tuberosum* L.). Ann.Bot. 48: 787-796.

Hussey G. and Stacey. 1991. Factors affecting the formation of *in vitro* tubers of potato (*Solanum tuberosum* L.). Ann. Bot. 53: 565-578.

Lentini, Z., Mussell H., Mutchler M.A. and EARLE E.D. 1988. Ethylene generation and reversal of ethylene effects during development of *in vitro* rapid cycling *Brassica campestris*. Plant Sci. 54: 75-81.

Segovia V. 2002. Optimización de la regeneración de lulo *Solanum quitoense* orientada a la transformación genética de plantas. Universidad Internacional de Andalucía. Sede Santa Maria de la Rabida – Huelva. España. 74 p

Roca W.M. 1984. Cassava. In: Sharp W.R., Evans D.A., Amirato P.V. and Yamada Y. (eds.). Handbook of Plant Cell Culture. p. 269-301.

Table 1 Farmers participating in the project

Tierradentro Site	Years of experience	# cultivated plants	Pescador Site	Years of experience	# cultivated plants
Luis Humberto Inseca	2	5200	Pedro Nel Herrera	15	1000
Saul Salazar	4	3000	Hermes Vitelio Menza	15	900
Casimiro Salazar	3	2000	Wilson Moriones	15	50
Orlando Valverde	3	2000	Nacho Herrera	15	2000
José Javier Valverde	3	1200	Diomar Patiño	9	260
Eduardo R. Salazar	7	1000	Nelson Orozco	5	400
Samir Salazar	2	1000	Alejandro Murillo	2	700
Miguel Astudillo	3	700	Urbano Sanabria	2	1000
Jeison Salaza	2	700	Arístides Chacue	1	2000
José Lisandro Yonda	2	400			
Félix M Cuello	4	400			

Table 2.- Establishment of *in vitro* clones from the original donor plants clonally propagated by farmers in the field

Farmer	Entry code	Field clonally propagated plants	Greenhouse plants	*In vitro* explants introduced	*In vitro* explants established	Efficiency %	Total *in vitro* plants
Pedro Nel Herrera	PH- E1	3	10	59	45	76.3	241
Pedro Nel Herrera	PH- SI	2	10	26	22	84.6	139
Diomar Patiño	DP- E1	5	9	39	26	**66.7***	31
Diomar Patiño	DP- E2	7	10	57	40	70.2	92
Wilson Moriones	WM- E1	13	10	52	24	**46.2***	120
Hermes Vitelio Menza	VM- E1	2	10	12	11	91.7	109
Hermes Vitelio Menza	VM- E2	2	10	23	14	**60.9***	84
Saúl Salazar	SS- E2	4	5	14	13	92.9	116
Saúl Salazar	SS- E1	4	0	17	16	94.1	121
José Lisandro Yonda	JY- E1	10	10	24	24	100.0	138
Orlando y José Valverde	OJV- E1	10	10	34	33	97.1	122
TOTAL		**62**	**94**	**357**	**292**	**81.8**	**1,313**

Entry code: The letters correspond to the first and last name initials of the collector farmer, followed by either E (clone with thorns) or S (clone without thorns), and then by the number of clones collected by each farmer. A passport of each clone was created including all the descriptors chosen to select the materials. (*) Donor plants with phytosanitary conditions when brought from the farmer's field.

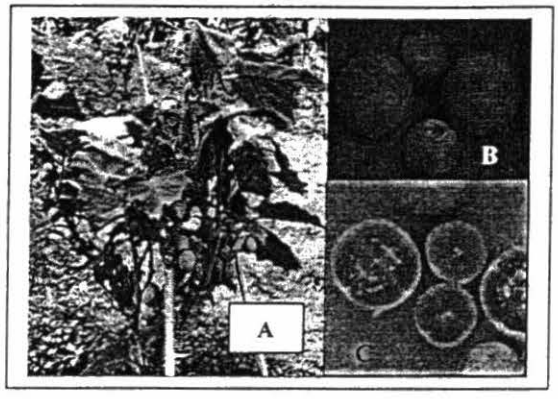

Figure 1. (A) Fruit setting in plants witht thorns. B) Fruits showing commercial and non-commercial sizes. (C) Dark green flesh that scored the highest acceptance by panelists in the quality trait assay.

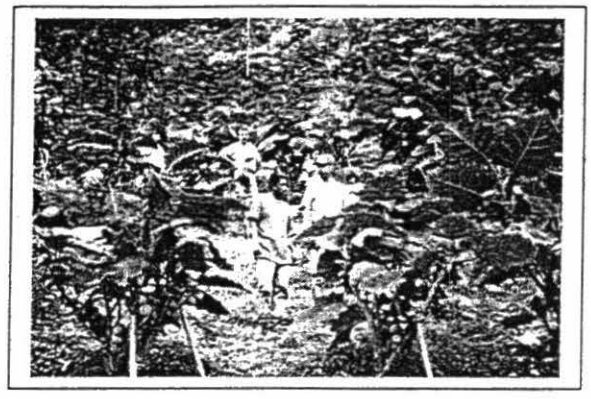

Figure 2. Selection of elite clones by farmers in the field..

CONTENT

ACTIVITY 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crops genetic base

2.2.1 Towards the Development of Tepary x Common Bean Interspecific Hybrid Lines, which are Cross compatible with Common Bean Cultivars and Competent to *Agrobacterium* Mediated transformation

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Introduction

Genetic transformation of plants through direct DNA delivery methods, including particle bombardment, are known to generate a number of rearrangements of the introduced foreign sequences, which include recombination between the introduced desired and undesired foreign DNA molecules, and insertions of multiple copies of the same in one or multiple loci (Somers and Makarevitch, 2004). This can led to the presence in the transgenic plants of undesired sequences of bacterial origin, including genes of antibiotic resistance, linked to the gene of agronomic interest. Such rearrangements may cause also the silencing of the desired agronomic important genes, or affect its correct expression. Their presence in a transgenic plant, that can be otherwise a success in the field, can hinder it to pass the biosafety evaluations, and reach the farmers or the markets. Contrary to direct transformation methodologies, *Agrobacterium* mediated transformation results in a higher proportion of "perfect" transgene loci with an integrated complete T-DNA and no damage to the recipient genomic DNA (Somers and Makarevitch, 2004). This transformation methodology is preferred today to produce "clean" transformed plants, which are expected to be commercialized.

The common bean (*Phaseolus vulgaris*), which has been recalcitrant to *in vitro* methodologies for plant regeneration, has only been genetically transformed by particle bombardment. *Agrobacterium* mediated transformation of this species has been attempted since the last 20 years without success (see review in Jacobsen 1999). Only in the related species tepary bean (*P. acutifolius*), the production of transgenic plants through *Agrobacterium* mediated transformation (Agro-transformation) has been possible (Dillen et al., 1997; Mejía-Jiménez et al. 2000).

Interspecific hybridization and gene introgression from tepary to common bean, although difficult to achieve, has been possible (see Singh 2001 for a review). The tepary bean has been proposed as a bridge species to introgress transgenes, introduced via *Agrobacterium*, to common bean. However, the only one genotype of tepary bean that has been competent to Agro-transformation, the wild accession NI576, has proven to be difficult to cross with

common bean. Besides, morphological marker genes such as the hypocotyl, flower and seed color, have been impossible to transfer (Mejía Jiménez *et al.* unpublished results). As an alternative of using NI576 as bridge, we are developing interspecific hybrid lines involving NI576 and other tepary and common bean genotypes selected for its *in vitro* culture response and Agro-transformation competence, to use them as bridge for gene transfer to common bean cultivars. During 2004 we advanced in the development of such hybrid-lines and produced advanced DCBC (Double congruity Backcross hybrids; see report of Mejía *et al.* on interspecific hybridization in this Annual Report), which are highly responsive to *in vitro* culture and amenable to Agro-transformation methods and, at the same time, cross compatible with common bean genotypes and cultivars.

Methodology

The screening of hybrids for improved competence to *Agrobacterium* mediated transformation is being carried out by applying the AMMSM-transformation methodology developed previously. In short, this methodology consists in the transformation of meristems found in mature seeds using *Agrobacterium* (Mejía-Jiménez et al. 2000 and 2002).

For developing common x tepary bean hybrid lines competent to *Agrobacterium* mediated transformation, a breeding program started in 2000. The program included the use of the genotype NI576 of tepary bean as source of the trait(s) for competence to Agro-transformation (Dillen et al. 1997), and other common and tepary bean genotypes selected on the basis of their response to morphogenic callus induction and plant regeneration. Because crossing of NI576 with common bean genotypes was not possible through different backcross methodologies, previously produced CBC hybrids (Congruity Backcross Hybrids; Haghighi and Ascher 1988) were used as bridge, and a novel backcross strategy was initiated (Double Congruity Backcrosses, DCBC). In contrast to the CBC, the DCBC are carried out in both, tepary and common bean cytoplasm and not genotypes of each species are alternately crossed but the most advanced fertile interspecific hybrids are used instead.

For improving the response to *in vitro* methodologies for plant regeneration and competence to Agro-transformation, the best hybrids identified after screening hundreds of lines of both cytoplasmic backgrounds, were intercrossed or crossed against the most advanced DCBC lines of the same or the opposite cytoplasm.

Results

Screening Advanced DCBC Hybrid-lines for improved in vitro culture response and competence to Agrobacterium Mediated Genetic Transformation

The Agro-transformation of tepary bean has been possible through the use of green nodular calluses produced from pedicel explants (Dillen et al., 1997), or by introducing directly the

transgenes to cells in meristematic zones present in mature seeds (AMMSM transformation; Mejía-Jiménez et al. 2000). During the development of both methodologies only the genotype NI576, or intraspecific hybrids involving it (G40065xNI576 and G40022xNI576), produced transgenic, regenerable, meristematic calli and plants. Experiments performed with hybrids involving the genotype NI576 as male parent, suggested that nuclear genes of it were responsible for the competence of hybrids to Agro-transformation. Since the stable introgression of tepary bean alleles into common bean has been possible, we are trying to transfer the genes for competence to Agro-transformation to common bean genotypes or common x tepary interspecific hybrid lines, through sexual crosses.

The genotype NI576 could not be crossed directly to common bean. Only after developing the DCBC backcross methodology, fertile common x tepary hybrids, involving NI576 could be produced. Several fertile hybrid generations have been produced that were screened during 2003 and 2004 for improved *in vitro* culture response, through the production of a long term cultivable meristematic callus (m-callus) and Agro-transformation competence (transient and stable GUS gene expression, and eventually transgenic plant regeneration). The best lines selected during 2003 were used to produce the lines tested during 2004.
The DCBC crossing experiments are producing hybrids with a high variability, as can be observed in their morphology, fertility, response to Agro-transformation, ability to produce a morphogenetic callus that can be cultured for longer periods *in vitro*, and also crossing compatibility to other hybrids of the opposed cytoplasm (for example V-DCBC x A-DCBC crosses).

During the last 12 months a total of 277 hybrid lines, 129 with the cytoplasm of common bean, and 148 with the cytoplasm of tepary bean were screened for competence for AMMSM transformation.Only 0.31% of the V-DCBC, and 0.66% A-DCBC explants produced m-callus after selection. None of the V-DCBC lines selected survived *in vitro* culture for more than 3 months, while 49 of the selected callus lines from A-DCBC hybrids survived longer. Some lines like BWZ-2F and BWG-10A1 produced m-callus with outstanding characteristics, prolific bud production and survival efficiencies higher than 33% (highlighted in the Table 2).
Once again, the hybrids with tepary bean cytoplasm clearly produced the calli with the best morphology and capacity to withstand longer in vitro culture, and which differentiated buds more prolifically.

Table 1 Results of the AMMSM-transformation experiments of DCBC-hybrid lines with the cytoplasm of *P. acutifolius* and *P. vulgaris*, performed between October 2003 and September 2004.

Cytoplasmic Background Hybrids	Number of Hybrid Lines Screened	Number of initial Explants Screened(?)	Average number of Explants per Line	# Lines Producing M-Calli (m-calli?) after selection[1]	Efficiency-1[2]	Efficiency-2[3]	% of lines with surviving M-Calli[4]
P. vulgaris	129	3260	25.3	10	0.31	7.7	0
P. acutfiolius	148	5908	39.9	39	0.66	26.3	12.1 (18)
TOTAL	277	9168	33.1	49	0.53	17.6	6.5 (18)

[1] Number of lines producing meristematic calli resisting selection with 50 mg/l geneticin for 1 month
[2] % of Inoculated Explants Producing M-Calli resisting selection with 50 mg/l geneticin for 1 month
[3] % of transformed lines which produced at least one M-Callus resisting selection with 50 mg/l geneticin for 1 month
[4]% of screened hybrid lines which produced geneticin resisting calli that withstood more than three months of culture *in vitro* after selection

It is not yet clear how many of the selected lines are transgenic, since we experienced problems detecting the GUS gene expression in small, meristematic callus tissue. In one out of nine callus lines analyzed with Real-Time PCR® the amplification of the npt II transgene was observed.
The screening performed during 2004 was useful to identify highly responsive genotypes best fitted for induction and survival of morphogenetic callus and possibly with competence to Agro-transformation. However, these experiments showed also that the efficiency achieved with the AMMSM –Transformation methodology continues to be low. It would be worth to test other transformation methodologies, as the recently optimized green nodular callus transformation developed in Gent, Belgium (Clercq 2002), with our best interspecific hybrid lines.

Development of New Populations of Advanced DCBC Hybrids involving other DCBC Hybrids as Parentals selected for their Improved response to *in vitro* culture and competence to Agro-Transformation

A low number (7 of 501) of the inoculated explants of the intraspecific tepary bean hybrids (G40022 x NI576 or G40065 x NI576) produced transgenic callus after the selection (Mejía et al. 2002), which suggests that the trait for Agro-Transformation competence, present in NI576, is likely to be multigenic. It is unlikely that a multigenic trait can be transferred to a DCBC hybrid in a single cross. Several cycles of DCBC may be necessary in order to accumulate different alleles involved in such a complex response in one genotype. Eight new fertile hybrid generations were produced (4 A-DCBC and 4 V-DCBC) during 2004 involving DCBC-hybrids of both cytoplasmic backgrounds as parentals, selected for increased competence to Agro-transformation. It is expected that

these lines will possess an improved *in vitro* culture response and Agro-transformation competence.

Conclusions

It is unlikely that direct transformation methodologies, such as particle bombardment, will produce the clean transformants that are needed for the development of transgenic common bean cultivars that will reach the poor small-scale farmers of the tropics. Also the use of tepary bean as bridge to transfer Agro-transgenes to common bean seems to be applicable, but promises very limited success. Results achieved during 2004 supports the hypothesis that it is possible to breed interspecific common x tepary bean hybrids which are competent to *Agrobacterium* mediated transformation, and which can be efficiently crossed to common bean genotypes . This kind of hybrids would represent a valuable tool for the production of clean transgenic common bean cultivars.

Table 2. DCBC hybrid lines which yielded morphogenetic (meristematic) calli after AMMSM-transformation and efficiency of recovery and survival of geneticin resistant meristematic calli.

Hybrid Code/ Generation[1]	Cytoplasm[2] and Hybrid Generation	Agrob. Strain[3]	Level of Transient GUS Expression[4]	Level of M-Callus Induction[5]	Inoculated and selected explants	# of *Geneticin* Resisting M-Calli[6]	# of M-calli *Surviving* and Differen-tiating[8]	% of M-calli Survival and Differen-tiation[9]
BWG-22A	A-DCBC$_{12B}$	I/II	---	13/13	13	3	1	7.7
BWG-17B	A-DCBC$_{12B}$	I/II	+++	14/14	14	2	1	7.1
BWG-10A	A-DCBC$_{12B}$	I/II	+++	65/96	65	20	1	1.5
BWG-25B	A-DCBC$_{12B}$	I/II	+++	58/58	58	17	3	5.2
BWG-25A	A-DCBC$_{12B}$	I/II	+	89/149	89	10	4	4.5
BWG-104	A-DCBC$_{12B}$	I	+++	40/42	48	1	1	2.1
BWZ-2F	A-DCBC$_{12B}$	I	+++	30/30	30	18	14	46.6
BWZ-3FL	A-DCBC$_{12B}$	I-II		22/25	22	1	1	4.5
ZXX-130002	A-DCBC$_{8D}$	I-II		19/19	19	10	1	5.2
BWG-22A1	A-DCBC$_{12B}$	I-II	+++	18/18	18	13	1	5.5
BWG-10A1	A-DCBC$_{12B}$	I-II	+	27/27	27	11	9	33.3
BWG-19A8B	A-DCBC$_{12B}$	I-II	+	53/53	53	10	1	1.8
BWG-19A8C	A-DCBC$_{12B}$	I-II	++	35/35	35	10	1	2.8
BWG-13A8A	A-DCBC$_{12B}$	I-II	++	25/25	25	9	1	4.0
BWG-13A11A	A-DCBC$_{12B}$	I-II	+++	28/30	28	7	3	10.7
BWTZBB-2M	A-DCBC$_{14B}$	I-II	+++	32/32	32	10	1	3.1
BWTZBB-2U	A-DCBC$_{14B}$	I-II	+++	45/49	45	10	5	11.1
Total						162	49	

1. For understanding the pedigree of the lines see Table DCBC Hybrids http://gene3.ciat.cgiar.org/blast/inicio.htm
2. Cytoplasm of the DCBC Hybrids; A = *P. acutifolius*; V = *P. vulgaris*
3. *Agrobacterium* strains: I = C58C1 pTARC; II = AGL1 pC3200
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 inoculated.
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 inoculated.
8. Number of M-calli with differentiating buds, which survived for more than three months after selection.
9. % of the inoculated explants which produced surviving and differentiating m-calli three months after selection.

Future Plans

- Lines of the most advanced A-DCBC hybrids produced during 2004, showing also the highest crossing efficiency with *P. vulgaris* genotypes, will be selected and the transformation methodology optimized for these lines to produce stable lines competent to Agro-transformation which are cross compatible with common bean.

- It will be further attempted to develop V-DCBC lines (hybrids with the cytoplasm of common bean) competent to Agro-transformation through further DCBCs with the best A-DCBC lines.

- Transgenes of agronomic importance will be included in separate plasmids and *Agrobacterium* strains in the transformation experiments, in order produce transgenic lines with traits of interest, while the transformation methodologies are optimized.

References

Clercq, J. De, M. Zambre · M. Van Montagu W. Dillen and G. Angenon (2002).An optimized Agrobacterium- mediated transformation procedure for Phaseolus acutifoliusA. Gray;Plant Cell Rep 21:333–340

Dillen W, De Clercq J, Goossens A, Van Montagu y M, Angenon G. Agrobacterium-mediated transformation of Phaseolus acutifolius A. Gray. Theoretical and Applied Genetics 1997; 94 (2): 151-158.

Jacobsen, H.J. (1999). Genetic Transformation. In: Common Bean Improvement in the Twenty-First Century. Edited by Shree P. Singh Developments in Plant Breeding 7. 420 pp. ISBN 0-7923-5887-

Mejía Jiménez, A., L. Galindo and J. Tohme. Towards the development of efficient genetic transformation protocols in common bean Annual report 2002. Agrobiodiversity and Biotechnology Project. CIAT

Mejía-Jiménez, A., Galindo, L.F., Hassa, A., Jacobsen, H.J. and Roca, W.M. Genetic transformation of Phaseolus beans. Annual report 2000. Biotechnology Research Unit-CIAT

Mejia-Jimenez, A., H. J. Jacobsen and W. M. Roca (1998). Development of an in vitro regeneration system in common bean suitable for genetic transformation. EUCARPIA International symposium on breeding of protein and oil crops, Pontevedra - Spain 1-4 April.

Mejía-Jiménez, A., Muñoz, C. Jacobsen, H.J., Roca, W.M. and Singh, S.P. 1994. Interspecific hybridization between common bean and tepary bean: Increased hybrid embryo growth, fertility, and efficiency of hybridization through recurrent and congruity backcrossing. Theor. Appl. Genet. 88: 324-331.

Singh, Shree P: Crop Science 41 (2001):1659–1675. Broadening the Genetic Base of Common Bean Cultivars: A Review

2.2.2 Successful crossing of hybrids carring desirable traits of common and tepary bean through Double Congruity Backcrossing

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Introduction

Additionaly to the many traits found in the tepary bean (*Phaseolus acutifolius*) that are important for common bean breeding (for a review see Singh 2001), this species has been the only one in the genus *Phaseolus* in which transgenic plants have been produced through *Agrobacterium* mediated genetic transformation (Agro-transformation; Dillen et al., 1997; Mejía-Jiménez et al. 2000). Interspecific hybridization between the tepary and common bean, although difficult to achieve, has been possible through the use of facilitator genotypes of both species, embryo rescue, and recurrent and congruity backcross methodologies (Waines et al. 1988; Haghighi and Ascher, 1988; Mejía-Jiménez et al. 1994). The introgression of tepary bean alleles into common bean, including traits of agronomic importance, has been demonstrated (Scott and Michaels 1992; Mejía-Jiménez et al. 1994; Singh and Muñoz 1999). Due to this, the use of NI576, a wild tepary bean genotype, competent to Agro-transformation, has been proposed as bridge for the production of transgenic cultivars of common bean by transferring the transgenes through sexual crosses (Dillen et al. 1997).
For transferring the traits responsible for Agro-transformation competence to the common bean, the genotype NI576 was included in year 2000 in an ongoing interspecific hybridization program (Mejía-Jiménez 2000). Since the genotype NI576 resulted incompatible with common bean through recurrent or congruity backcrosses, it was necessary to develop a novel backcross strategy called double congruity backcrosses (DCBC; Mejía-Jiménez et al. 2000).
During 2004 the DCBC strategy continued using advanced DCBC hybrids of both cytoplasmic backgrounds as parentals, selected for their response to *in vitro* culture and genetic transformation methodolgies, and for resistance to insects. This resulted in the production of vigorous and cross-fertile interspecific hybrids showing introgression of tepary bean alleles.

Methodology

The DCBC strategy is described in http://gene3.ciat.cgiar.org/blast/inicio.htm (Mejía-Jiménez et al. 2000). Fifteen new backcrosses to hybrids with the cytoplasm of common bean (V-DCBC hybrids), five of which were interspecific (V-DCBC x A-DCBC hybrids), were performed during 2004 (Table 1). Also seven new backcrosses were performed to hybrids with the cytoplasm of tepary bean (A-DCBC Hybrids), two of which were interspecific (A-DCBC x V-DCBC; Table 2). In general, an interspecific DCBC generates self-sterile hybrids, which have to be backcrossed with a genotype or hybrid of its own cytoplasm to produce fertile progeny. The as parentals of the backcrosses were tepary or common bean genotypes or advanced V-DCBC or A-DCBC hybrids selected for competence to *Agrobacterium* mediated transformation. Also A-DCBC hybrids identified by the project PE-1 of CIAT as resistant to bruchids (*Acanthoscelides obtectus;* Valor *et al.* 2003) or tolerant to leafhopper (*Empoasca kraemeri*; Bueno *et al.* 2003) were included (for the complete pedigree of the hybrids and references, see the table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/inicio.htm)
All embryos resulting from interspecific DCBCs and from most of the intraspecific DCBCs, were rescued and cultured *in vitro.* All plants resulting from a backcross on fertile hybrids (interspecific DCBCs), were verified as hybrids by the presence of morphological markers derived from the male parent (hypocotyl and flower color or primary leaf petiole size).

Results

Breeding interspecific tepary x common hybrid lines that are competent to *Agrobacterium* mediated transformation and cross compatible with common bean genotypes or cultivars.

The probabilities of success in the use of tepary bean accession NI576 as a "bridge" for the introgression of transgenes introduced via *Agrobacterium* into common bean cultivars, as has been proposed (Dillen *et al.* 1997), depends on the efficiency of transformation methodology itself, on the hybridization efficiency of the transformed genotype with common bean and on the amount of introgression of tepary bean genes into the common bean that can be achieved through the different backcross methodologies. The transformation efficiency reported with this genotype was initially low (Dillen *et al.* 1997; Mejía Jiménez et al. 2000 and 2002), but has been improved considerably (Clercq 2002). However producing cross-fertile hybrids from hybridizations between common bean cultivars and NI576 (using NI576 as male parent) has been impossible in our hands. In addition gene introgression between common and tepary bean has been shown recently to be lower than expected (an average of 5.2% in recurrent backcrosses and 8.8% in congruity backcrosses; Muñoz *et al.* 2004). Thus, this approach seems to be applicable, but promises very limited success.
As an alternative, we are working in the development of hybrid lines between common and tepary bean that are competent to Agro-transformation and cross compatible with common

bean cultivars. We produced fertile complex hybrids involving NI576 with the tepary bean cytoplasm, which we hybridized with CBC hybrids with common bean cytoplasm, for producing fertile and cross-fertile plants, carrying alleles of NI576. Since the trait that we are trying to transfer is likely to be multigenic, we started a novel backcross methodology, which is expected to maximize the level of introgression of the desired traits.
For accumulating alleles involved in competence to Agro-transformation, we screened each fertile population developed, selected the best responding hybrids and then used them as parentals in the next DCBC (see report on bean genetic transformation). We are aiming at improving both, crossability between hybrids of both cytoplasms, and competence to Agro-transformation.
Most of the DCBC hybrids produced during 2004 involve in their pedigree, lines selected for improved competence for Agro-transformation (Tables 1 and 2)

Using advanced DCBC hybrids for crossing other "incompatible" genotypes of tepary bean with common bean for introgressing resistance to insects

Additionally to genotype NI576 other genotypes of tepary and common bean and of other *Phaseolus* species have been included in the DCBCs.
Several DCBC hybrid populations with the cytoplasms of common and tepary bean have been evaluated by the IP-5 project for insect resistance. Some of them have proven to be resistant to bruchids (Valor *et al.* 2003), and *Empoasca* (Bueno et al 2003).

Table 1. New DCBC (Double congruity backcross) hybrid progenies with the cytoplasm of common bean (V-DCBC) developed during 2004. A-DCBC=DCBC with the cytoplasm of tepary bean. The shaded boxes correspond to interspecific DCBC (V-DCBCxA-DCBC). (For the complete pedigree of the hybrids see the table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/inicio.htm).

Cross Abbreviation/ Hybrid Code	Female Parent/ Code	Male Parent/ Code	Verification of Hybrids	Nr. of Fertile or Cross-Fertile Hybrids
V-DCBC$_{8E}$ TZTTZ	V-DCBC$_{7A,-8A,-8B,-8C}$ AT7, TZTA, TZTE, TZT	V-DCBC$_{7A,-8A,-8B,-8C}$ TZT, TZTU, TZTA, TZTE,	Red hypocotyl and flower color from the male parent	127
V-DCBC$_{8E}$ TSC	V-DCBC$_{8C,-8D}$ TZTA, TZTTZ	V-DCBC$_{7A,-7B,-8C}$ A36Y, EMPZ, TZTE	Seed color from the male parent	129
V-DCBC$_{8G}$ ST	G12922A x DOR364	V-DCBC$_{8D}$ TZTTZ	Red hypocotyl and flower color from the male parent	6
V-DCBC$_{9A}$ TZTEB	V-DCBC$_{8C}$ TZTE	A-DCBC$_{12A,-12B}$ BW, BWZ	Red hypocotyl and flower color from the male parent	2
V-DCBC$_{9B}$ STZ	V-DCBC$_{8G}$ ST	A-DCBC$_{8D}$ *ZX99-15.2.14A*	Red hypocotyl and flower color from the male parent	1
V-DCBC$_{10A}$ R	V-DCBC$_{9A}$ TZTEB	V-DCBC$_{-8E}$ TZTTZ, TZTEU	Female parent self-sterile	6
V-DCBC$_{10B}$ RR, RT	V-DCBC$_{10A}$ R	V-DCBC$_{8D,-10A}$ TZTTZ, R	Female parent self-sterile	54
V-DCBC$_{10D}$ RTU	V-DCBC$_{10B}$ RT	V-DCBC$_{8D}$ TZTU	Scarlet flower color from the female parent	48
V-DCBC$_{10D}$ RS	V-DCBC$_{10A}$ R	G12922AxDOR364	Red hypocotyl and flower color from the male parent	21
V-DCBC$_{10E}$ STR	V-DCBC$_{8G}$ ST	V-DCBC$_{10c}$ RTU	Scarlet flower color from the female parent	7
V-DCBC$_{11A}$ RG-3	V-DCBC$_{10A}$ R	A-DCBC$_{12A,-14A,-14B}$ G102BB	Small primary leaves	9
V-DCBC$_{11B}$ STRB	V-DCBC$_{10D}$ STR	A-DCBC$_{14C}$ BB, GB	Red hypocotyl and flower color from the male parent	12
V-DCBC$_{12A}$ RGR	V-DCBC$_{11A}$ RG-3	V-DCBC$_{10B}$ RR	Female parent self-sterile	3
V-DCBC$_{12B}$ RGRR, RGRT	V-DCBC$_{12A}$ RGR-1	V-DCBC$_{8F,-10D}$ RTU-43, TSC-19	Female parent self-sterile	7
V-DCBC$_{13}$ STRB, STRG, RGRTC	V-DCBC$_{12A}$ RGRT, STR	A-DCBC$_{8F,-16B}$ BB, GB, CWB	Red hypocotyl and flower color from the male parent	12
TOTAL				444

The identified resistant hybrids, and also other sources of resistance from tepary bean (G40019 and G40036 for *Empoasca* and G40199 for *Acanthoscelides obtectus* resistance) were then included in subsequent DCBCs. Fertile hybrids of both cytoplasmic backgrounds have been produced and made again available to the project IP-5 for evaluation.

The results achieved with these lines can be read in the annual reports of the IPM project.

During the second half of 2004 especially vigorous and cross-fertile hybrids involving A-DCBC lines resistant to *Acanthoscelides* could be produced with high efficiency. Thus, possibly for 2005 new fertile V-DCBC hybrids carrying resistance alleles to bruchids will be available.

Table 2. New DCBC (Double congruity backcross) hybrid progenies with the cytoplasm of tepary bean (A-DCBC) developed during 2004. V-DCBC=DCBC with the cytoplasm of common bean. The shaded boxes correspond to interspecific DCBC (A-DCBCxV-DCBC). (for the complete pedigree of the hybrids see the table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/inicio.htm).

Cross Abbreviation/ Hybrid Code	Female Parent/ Code	Male Parent/ Code	Verification of Hybrids	Nr. of Fertile or Cross-Fertile Hybrids
A-DCBC$_{13A}$ BWTZ	A-DCBC$_{12A}$ BW	V-DCBC$_{8C,-8D}$ TZTK, TZTE, TZTU	Red hypocotyl and flower color from the male parent	2
A-DCBC$_{14A}$ BWTZB	A-DCBC$_{13A}$ BWT	A-DCBC$_{12B}$ BWG, BWZ	Female parent self-sterile	2
A-DCBC$_{14B}$ BWTZBB, BWTZBG	A-DCBC$_{14A}$ BWTZB	A-DCBC$_{8B,-12A,-12B}$ GNVAV, GKX, BWG, SCO	Red hypocotyl and flower color from the male parent;	25
A-DCBC$_{14C}$ GB, BB, BG	A-DCBC$_{14A}$ GNVAV, GVV, BWTZB	A-DCBC$_{8B,-12B,-14A,-14B}$ BWTZBB, BWTZBG, BWTZB, GKX, BWG	Red hypocotyl and flower color from the male parent	83
A-DCBC$_{15}$ BS, BR	A-DCBC$_{14A,-14B}$ BWTZB, BWTZBB	V-DCBC$_{10C,-8F}$ RS, ST	Red hypocotyl and flower color from the male parent	19
A-DCBC$_{16A}$ CW	A-DCBC$_{15}$ BS, BR	A-DCBC$_{-14B}$ BWTZBB, BWTZBS	Female parent self-sterile	2
A-DCBC$_{16B}$ CWB	A-DCBC$_{16A}$ CW	A-DCBC$_{12B,-14B,-14C}$ BWZ, BWTZBB	Red hypocotyl and flower color from the male parent or emale parent self-sterile	17
Total				150

Conclusions

Double congruity backcrosses and advanced A-DCBC and V-DCBC hybrids used as bridge, are helping to overcome crossing incompatibility existing between genotypes of common and tepary bean carrying important traits [competence to Agro-transformation, resistance to bruchids (*Acanthoscelides obtectus)* and leafhopper (*Empoasca kraemeri*)]. The hybrid populations produced may be a source for other desirable traits of tepary bean, for example resistance to drought and heat.

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 and *Empoasca,* or *A. obtectus* resistance.

- To study the introgression of DNA fragments, morphological and biochemical markers from the genotypes and species involved in the DCBCs in the different hybrid populations, using AFLP and other techniques.

References

Bueno, JM, C. Cardona, A. Mejía, J. Tohme. Annual Report 2003-Project PE-1. CIAT. www.ciat.cgiar.org/ipm/report2003/ipm_2003_04.pdf

Clercq, · J. De, M. Zambre · M. Van Montagu W. Dillen and G. Angenon (2002)An optimized Agrobacterium-mediated transformation procedure for Phaseolus acutifoliusA. Gray;Plant Cell Rep 21:333–340

Dillen W, De Clercq J, Goossens A, Van Montagu y M, Angenon G. *Agrobacterium*-mediated transformation of Phaseolus acutifolius A. Gray. Theoretical and Applied Genetics 1997; 94 (2): 151-158.

Haghighi, KR. and PD. Ascher. Fertile, intermediate hybrids between *Phaseolus vulgaris* and *P. acutifolius* from congruity backcrossing. Sexual Plant Reproduction 1 (1988): 51-58.

Mejía Jiménez, A., L. Galindo and J. Tohme. Towards the development of efficient genetic transformation protocols in common bean Annual report 2002. Agrobiodiversity and Biotechnology Project. CIAT

Mejía-Jiménez, A., Galindo, L.F., Hassa, A., Jacobsen, H.J. and Roca, W.M. Genetic transformation of *Phaseolus* beans. Annual report 2000. Biotechnology Research Unit-CIAT

Mejía-Jiménez, A., Muñoz, C. Jacobsen, H.J., Roca, W.M. and Singh, S.P.. Interspecific hybridization between common bean and tepary bean: Increased hybrid embryo growth, fertility, and efficiency of hybridization through recurrent and congruity backcrossing. Theor. Appl. Genet. (1994) 88: 324-331.

Singh, Shree P. and Carlos G. Muñoz. Resistance to Common Bacterial Blight among Phaseolus Species and Common Bean Improvement. Crop Science 39 (1999): 80-89.

Singh, Shree P: Crop Science 41 (2001):1659–1675. Broadening the Genetic Base of Common Bean Cultivars: A Review

Valor, JF. C. Cardona, A. Mejía, S. Beebe, and J. Tohme. Annual Report 2003-Project PE-1. CIAT. www.ciat.cgiar.org/ipm/report2003/ipm_2003_04.pdf

Waines, J.G., R.M. Manshardt, and W.C. Wells. 1988. Interspecific hybridization between Phaseolus vulgaris and P. acutifolius. *In* P. Gepts (ed.) Genetic resources of Phaseolus beans. Kluwer, 940–946. Dordrecht, the Netherlands.

2.2.3 Comparing solid and liquid media for the induction of somatic embryos in cassava

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Introduction

The induction of somatic embryos in cassava *Manihot esculenta*, Crantz, has been generally carried out on solid media using lobes of immature leaves, axillary buds and apexes with acceptable results as for quality and quantity. The time of induction oscillates between 15 to 30 days. The following report summarizes the findings on the effect of the physical state of media, solid or liquid, and the explant type, buds and immature leaves, on the production of somatic embryos in eight cassava genotypes.

Materials and methods

The cassava genotypes used for the induction of somatic embryos were: 60444 (MNig11), MTAI 8, MCOL 2215, CM 3306-4, CM 523-7, MPER 183, CG 489-31 and CM 6740-7, from the *in vitro* bank of the International Center of Tropical Agriculture – CIAT. They were propagated *in vitro* from shoots and buds in 4E: MS salts (Murashige and Skoog,1962), sucrose 20 g/l, thiamine-HCl 1 mg/l, M-inositol 100 mg/l, BAP 0.04 mg/l, GA_3 0.05 mg/l, NAA 0.02 mg/l, CuSO4 2µM and agar (Duchefa) 4.5 g/l (Roca et al, 1984).

Immature leaves and axillary buds from approximately two months old plants were used for the induction of embryos. Young leaves were placed directly on the solid media, while the nodal segments, that contained the buds, were grown for two to three days on 4E before removing the buds. The media for the induction of embryos was MS4: MS basal salts, vitamins B5 (Gamborg et al, 1968), sucrose 20 g/l, casein hidrolysate 50 mg/l, $CuSO_4$ 2µM, Gel-rite 2g/l, 4 mg/l (18 M) of 2,4-D, at pH 5.6-5.7. Solid medium was served in 25 ml petri dishes. Glass vessels were used to pour 5 ml of liquid medium, which formed a film at the bottom of the vessel, on top of which explants were placed. This system was designated Liquid Film Stationary Culture. It was kept still during embryogenesis. For solid media, 20 explants were placed by petri dish, while in liquid media 10 explants were placed by glass vessel. The environmental conditions of the induction went from 26 to 31°C, in darkness for 30 days. The variable evaluated was percentage of explants that generated somatic embryos for each genotype.

Results and discussion

The embryogenic potential of the evaluated genotypes was variable, and basically depended on the genotype. The type of explant, and the physical state of media (solid vs liquid) also seemed to have an effect in embryogenesis. Table 1 condenses the results, in

terms of percentage, for the induction of somatic embryos in liquid or solid media, from buds or leaves, in eight genotypes.

Table 1. Comparison between solid and liquid medium, and buds or leaves as explants, for the induction of somatic embryos in eight cassava genotypes. As an average, buds responded better in liquid medium; although there were exceptions with two clones were 70% or more of the leaves produced embryos

	SOLID		LIQUID
Genotypes	Explant: Buds	Explant: Leaves	Buds
60444	79/223 (35.4%)	13/109 (12.0%)	155/230 (67.4%)
Mtai 8	19/111 (17.1%)	25/53 (47.1%)	85/110 (77.2%)
Mcol 2215	42/60 (70.0%)	20/41 (48.8%)	18/50 (36.0%)
CM 3306-4	94/120 (78.3%)	4/43 (9.3%)	81/130 (62.3%)
CM 523-7	70/120 (58.3%)	25/80 (31.2%)	84/120 (70.0%)
Mper 183	298/400 (74.5%)	36/133 (27.0%)	271/380 (71.3%)
CG 489-31	174/317 (54.8%)	21/123 (17.0%)	223/290 (76.9%)
CM 6740-7	152/275 (55.2%)	77/200 (38.5%)	206/275 (74.9%)
Average	55.45%	28.86%	67.0%

As an average, the liquid medium seemed to be better for the induction of embryos using buds as explants. Table 1 shows that five out of eight clones surpassed 70% of induction (numbers in red). However clones Mper183 and Mcol2215 also produced embryos above the 70% minimum on solid medium, when buds were used as explants. In general leaves were not as embryogenic as were buds, on solid medium. Leaves were not treated in liquid medium since preliminary experiments revealed that they become translucent, friable, non-embryogenic callus. Some leaves did not change at all, and many sunk, due to their inability to float.

One more advantage of the liquid medium was the reduction of time to induce embryos (20 days instead of 30 days on solid medium), and the quality of embryogenic tissues, which appeared cleaner, free of non-embryogenic callus that sunk into the medium, leaving the embryo cluster floating by itself. Furthermore liquid medium offers an economic advantage by reducing the consumption of media (1/5 of the volume of the solid media), and by not having to use agar and to disposable petri dishes.

Conclusions

The ability of cassava to produce embryos is genotype dependent, and it is influenced by the medium state, being liquid medium a better system to induce embryos from axyllary buds of most clones.

The induction of embryos on liquid medium offers advantages in terms of quality and quantity of embryogenic tissue, easiness of handling of tissues, and reduction of costs and time of induction.

Future activities

- Improve embryo to plant conversion in several cassava clones

References

Gamborg O. L.; Miller R. A.; Ojima K. (1968). Nutrient requiriments of suspension cultures of soybean root cells.Exp. Cell Res. 50: 151 – 158.

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassays with tabaco tissue cultures. Physiol. Plant 15: 473 – 497.

Roca, W.M., Rodriguez, J.A., Mafla, G., Roa, J.C. 1984. Procedures for recovering cassava clones distributed *in vitro*. CIAT, Cali, Colombia. 8p

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

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Introduction

Cassava contributes in a great deal to small farmer's food security and income at Africa, Asia, Latin America and the Caribbean, where it is one of the most important sources of calories. Its world output in 2002 is estimated at 184 million tones of fresh root equivalent (FAO). Such an important staple crop could play a major role in improving the living standards of poor farmers under an adequate breeding program. Traditionally, cassava has been produced only on small-scale family farms as a subsistence crop and for village markets, but it could develop as a major feed and industrial crop. However, its short post-harvest storage life is one of the major constraints towards achieving this goal. Its perishability is due to a post-harvest physiological deterioration (PPD) of cassava roots that begins within 24 hours after harvest. Thus the reduction of PPD has been identified as a priority target for strategic research.

The objective of this project is to identify the full set of genes involved in cassava PPD using cDNA microarrays to perform a genome wide expression analysis of this process. A PPD time-course and time-range experiment was designed as described earlier (Cortes) and a biological replica was hybridized. In order to identify gene expression differences less than 2-fold, which can also elicit meaningful biological effects, it is necessary to construct statistical tests on the basis of several measurements, which show a variance. In this sense it is very important to realize there are many sources of variation in a microarray experiment, and they can be partitioned in to biological and technical ones. If we are

interested in determining how a specific condition affects different biological populations represented in our samples, statistical tests should be based on the biological variance.significance (Churchill). This is why two additional biological replicates of the microarray hybridizations had to be carried out. In the mean time, analysis of the first biological replicate enabled the identification of a number of regulated clones, which were sequenced, and based on their putative function, a group of them was chosen to be confirmed with Northern analysis. Lastly, since PPD on cassava roots has been perceived as an unstopped wound response, a gene expression analysis comparing leaves and roots has been planed in order to identify root specific genes, and genes which are expressed during the healing of wounded leaves, and are not expressed during PPD response.

Methodology

PPD-related microarrays: Microarrays were made as reported earlier (Cortés). Briefly, clone spotting of the PPD-related cDNA library was carried out using the Hitachi SPBIO 1.55 system available at CIAT, obtaining 7 slides for each hybridization, where slides 1-5 are spotted with 7680 clones from early library, and slides 6–7 are spotted with 3072 clones from the late library. Every slide has 4 technical replicates of each library clone and of each control spot.

Microarray hybridizations: PPD was established on cassava roots from two individuals of CM2177-2 genotype as described by Wheatley *et al.* (1985), and samples were taken at 0, 12, 24, 48, 72 and 96 hours after harvest. Leaf tissue was collected from the same individuals and flash frozen in liquid nitrogen, or leaves were wounded and tissue was flash frozen after 24 hours. Total RNA was extracted from cassava roots and leaves (Chang). SV total RNA isolation system from Promega was used to eliminate DNA and clean total RNA. mRNA was isolated with Oligotex kit from QIAGEN. cDNA was PCR-synthesized using SMART system from Clontech. Root cDNA was labeled with Cy3 or Cy5 to perform two biological-replicas of the time-course hybridizations as described earlier (Cortés *et al.*, 2003). Microarrays were scanned using the VersArray Chipreader scanner from BioRad. Tagged Image File Formats for each slide of the second and third biological replicas were sent to Bath University (UK) for expression analysis.

Microarray and functional analysis: Microarray gene-expression pattern analysis is carried out at Bath University (UK) using ArrayVision, Cluster and Treeview software. A preliminary analysis based on data from the first biological-replica has all ready been done. Based on the preliminary analysis, clones that were up-regulated at least 2 times or down-regulated at least 2.8 times, in at least two time-point comparisons were transferred to Bath University and sequenced. Their putative function was established according to homologies found in the Gene Bank databases.

Northern confirmation: Based on the revision of the relevant literature of the regulated clones, a subgroup of them was selected to confirm their expression pattern over time, and to determine their expression pattern on leaves using standard Northern experiments.

Results

Data for two biological replicas of the time-course hybridizations is now available to perform a more biologically meaningful analysis of gene expression pattern a long PPD on cassava roots. So far, analysis of data from the first biological replica has enabled the selection of 114 up- and 70 down-regulated clones. These have all been partially sequenced and compared to sequence databases in order to establish their putative identity. Some clones were redundant as was expected from the random selection of clones from the cDNA library to construct the microarrays. These clones include genes with roles in: cell wall strengthening; glucosinolate biosynthesis; programmed cell death; control of transcription and translation; oxidative stress; signal transduction or perception; ion, water and metabolite transport; and of unknown function. Clustering analysis coupled to sequencing and homology search, allowed the grouping of up-regulated clones with a putative function according to their gene-expression pattern during PPD (Figure 1) in to "early genes", whose expression is augmented before 24 hours, before 48 hours, or at 72 hours, and "late genes" whose expression is augmented at 72 and 96 hours.

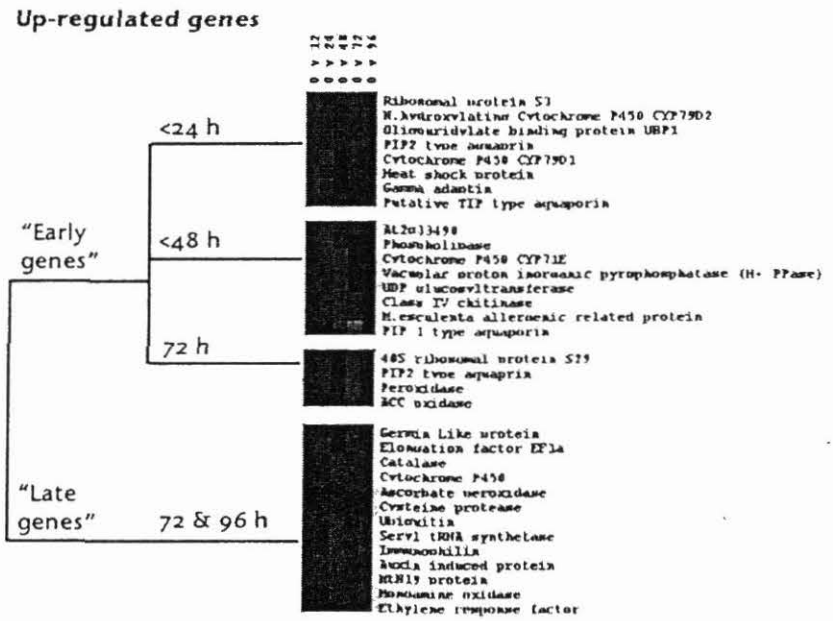

Figure 1. Clones organized in to clusters according to the moment (before 24, 48, 72 or 96 hours) when they are up-regulated during post harvest deterioration of cassava roots.

The same clustering and sequence analysis for down-regulated clones allowed their grouping as shown in figure 2, in to genes whose expression decreased before 12 hours or after 12 hours post-harvest.

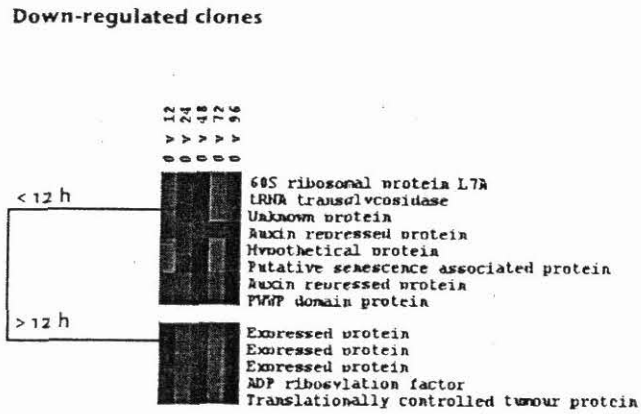

Figure 2. Clones organized in to clusters according to the moment (before 24, 48, 72 or 96 hours) when they are down-regulated during post harvest deterioration of cassava roots.

Taking in to account the temporal gene-expression pattern and functional information, the following regulated clones of *Manihot esculenta* (Mec) were selected to confirm their temporal gene expression pattern, and to asses their gene expression pattern in leaves using northern analysis at Bath University: aminocyclopropane-carboxilic acid oxidase (MecACCO2), auxin induced protein (MecAKR), ascorbate peroxidase 1, 2 and 3 (MecAPX1, MecAPX2, MecAPX3), auxin repressed protein 1 and 2 (MecARP1, MecARP2), catalase (MecCAT2), cysteine proteinase (MecCP1), ethylene response factor (MecER), germin-like protein (MecGLP), proline-tryptophan-tryptophan-proline protein (MecPWWP) and a Peroxidase (MecPX3). From these analysis we know a peroxidase is highly expressed in roots but not in leaves.

Current activities and future work

The corresponding genomic clone of the chosen sequences is being isolated from the cassava genomic library available at Bath University, in order to identify their promoters and other regulatory elements.

The time course hybridizations are being done on a microarray encompassing a unigene set of 6046 EST's of different tissues and cultivars of cassava (López).

Hibridizations of three biological replicas on the PPD and unigene cassava microarrays, comparing roots and leaves as follows:

T=0 root vs. T=0 leaf to identify root specific genes.
T=0 root vs. T=24 h wounded leaf (gently crushed leaves) to identify root specific genes that are not required for leaf wound/defence response.
T=24 h wounded root vs. T=24 h wounded leaf to identify any possible genes involved in root wound/PPD response but not in leaf wound response.

The genes corresponding to the chosen clones will be mapped via CAPs (Cleaved amplified polymorphisms) in the cassava molecular genetic map in order to see if there is any association with QTLs.

References

López, C., Mba, C., Cortés, D., Restrepo, S., Soto, M., Tohme, J., Verdier V. 2003. Generation and analysis of cassava ESTs: towards the identification of a unigene set. In: CIAT (Centro Internacional
de Agricultura Tropical). Assessing and utilizing agrobiodiversity through biotechnology. Annual report 2003, Project SB-2. Cali, CO

FAO: Food And Agricultural Organization of the United Nations Commodities and Trade. Economic and Social Department. June 2003 cassava market assessment.
http://www.fao.org/es/ESC/en/20953/21029/highlight_28829en.html

Churchill, G. A. ,2002. Review: Fundamentals of experimental design for cDNA microarrays. Nature Genetics Supplement, vol. 32, december 2002

Chang, S., Puryear, J., Cairney, A. 1993. Asimple and efficient method for isolating RNA from pine trees, Plant Mol. Biol. Rep. 11:113-116

2.2.5 Embryo Rescue of Sexual Seeds from BC_2 Families for Molecular Marker-Assisted Selection (MAS) for Resistance to Cassava GreenMites (CGM) and the Cassava Mosaic Disease (CMD)

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CIAT

Funding: Rockefeller Foundation, CIAT

Important Outputs

1) *In vitro* establishment of embryo axes of 1490 mature seeds derived from 43 BC_2 families, that combine resistance to the cassava green mites (CGM) and the cassava mosaic disease (CMD).
2) Shipment of progenies selected by MAS to Tanzania for use as parents in the RF cassava MAS project in Tanzania

Introduction

Embryo rescue of populations for molecular breeding for resistance to the cassava mosaic disease (CMD) was initiated last year for easy movement of this germplasm to partners in Africa and India. In the second phase of the MAS project at CIAT, resistance to CMD is to be combined with resistance to the cassava green mites (CGM) derived from a close

wild relative of cassava, *Manihot esculenta* sub spp *flabellifolia*. This germplasm is primarily to serve as parents for a MAS project to improve CMD and CGM in Tanzania funded by the Rockefeller Foundation, and for use in the CIAT breeding program. A total of 1490 seeds were obtained from more that 2000 controlled crosses between BC_1 progenies having excellent resistance to mites and CMD, and resistance parents at CIAT, 1291 genotypes were germinated by means of embryo rescue and propagated for MAS. Genotypes that were shown to have CMD and CGM resistance genes by MAS were further propagated and a set of 8-10 plants shipped to partners in Tanzania or transferred to the greenhouse here at CIAT for further field evaluation.

Methodology

Embryo rescue of 1490 mature seeds of cassava from 43 BC_2 progenies was done following a protocol developed earlier at CIAT (CIAT 2003). Briefly, seeds were put in a beaker of water and those that floated were discarded, viable seeds were soaked in 97% suphuric acid for 50 minutes followed by several washings with water to remove the acid. The seeds were then disinfected by soaking in 70% ethanol for 2 minutes followed by 0.5% sodium hiphoclorite for 12 minutes and three washings with sterile deionized water. The embryo axes with cotyledons were removed and placed on 17N medium. They were incubated in the dark for 5 days in the growth room and then exposed to light under photoperiod conditions of 12/12 hours night/darkness at a temperature of 27-30°C. The plantlets were coded "AR" to distinguish them from the CR series having only resistance to CMD. After 3-4 weeks of growth, leaf tissue was removed from individual plants for molecular analysis with markers associated with CMD and CGM resistance. Genotypes shown to carry CMD and CGM resistance were propagated and moved to the screen house or shipped to partners in Tanzania.

This year a different method of screen house hardening was used to increase percentage of survival of tissue culture plantlets. Briefly, plantlets were transferred to polyethelene bags filled with a mixture of soil and sand (3:1) prepared by washing three times with water, drying under the sun, sieving, and sterilizing for three hours by steam. A solution of Barot (0.7 gr/l in deionized water) was applied to the soil to prevent fungal attack especially *Phytophthora, Phytium,* and *Fusarium*. The transplanted seedlings were placed in deep plastic trays inside a big transparent plastic bag, to create an artificial high humidity chamber but still permit entrance of light, for 15 days. Water was applied as necessary. After 15 days the tray was removed from the bag and a fertilization plan started (micro and macronutrients were applied every 8 days until moved to the field). At one month the plants were watered every two days and at the three months they were carried to field. Plants at 60 and 90 days old with good foliage were transferred to a well-watered field and planted at a spacing of 0.8m between plants and 1.60m between rows.

Results

Of the 1490 seeds obtained from crosses this year 1291 were planted, 199 seeds were discarded as 'vain'. A total of 824 plantlets, or 64%, were successfully established (Table 1), a 10% increase over results obtained last year for genotypes having resistance to CMD, the "CR" series (CIAT, annual report 2003). Of the 824 plants evaluated by MAS, 335 genotypes were found to combine resistance to CMD and CGM the rest susceptible. Genotypes that were resistant were propagated and approximately 10 copies each per genotype was sent to partners in Tanzania, Nigeria, and transferred to the greenhouse/field at CIAT respectively. Other shipments this year were 207 CR

Table 1. Embryo rescue of AR genotypes from 43 BC_2 families

Code	Mother	Father	No. of seeds produced	No. of vain seeds	seeds planted	No. of plants obtained	% recovery of plants
AR-1	C-127	CW257-12	211	20	191	181	95%
AR-2	C-4	CW236-14	102	0	102	28	27%
AR-3	C-127	CW259-43	100	8	92	67	73%
AR-4	C-19	CW234-2	1	0	1	1	100%
AR-5	C-19	CW259-3	1	0	1	0	0%
AR-6	C-4	CW235-72	48	2	46	30	65%
AR-7	C-127	CW234-2	78	24	54	46	85%
AR-8	C-243	CW234-2	11	4	7	4	57%
AR-9	C-243	CW257-12	86	6	80	70	88%
AR-10	C-243	CW259-3	5	2	3	1	33%
AR-11	C-243	CW259-43	37	7	30	20	67%
AR-12	C-33	CW234-2	154	33	121	60	50%
AR-13	C-33	CW235-2	5	0	5	0	0%
AR-14	C-33	CW257-10	25	2	23	15	65%
AR-15	C-33	CW257-12	26	5	21	10	48%
AR-16	C-33	CW259-3	55	7	48	27	56%
AR-17	C-33	CW258-17	53	2	51	28	55%
AR-18	C-377	CW257-12	17	4	13	4	31%
AR-19	C-39	CW234-2	5	3	2	0	0%
AR-20	C-39	CW257-12	22	0	22	1	5%
AR-21	C-39	CW258-17	7	0	7	2	29%
AR-22	C-39	CW259-3	3	2	1	1	100%
AR-23	C-39	CW259-43	9	2	7	1	14%
AR-24	C-413	CW257-12	12	2	10	0	0%
AR-25	C-413	CW259-3	5	2	3	1	33%
AR-26	C-413	CW259-43	5	0	5	3	60%
AR-27	C-4	CW258-17	22	3	19	16	84%
AR-28	C-243	CW258-17	2	0	2	2	100%
AR-29	C-127	CW235-72	2	0	2	0	0%
AR-30	C-413	CW258-17	5	0	5	5	100%
AR-31	C-127	CW258-17	14	6	8	4	50%
AR-32	C-33	CW259-10	8	3	5	3	60%
AR-33	C-39	CW259-10	18	2	16	2	13%
AR-34	C-19	CW259-10	6	3	3	2	67%
AR-35	C-243	CW257-10	20	5	15	3	20%
AR-36	C-127	CW259-10	37	1	36	29	81%
AR-37	C-33	CW259-42	184	27	157	111	71%
AR-38	C-377	CW259-42	21	6	15	7	47%
AR-39	C-4	CW259-42	11	1	10	7	70%
AR-40	C-39	CW259-42	37	2	35	23	66%
AR-41	C-19	CW259-42	7	2	5	2	40%
AR-42	C-413	CW259-42	9	1	8	6	75%
AR-43	C-19	CW259-43	4	0	4	1	25%
TOTAL			1490	199	1291	824	64%

genotypes to Tanzania, 205 CR genotypes to Nigeria and 166 CR genotypes to India, of approximately 10 plants/genotype.

Conclusions and Perspectives

In vitro establishment of embryo axes of mature seeds derived from 43 BC_2 families that combine resistance to cassava green mites (CGM) and to the cassava mosaic disease (CMD) were conducted this year. An increase of 10% was obtained in recovery of full plants from embryo rescue. Plants shown to be resistant to CMD and CGM by MAS were shipped to Tanzania, Nigeria and transferred to the screen house/field. Pending are shipments of the AR and CR plants to Uganda and South Africa. Other activities planned this year are embryo rescue of 3000 seeds from the CR series and MAS for CMD resistance.

References

CIAT 2003. Annual Report IP3. Improved cassava for the developing world. Pp8-85 to 8-90

2.2.6 Dissemination of Improved Cassava Varieties and Management of Genetic Stocks as Tissue Culture Plantlets

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CIAT

Funding: CIAT

Important Outputs

1) More than 1,000 plantlets of improved cassava varieties were multiplied and shipped to partners in Meso America, South America, and Europe
2) A BC_1 mapping population and several wild and cultivated parental lines were established in vitro for safe guarding
3) One person from a Mexican National program, 2 students from the Universidad Nacional, Sede Palmira and one from CIAT partners were trained in tissue culture methods

Introduction

The vegetatively propagated nature of cassava makes tissue culture a key method for safe exchange of germplasm and for safe guarding materials in the field. Last year cassava tissue culture group received from the genetic resource unit (GRU) 37 improved varieties and partly assumed responsibility for dissemination these varieties to partners in Latin America, Asia and Africa. Other activities last year were the establishment of breeding and gene tagging populations as well as transfer of valuable genotypes in the field to *in*

vitro cultures. This year the group received 2 sets of the Core collection and several hundred other genotypes for the CIAT world germplasm collection for activities in the generation challege program. These materials were multiplied and eventually transferred to the screen house. Other activities carried out this year include dissemination of improved varieties of cassava to partners in Meso America, South America, and Europe, establishment as embryo cultures of 2 BC_1 gene tagging populations for protein content and the transfer *in vitro* of several genotypes with valuable traits. The group also carried training activities for partners from a National program, local University and CIAT in tissue culture methods this year.

Methodology

The core collection of the world cassava germplasm collection at CIAT is to be evaluated as part of activities in the challenge program. The core collection and more than 1000 accessions are to be genotyped with SSR markers this year. Between 2-4 *in vitro* plantlets per genotype of the above germplasm was received and multiplied to give at least 6-8 plants according to standard methods at CIAT (Roca et al. 1984). Between 4-6 plants were transferred to the screen house for hardening while 2 were kept *in vitro*. A group of 37 improved varieties held at the cassava tissue culture facility was also multiplied in 4E culture media for distribution to partners using CIAT's multiplication protocol (Roca et al. 1984).

Two BC_1 populations of between 200 and 300 progenies for gene tagging of protein content were established *in vitro* from embryo axes of mature seeds. The populations were obtained from crossing the high protein inter-specific hybrids CW205-4 and CW205-7 to MTAI8. Methods used for embryo rescue of mature cassava seeds was as described earlier (CIAT 2003). Wild accessions being used for the introgression of high protein, resistance to green mites, and high dry matter content from wild species to cassava often have problems of poor vegetative propagation. This year many of these wild parents were transferred *in vitro* for safe-keeping. They include: OW146-1, OW181-2, OW231-2, OW234-2, OW240-8, OW280-1, OW280-2, OW284-1, OW589-2, OW180-4, OW230-6 y CW429-1, MCra-013, OW240-6, OW181-2, OW235-3, OW230-3 and OW180-4. Shoot tips from plants growing the field or screen house were transferred to tissue culture as described earlier (CIAT 2003).

One person from a Mexican National program, 2 students from the Universidad Nacional, Sede Palmira and one from CIAT partners were trained in tissue culture methods. The training covered an introduction to equipments in the tissue culture facility and their proper use, small and large scale *in vitro* propagation, preparation of tissue culture media, necessary precautions and general safety in the laboratory, and tissue culture bibliography.

Results

Two sets of the core collection a total of 1260 genotypes and another 775 accessions were received from CIAT's GRU and successfully multiplied and 4-6 copies sent to the screen house for activities in the GCP. A total of 3506 plants from a list of 37 improved genotypes were shipped to collaborators in Nicaragua, Peru, Mexico and Austria this year (Table 1). A total of 160 sexual seeds out of 170 have been processed so far from more than 400 for 2 BC_1 gene tagging populations for protein content, embryo rescue of the other seeds are continuing. Of the 20 wild and inter-specific parents transferred *in vitro* this year only 12 were successfully established, the others had problems of contamination and poor growth. New attempts are being made using different media to transfer these

Table 1. Summary of shipments of *in vitro* plants of cassava (*Manihot esculenta* Crantz) made to several countries from February to September 2003

ITEM	GENOTYPE	NICARAGUA	AUSTRIA	PERU	MEXICO	TOTAL
1	CG 1141-1	30	-	30	64	124
2	CG 1450-4	30	-	30	-	60
3	CM 1335-4	-	10	-	-	10
4	CM 2177-2	30	-	30	55	115
5	CM 2600-2	30	-	30	-	60
6	CM 2766-5	30	-	30	-	60
7	CM 2772-3	-	10	-	-	10
8	CM 3306-4	-	10	30	-	40
9	CM 3750-5	-	10	-	-	10
10	CM 4574-7	-	10	-	-	10
11	CM 4843-1	-	6	-	-	6
12	CM 489-1	-	10	-	-	10
13	CM 4919-1	-	10	-	-	10
14	CM 507-37	-	10	-	15	25
15	CM 523-7	-	10	-	-	10
16	CM 5306-8	-	10	-	-	10
17	CM 6119-5	-	10	-	-	10
18	CM 6438-14	-	10	-	-	10
19	CM 6740-7	-	10	-	-	10
20	CM 6754-8	-	6	-	60	66
21	CM 6921-3	-	10	-	-	10
22	CM 7033-3	-	10	-	-	10
23	CM 7073-7	-	10	-	-	10
24	CM 7514-8	-	10	-	-	10
25	CM 7951-5	-	10	-	-	10
26	CM 8027-3	-	10	-	-	10
27	HCM 1	30	10	30	39	109
28	MBRA 383	30	10	-	-	40
29	MCOL 1468	-	10	-	-	10
30	MCOL 1505	-	-	-	20	20
31	MCOL 1684	-	10	-	-	10
32	MCOL 1734	-	10	-	-	10

33	MCOL 2063	-	-	-	15	15
34	MCOL 2215	30	10	30	-	70
35	MCR 30	30	-	30	-	60
36	MCR 31	30	-	30	-	60
37	MCUB 72	-	-	-	9	9
38	MPER 183	-	10	-	-	10
39	MTAI 8	-	10	-	-	10
40	SM 1411-5	-	10	-	-	10
41	SM 1433-4	-	10	-	-	10
42	SM 1460-1	-	10	-	-	10
43	SM 1565-15	-	10	-	-	10
44	SM 1741-1	-	10	-	-	10
45	SM 1821-7	-	10	-	-	10
46	SM 667-1	-	-	-	10	10
47	SM 805-15	-	5	-	-	5
48	SM 909-25	-	10	-	-	10
	TOTAL	300	357	300	287	1244

materials into *in vitro*. An unusual development observed this year was the production *in vitro* of a female flower in the genotype AR37-15, a BC_2 progeny for breeding resistance to CMD and CGM (Fig 1). A Mexican national program scientist Ms Fanny Cruz from Agropecuaria Santa Genoveva, and Ms Ximena Moreno from cassava breeding were trained in tissue culture methods. Others include Milena Sepúlveda y Angie Ayala, undergraduate students from the National University of Colombia in Palmira trained in multiplication and hardening of *in vitro* plants.

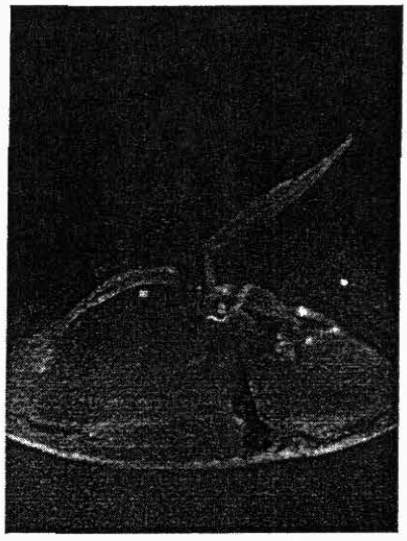

Figure 1. A female flower formed in a 4 week old *in vitro* plantlet of the genotype AR37-15, a BC_2 progeny for breeding resistance to CMD and CGM

Conclusion and Future Perspectives

The cassava group processed more than 2000 accessions from the world germplasm collection for activities in the GCP this year. They also continued shipment of improved varieties of cassava to partners in Meso America, South America, and Europe, and tissue culture establishment of genetic stocks with valuable traits. Four partners from a Mexican national Program, the local university in Palmira and from CIAT were also trained this year.

The tissue culture facility has a growth with a capacity of around 28,000 plants at the moment it has more than 30,000 and requires additional space. Plans are ongoing to convert another of the laboratories in the cassava physiology section to a larger growth room. Future activities include the continued dissemination of improved varieties, establishment of genetic stocks and the implementation of a bar-code method for proper inventory of all plants entering the growth room.

References

Roca, W.M., Rodriguez, J.A., Mafla, G. Y Roa, J. (1984) Procedures for recovering cassava clones distributed *in vitro*. Centro Internacional de Agricultura Tropical (CIAT). 8 pp.

2.2.7 A Simple Method for the Rapid Multiplication of Clean Cassava Planting Material

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Funding: CIAT core funds and NSM

Important outputs

1) The establishment of a 20ha seed bank (200,000 plants) from plants obtained from 2-node cuttings of 4 improved varieties.

Introduction

The Nigerian Starch Mill (NSM) embarked upon a commercial cassava production last year to provide a reliable source of raw materials for its starch mill. Following a visit of NSM's chief executive, Dr Chris Okeke, to CIAT Colombia last year and a consultancy visit by a CIAT-CLAYUCA team, a road map was outlined for commercial scale cassava production. Two Cubans technical experts from INIVIT, the Cuban national root and tuber

institute, were seconded to NSM to implement the road map. They arrived in Nigeria in February and had started work at the NSM farm in Opuoma. Their terms of reference were to set up a 200ha seed bank via a rapid 2-node multiplication of tissue culture introductions for the supply of healthy planting material to the farm amongst other things. Certified planting material has been shown to increase yield by as much as 40% (Guritno 1985). We describe here activities in the first year of the rapid multiplication of improved varieties for

Methodology

The rapid multiplication seedling nursery is supposed to establish a seed bank of high quality for NSM and it consists of 2 parts, the 2-node nursery and a screen house hardening of introduced *in vitro* germplasm. A 2-node multiplication scheme was initiated using carefully selected stems of 4 improved varieties: NR8083, TMS53, TMS4(2)1425, and TMS30572 for the establishment of a certified gene bank (stakes free of pest and disease). The micro-stakes were planted in plastic bags containing a mixture of sand and top soil (ratio 3:1) that had previously been sterilized by placing in a dry bath and heating on an open fire for 2-4 hours. Later, the sand and top soil mixture was replaced by top soil alone, the soil at Opuoma is very sandy, and the sterilization step was eliminated. At 4-6 weeks after planting the plants were transferred to the field. The multiplication nursery is capable of holding > 200,000 plants at any time.

Tissue culture plantlets of 44 IITA improved germplasm were placed *in vitro* as described earlier (CIAT 2003) and multiplied at the University of Legon Ghana. This first batch of around 3000 plantlets was transferred to Nigeria at the end of January 2004. The non completion of the NSM screen house led to their being held temporarily at the tissue culture facility at the Institute for Agricultural Research and Training (IAR&T), Ibadan. They were later transferred in April to NSM farm at Opuoma. Two additional batches of 1530 and 2,800 plantlets were shipped to Opuoma in May and August respectively. The plants were transferred to the completed screen house for hardening as described earlier (CIAT 2003).

Results

A total of 10ha have been planted in with seedling raised from carefully selected 2-node stakes from the varieties NR8083, TMS53, TME30572 and TMS4 (2)1425. There is also another 69,900 plants ready for transfer to the field, enough to plant another 7ha (Fig1). A third set of 30,000 plants has been planted and will be transferred to the field before the end of the season, a total of 20ha of cassava seed bank will be planted this year to provide clean and healthy planting material for seeding 200ha next year.

The screen house hardening section of the nursery suffered severe technical and management problems that lead to the loss of close to 90% loss of germplasm introduced as tissue culture plantlets. The 1st set of plants arrived April 15/04, they came from University of Legon Ghana via IAR&T, Ibadan, these plants were old, between 3-4

months. Of a total of more than 2000 plantlets introduced, 825 could be successfully transferred to the screen house and only 65 survived. The reason for the high failure rate was adduced to the high temperature which is extremely high at that time of the year (April, May, June), a minimum of 24.8-26.12°C and a maximum of 33.2-36.6°C between 6am and 4 pm (Table1). A second set of 1053 *in vitro* plants arrived June 4, a total of 853 were transferred successfully but only 163 survived, temperature was still high but was better controlled with additional netting and misting of the screen house with the irrigation sprays.

Fig 1. Cassava seedling nursery raised from carefully selected 2-node stakes from the varieties NR8083, TME30572 and TMS4 (2)1425

A third group of 2800 *in vitro* plants came in July, of this 2253 were be transferred to the screen house, the others were damaged or contaminated during the trip. Of this group just 72 survived, the very high mortality was blamed on lack of water over a period of 15

Table 1. Temperature regime in the screen house at NSM Farm, Opuoma

Month	6 am	8 am	10 am	12 am	1 pm	2 pm	4 pm	6 pm	8 pm
April	24.8	26.6	32.55	37.3	39.11	36.6	33.6	32.3	32.85
May	25.12	29.19	32.5	37.96	39.16	36.5	34.1	31.2	32.96
June	26.32	28.8	32.6	36.2	37.1	36.3	33.2	31.12	32.7
July	25.36	28.7	31.3	35.9	36.92	36.21	32.9	31.1	32.29
August	25.21	27.92	31.21	35.52	36.63	36.15	32.53	30.92	31.9

days. Poor water quality was also pointed out as one of the reason for the high mortality in the 3 introductions, Opuoma is a petroleum producing area and ground water from a borehole that supplies the screen house is contaminated with a reddish substance.

Conclusions

A 20ha seed bank (200,000 plants) from plants raised from 2-node cuttings of 4 improved varieties has been established at the NSM Opuoma farm. This seed bank will provide healthy planting material to plant 200ha of a seed bank next year. The screen house hardening of in vitro plants on the other hand did not succeed as expected, the Cuban agronomist handling the task blamed the high mortality on temperature, but the maximum and minimum temperatures at the screen house are comparable to those in several other places including some African countries where we have successfully shipped *in vitro* plants to (see section 8.2, this report). The water quality and the sub-optimal management of the *in vitro* plants are more plausible reasons.

Future Perspectives

A plan has been put in place to identify and resolve problems associated with hardening of *in vitro* plantlets at the NSM screen house in Opuoma. The effort consists of visit by CIAT technical personnel to the NSM farm for between 3 to 4 weeks to assist with hardening of *in vitro* plantlets

References

CIAT 2003. Annual Report. Improved cassava for the developing world. Project IP3. pp8-89 to 8-92

Guritno J.H. 1985. Influence of planting material on plant performance in cassava. Ph.d. thesis, University of Brawijaya, Malang, Indonesia. 158pp.

2.2.8 Improved sterilization and direct-grafting methodology for starting *in vitro* cultures of clones or selected elite soursop (*Annona muricata* L.) trees

A.Mejía-Jiménez[1-2], J.P. Villamizar[2], J.Tohme[1], J. Cock[2]
[1]Project SB-02; [2]IP-06 Tropical Fruits Project –CIAT

Introduction

Since the origins of agriculture, grafting has been an important tool for the vegetative propagation of trees, including fruit trees. Difficulties in rooting shoots of valuable trees have been overcome by grafting of buds over rootstocks of the same species or of different related species. Grafting of very small pieces of buds *in vitro*, a methodology called micrografting, has also found wide application in tissue culture, mainly for the production of clean clones from plants contaminated with viruses or other pathogens (George 1993).
Based on the application of an optimized micrografting methodology, CIAT and Corporación BIOTEC developed a methodology for the *in vitro* clonal propagation of elite selections of soursop (or guanábano in Spanish, *Annona muricata* L.; Royero et al., 1998;

Mejía *et al.* 2000). This propagation methodology consists of the succesive micrografting *in vitro* of buds produced by other micrografts that are maintained under aseptic conditions also *in vitro*. In general, tropical woody species are considered difficult to propagate *in vitro* (Litz and Jaisval 1991; George 1994).

In 2004 Corporación BIOTEC and Profrutales, one of the most important nurseries in Colombia, started a project funded by COLCIENCIAS to scale up this propagation methodology to the commercial level. The roll of CIAT in this project is to supply mother plants of four soursop clones growing under aseptic conditions, ready to be used as source of clean buds for starting clonal propagation.
During 2004 we optimized the disinfection and direct grafting methodologies developed in previous years for the efficient and rapid production of clones of selected trees and cultivars growing under aseptic conditions *in vitro*.

Methodology

The source of explants used for starting *in vitro* cultures were clones older than 4 years growing in the field at CIAT or in a greenhouse. These clones were of one cultivar (Elita; Ríos-Castaño and Reyes, 1996) and three elite selections of soursop. These selections, made by Ing. Agr. Francisco Arboleda, correspond to the best trees with respect to fruit quality and quantity from commercial plantations established from seeds in the state of Huila (clones Francia, Rosa and Cristina).
As rootstocks for direct grafting, *in vitro* germinating seeds of one accession of *Annona montana* (AmonV54) or two clones of soursop planted at CIAT (Elita and Cristina) were used.
The methodologies for *in vitro* culture of seeds, sterilization of explants, micrografting and *in vitro* culture of micrografts, were described previously (Royero et al. 1998; Mejía et al. 2000, Alzate 2002).
The treatment solution for reducing the presence of contaminants in the explants, and for stimulating the growth of buds after isolation, was composed of: Cosmoaguas™, 1 g/l, Inex - A™, 1 ml/l; Benlate™, 2 g/l, copper sulfate ($CuSO_2.5H_20$) 100 mg/l and Cosmocel™ 1 g/l. Mother plants in the greenhouse or in the field were sprayed every two days with this solution, at least 5 times before the shoots were taken. Two sterilization methodologies of shoots were tested. In both, the shoots were sterilized by two wash steps with 70% ethanol, each of them for 1 min, followed by an immersion in 1% NaOCl with a drop of liquid soap, for 8 minutes and a wash in sterile water. The second method also included the application of vacuum (500 mm Hg) for five minutes during the sterilization with NaOCl.

In addition, two direct-grafting methodologies were tested. Buds were cut from sterilized shoots and (i) grafted directly onto rootstocks obtained from seedlings germinated *in vitro* or (ii) grafted after one, two or three weeks of culture on a medium containing 1 mg/l BAP (RO 1/2 1BAP medium; Alzate 2000). For all treatments the grafting solution was 1/8 Murashige and Skoog salts, PVP (1 g/L), and Benlate 100 mg/l.

Results

Improved sterilization of shoots of elite clones or selected trees growing in a greenhouse or in the field

Plants growing *in vivo* may carry on their surface a large quantity and diversity of microorganisms. Their presence represents a serious obstacle for starting *in vitro* cultures of vegetative material taken from them.

In previous years, standard sterilization methodologies were adapted to decontaminate explants taken from clones of soursop maintained in a greenhouse, or trees growing in the field (Royero *et al.* 1998).

In order to achieve proper sterilization of the explants and to increase the efficiencies of regrowth *in vitro* of the isolated soursop buds from adult trees, a periodical treatment of the plants with antibiotic substances and foliar fertilizers, days before the isolation of the buds, is beneficial (Mejía 2000).

This year we simplified the treatment solution developed previously by replacing the antibiotics tetracicline and streptomycine with copper sulfate (100 mg/l). Copper sulfate has been used recently as a wide spectrum antibiotic and antimicotic for *in vitro* applications (see http://plant-tc.coafes.umn.edu/listserv/).

In addition, the sterilization process in the lab was modified by including the application of vacuum during part of the time that the explants were in contact with the bleach solution. Moderate vacuum allows the solution to enter spaces of the explants filled by trapped air bubbles and come in contact with spores of bacteria and fungi that may be present there (http://plant-tc.coafes.umn.edu/listserv/).

Both modifications, the treatment of the plants *ex vitro* and the application of vacuum during sterilization, allowed improvement in decontamination of shoots from field-grown plants from 9.5 to 43.7 % and from plants growing in the greenhouse from 41.9% to 94.3% (Fig. 1).

No deleterious effect of the application of high concentrations of copper sulfate were observed in the treated plants in the field or greenhouse, or in the produced direct grafts.

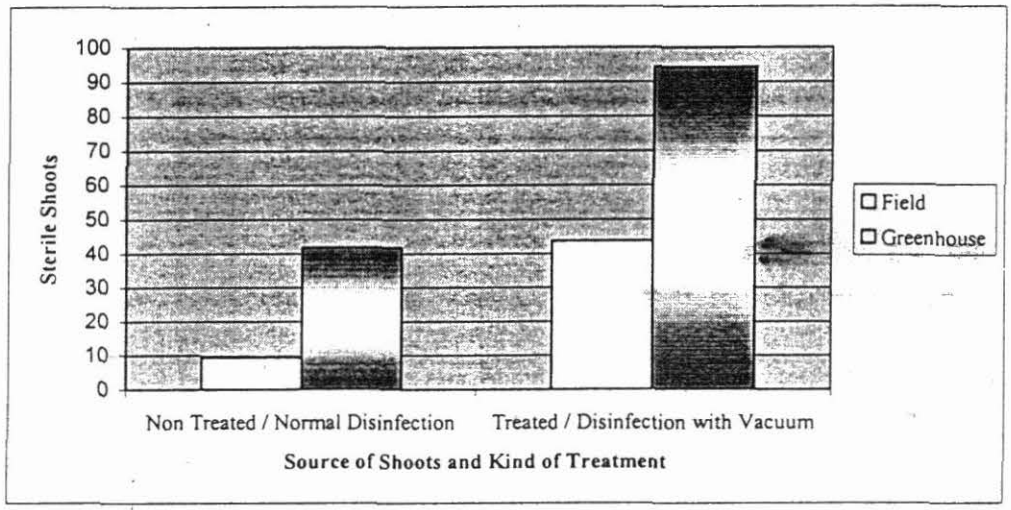

Figure 1. Efficiency of sterilization of young shoots from trees of soursop (*Annona muricata* L.) maintained in a greenhouse or in the field, and treated or not with an antimicrobial solution.

Improved direct grafting *in vitro* of buds isolated from adult trees or clones of soursop growing in the field or in a greenhouse

Direct grafting of buds of greenhouse-grown clones, or of adult trees growing in the field, was used in previous years to produce mother plants for starting *in vitro* propagation. The efficiency achieved with this methodology was, however, low (5.3% measured as the % of clean grafts that developed further *in vitro*) mainly because of contamination of the explants, the lack of union between rootstock and scion, or the lack of development and regrowth of the grafted buds (Mejía-Jiménez *et al.* 2000).
Similar low efficiency (7.8 %) was achieved during 2004 by attempting to graft shoots directly without an *in vitro* treatment. Only after using treated shoots through a pre-culture *in vitro*, in a BAP containing medium, for 7, 14 and 21 days, direct grafting efficiencies increased to 45.4, 59.3 and 62.2 % respectively (Fig. 2).

The modification of the direct-grafting methodology by including a treatment step *in vitro* has the advantage that during this treatment the contaminated shoots can be identified and discarded, and only the shoots that remain sterile after the treatment are grafted, thus reducing losses due to contamination.
Buds from direct grafts produced by this method grew vigorously *in vitro*, and 4 to 6 weeks after grafting could be already used as source of new buds for initiating *in vitro* propagation through cyclic micrografting (Fig. 3).
At least 200 direct grafts (50 of each clone) growing under aseptic conditions were provided to Corporación Biotec and Profrutales for initiating the scaling-up of the propagation methodology.

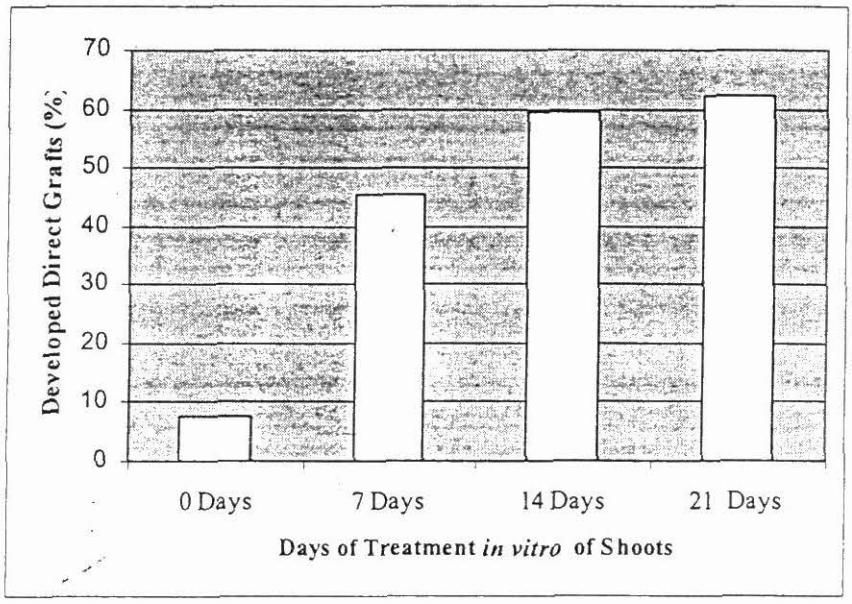

Figure 2. Efficiency of production of direct *in vitro* grafts of buds taken from adult trees growing in the field, after different time of treatment of the buds *in vitro*, in R01/2 medium containing 1mg/l BAP.

Conclusion

Explant sterilization and direct-grafting methodologies were developed or optimized that allow the fast and efficient initiation of *in vitro* cultures of buds isolated from selected adult trees growing under *ex vitro* conditions, in the field or in a greenhouse. These methodologies are also useful for collecting buds from selected trees located in distant places, for producing a limited number of clones of a selected tree for performing evaluations in multiple locations, and for producing clean clones of valuable trees to facilitate their international transport.

Future plans

To apply the methodologies developed for soursop *in vitro* propagation to other tropical fruit tree species.

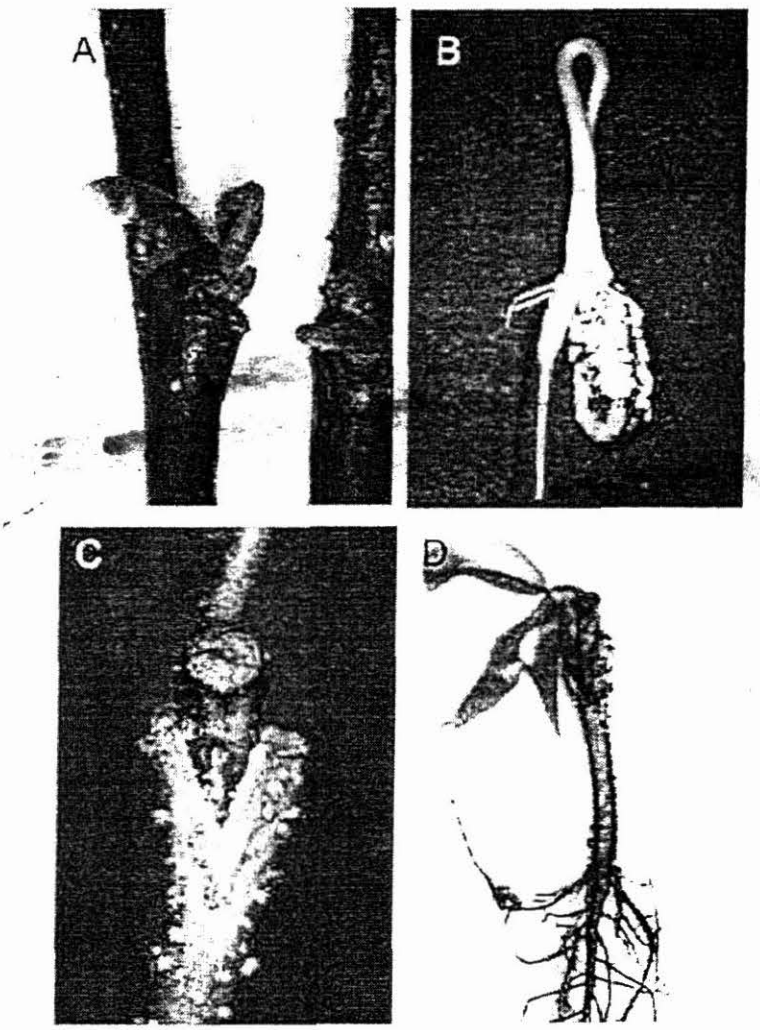

Figure 3. Process of production of direct grafts *in vitro* using buds isolated from selected soursop (*Annona muricata*) elite trees or clones, growing in the field or in a greenhouse. (A) Axillary bud development after 3 weeks of treatment *in vitro* in a culture medium containing 1 mg/l BAP; (B) seedling of the cultivar Elita four weeks after germination *in vitro*, ready to be used as rootstock for direct grafting of buds isolated from selected trees; (C) and (D) direct grafts of the clone Francia after four weeks of culture *in vitro*.

References

Alzate, A. M. 2002. Final Report. Joung Researcher Program of Colciencias-CIAT-Corporación BIOTEC. International Center of Tropical Agriculture (CIAT). 32 p.

Escobar, W. and L. Sánchez. Guanábano. Fruticultura Colombiana, ed. A. Morales. Santafé de Bogotá: Instituto Colombiano Agropecuario-ICA, 1992.

George, E.F. Plant Propagation by Tissue Culture. Part 1 The Technology. Second (revised) edition ed., Edington, Wilts: Exegenetics Ltd, 1993.

Litz R.E.; V.S. Jaiswal. Micropropagation of tropical and subtropical fruits. En: Micropropagation technology and application. P.C Debergh y R.H. Zimmerman (eds.). Dordrecht: Kluwer Academic Pub., 1991.

Mejía-Jiménez, A., Royero, N., Cabra, J. and Roca, W.M. Patent application to the SIC- Colombia. Corporación BIOTEC and the International Center of Tropical Agriculture -CIAT: "Proceso para la propagación clonal *in vitro* de plantas de guanábano (Annona muricata L.), empleando la microinjertación cíclica". (registro No SIC 00076031, 06-10-2000). http://www.sic.gov.co/ http://200.91.231.199/SCRIPTS/is34clatit.dll/detalle?Vano=1&Vnum=35948&vcont=

Nelson Royero, Alvaro Mejía, Gladys Perdomo, Jorge Cabra, William Roca (1999). Development and standardization of an *in vitro* clonal propagation method of soursop (Annona muricata l.). Annual Report CIAT

Ríos-Castaño D., Barona and C.E. Reyes. Guanábano Elita. Agricultura Tropical 33, 3 (1996):97-101.

Royero N., Muñoz L., Mejía A., Cabra J., Roca W. (1998). Development and standardization of an *in vitro* clonal propagation method of Soursop (Annona muricata L.). Annual Report CIAT.

2.2.9 Logistic aspects involved in the management of an *in vitro* eassava collection in liquid nitrogen.

R. Escobar[1], N. Manrique[1], A. Rios[1], A. Velasco[2], G. Mafla[1], M. Duque[1] and J. Tohme[1]
1 SB-2 Project, CIAT; 2 Ants Web

Introduction

Before putting the cryopreservation protocol into practice on entire collection we carried out a pilot project on the core collection. This allowed us to adjust the logistical aspect involved in the management of a complex collection and to give a general idea about how much effort, input, resources and manpower will be necessary for its implementation.

Material and Methods

The encapsulation-dehydration methodology was implemented (Annual Report, 2000) using in vitro plants supplied by the GRU; and previously intensively micropropagated on 4E medium (Roca, 1984). This process must be done until enough shoots/clones, 80-100 explants, are obtained.

Results

This year we received 153 clones from GRU. Starting with this material and after 2-3 subcultures we obtained 15,120 new explantes to be incorporated in the freezing scheme. We maintain a continuous process that incorporates 8-12 new clones into the process per week.
Currently we normally include 6 tubes per clone with 10 shoots per tube. Two of these tubes are use as controls (to take out after 1 and 3 months of conservation in liquid nitrogen) and 4 tubes for long-term conservation.

Five hundred and fifty-seven (557) clones from the core collection were maintained in a cryo-tank. Four hundred and fifty-two clones had been frozen and tested for conservation during different times. We consider that by the end of 2004 we could put the entire core collection under L.N. conditions.

As part of the logistical aspect, we constructed a database written in PHP and JavaScript languages with Mysql data base programming. This software facilitates information management across experiments and gives an easy tracking method per tube in the cryo tank.

Additionally it gives specific information about the status of each clone (passport data, morphological and isozyme characterization, phytosanitary, etc) and consolidated reports of stock per freezer, behavior of each clone after frozen conditions and a list of the lowest-responding clones that must be repeated again in a new experiment.

Nine CEF clusters from African clones supplied by Nigel Taylor (Danford Center. Mo-USA) were put under liquid nitrogen conditions following methodology described by Santos in 2002. Currently those clusters are growing on recovery media to test the recovery rate after 1 month on L.N. Controls are growing on GD250Pi.

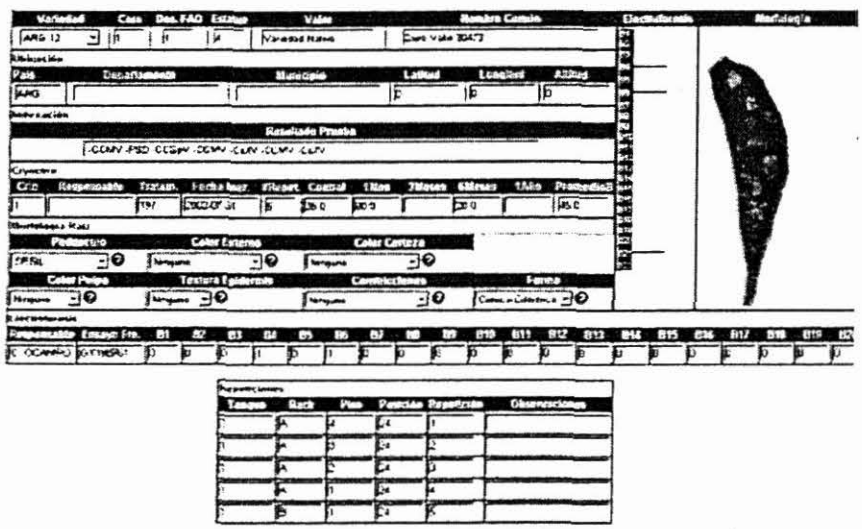

Figure 1: Details of cassava cryo-information management system software interface.

DNA analysis by AFLP was done using frozen tissues [they were maintained under L.N for different periods (from 1hour to 12 months) of conservation] and their control without freezing. Non-significant differences were observed with 6 polymorphic combinations (Figure 2). Clones have more than 96% similarity between non- and frozen tissues.

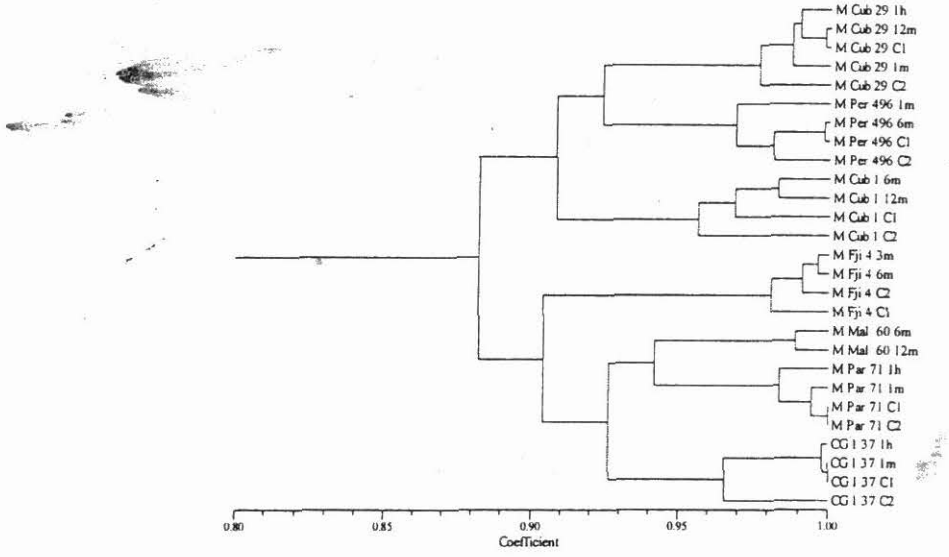

Figure 2: Dendrogram of similarity among frozen clones recovered after different times and its control without freezing.

Conclusions

If putting the entire cassava collection under L.N. conditions is under consideration, how to adjust the propagation facilities must be given through.
We maintain more than 91.6% of the core collection in a state of cryopreservation.
It was possible to establish a basic protocol for the management of a cryobank using the core collection as a model.
Different markers were analyzed (morphology, isozyme and DNA based markers) and did not show changes associated with frozen steps *per se*.

Future activities

Discuss the implementation of cryopreservation on the entire cassava collection.
Adjust the protocol to the lowest respondent clone.
Recover CEF after freezing.

References

Escobar, R.H.; Manrique, N.C.; Debouck, D.G.; Tohme, J.; Roca, W.M..2000. Cryopreservation of cassava shoot tips using encapsulation-dehydration technique. In: Annual Report, Project SB-01: Assesing and utilizing grobiodiversity through biotechnology. CIAT (Centro Internacional de Agricultura Tropical), Cali, CO. p. 178-181.

Roca W.M.1984. Cassava: In: Sharp, W.R.; Evans, D.A.; Ammirato, P.; Yamada, Y. (eds). Handbook of plant cell culture. V.2. Crops species. MacMillan Publishing Co. New York. P. 269-301

Santos L.G. Estudio exploratorio para desarrollar una metodologia de crioconservacion de callo embriogenico friable (CEF) de yucca (Manihot esculenta Crant) variedades Mcol 2215 y Mnig 11.2002, Tesis Universidad Nacional, Sede Palmira.

2.2.10 Somatic embryogenesis in cacao (*Theobroma cacao*) using Colombian clones

C.A. Chaparro[1], R.H. Escobar[1], R. Saavedra[2], J. Jaramillo[2] and J. Tohme[1]
1. CIAT; 2.CORPOICA

Introduction

In Colombia, cacao is an important crop, principally due to high internal consumption and great potential for exportation; small and medium sized farmers supply the largest part of the production. The Colombian government proposed a productive channel for cacao, however crop diffusion presents great challenges, the most important being the lack of planting material of selected clones (by quality and yield) and materials with tolerance to diseases.

The development of an efficient propagation system using tissue culture for cacao holds the potential to contribute to the improvement of this crop by providing a rapid and efficient multiplication system of elite clones. The somatic embryogenesis pathway provide the possibility to multiply selected clones and for transformation research.

Materials and Methods

An agreement with CORPOICA to obtain access to the cacao collection was requested. Six cacao clones were selected from the field gene bank maintained at CORPOICA, based principally on their agronomic characteristics of productivity, quality, and tolerance to diseases. Material tested includes: SCC13, SCC6, IMC67, CAP34, SPA121 and SCA6. The last one has been selected as a control material for the somatic embryogenesis process and it has been chosen for flavor quality but not for yield.

The somatic embryogenesis protocol developed by Pennsylvania State University was followed. Explants from immature flowers (staminoid and petal) were used. Primary

embryos were used for plant recovery, however induction of a secondary somatic embryo pathway was initiated.

Results

Under tissue culture conditions, initial explants need to be maintained in quarantine period during the initial 14 days before a continuation of process. At the initial stages the highest fungus contamination occurs. Callus initiation occurs in both explants using a DKW salts-based primary callus growth medium supplemented with TDZ for 14 days. An additional subculture on media without TDZ and supplemented with BAP was necessary for tissue induction. Somatic embryos arise after 42 days of culture onto a hormone-free DKW salts-based embryo development medium containing sucrose and glucose (Fig 1a).

Table 1: Somatic embryo induction on different cacao clones and tissue.

	Explants type		Embryos type		
Genotype	Staminoid	Petals	IPE*	Normal	Abnormal
IMC 67	347	387	28	24	4
SPA 121	141	164	5	0	5
CAP 3	129	128	12	12	0
SCC 64	234	249	2	2	0
SCC 16	241	289	7	1	6
SCA 6	511	535	117	83	34

Induce primary embryos structures

In all clones tested it was possible to induce callus formation (Table 1), however clone differences were observed in frequency and time of appearance and embryo quality as has been reported at CIRAD (Maximova *et al* 2002). Phenolic oxidation was evident in all tissue tested.

Callus induction rate average was 97.7% by CAP34 and 78.3% by IMC67, being more frequently in petals.

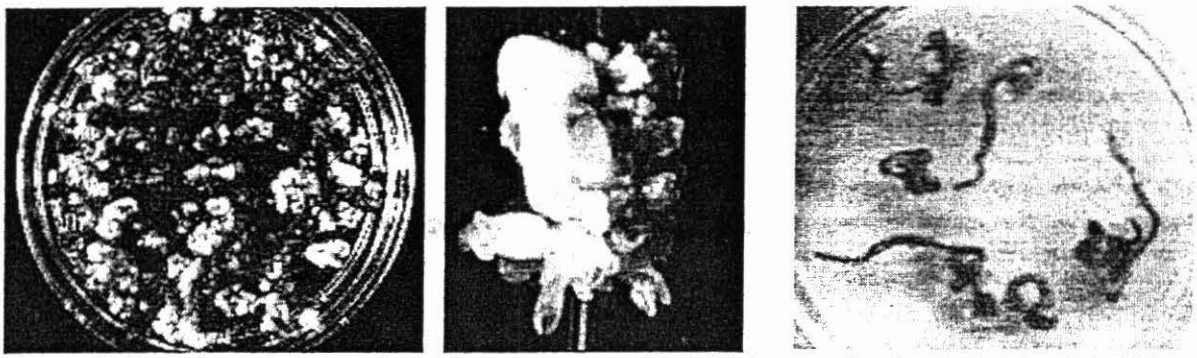

Figure 1: Somatic embriogenesis pathway in cacao. (a) Initial step of callus induction (b) different stages of plant regeneration, (c) initial regenerated plants.

A secondary embryogenesis cycle was necessary to improve the quality of the tissue induced. After 56 days of culture, different quantity and quality of primary embryos was possible to observe from both explants, as well as from non- and oxidative tissue. Globular, hearth and torpedo embryo stages of SCA6, IMC67, SPA121, SCC6, SCC13 and CAP34 were observed in the tissues but with variation in quantity (Fig 1b). SCC6 has produced non-embryogenic tissue already. According basic protocol the maximum embryo production is around the 24th week and the tissues were induced 12 weeks ago.

Normal and abnormal primary embryos have been recovered from different clones (Table1 and Fig 1c). In some cases abnormal embryos present two axes, a huge number of cotyledons or they are missing. We considered some characteristics as normal embryo appearances. These include white or pink cotyledons, axis differentiation and 2 or 3 cotyledons. Those characteristics provide assurance of normal plant regeneration and the embryos could be used as explants for secondary embryogenesis

Conclusions

In all clones tested it was possible to obtain somatic embryos. However SCA6, used as control, showed the best embryo induction and SCC6 the lowest rate.
Although both structures (staminoids and petals) produce callus, petals give better primary embryo induction. Primary somatic induction is clone dependant.
Quality of somatic embryos, after the second round of somatic embryo induction, coming from staminoid explants is better than petal structures.
High frequency of normal embryo structures will be possible from the initialized cultures, however some abnormal embryos have been observed.

Future Activities

Compare the effect of the primary and secondary embryogenesis cycles on plant regeneration.
Adjust the conventional method and evaluate other procedures for embryo induction (using RITA system or liquid cultures) to reduce time and increase the embryo production rate.

References

Li, Z.; Traore, A.; Maximova, S.; Guiltinan, M. J. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao L.*) using thidiazuron. In Vitro Cell. Dev. Biol. Plant 34:293–299; 1998.

Maximova, S.; Alemanno, L.; Young, A.; Ferriere, N.; Traore, A.; Gultinan, M. Efficiency, genotypic variability, and cellular origin of primary and secondary Somatic embryogenesis of *Theobroma cacao*. In Vitro Cell. Dev. Biol.—Plant 38:252–259, 2002.

Young, A.; Miller, C.; Antunez de Mayolo, G.; Swanson, J.D.; Pishak, S.; Maximova, S.; Guiltinan, M. Cacao tissue culture protocol book version 1.4, 2003.

2.2.11 Avocado (P. americana Mill) somatic embryogenesis using immature flower tissues

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Introduction

The avocado is cultivated in tropical and subtropical regions of more than 50 countries. In recent years, the Colombian government has established an agro-enterprise channel for avocado with potential for exportation, however one the biggest problems of avocado scaling-up is that it does not allow a rootstock production program to incorporate to the production scheme.

Rootstocks are important for productivity and tolerance against some crop diseases, and are also involved with yield and fruit quality. A rootstock material used by farmers, named Lula, has a fungus tolerance to *Phytophtora cinnamoni* and *Roselinia necatrix*, tolerance to soil stress and has a late flowering period. Conventionally farmers use ripening fruits they pick-up from the soil.

Our goal is to try to use tissue culture techniques to establish a mechanism to produce avocado planting material to use as rootstock by an embryogenesis pathway. This methodology could be used as propagation systems or transformation methods. Previously, some researchers have been published an embryogenesis methodology using nucelar tissues (Witjaksono et al, 2003; Vidales-Fernadez et al 2003). This research is a continuation of a Master thesis on plant biotechnology for avocado genetic transformation started at University of Malaga-Spain in 2003.

Materials and Methods

Lula rootstock was collected from the nursery located in Profrutales Ltda.; immature flowers and fruits (≤ 1.0cm) were used as explants sources. Stamens, ovaries and tepals from the immature flowers and the nucellar coat of embryos from immature fruits were dissected and introduced in vitro. Cultures were maintained at 25°C in dark conditions. We evaluated a total of 8 different media, supplemented with different concentrations of 2,4-D (2.26-4,52 uM), TDZ (0.9-9uM) or Picloram (0.41-8.2uM). Four replications with 50 explants/type tested per media were used.
After 14 days of culture, tissues were transfer to callus induction media that had been supplemented with BAP (4.43 uM), 2-4D (2.26-4.52uM) and Picloram (0.41-8.2uM). Development of embryogenic tissues from the material was evaluated at different times.

Results

Avocado tissue culture is difficult because tissues have the tendency to produce browning and later necrosis; additionally initial explants under culture show a high percentage of fungus contamination (51.5-97.8%). Stamens and ovaries have been shown to have less contamination by fungus and less contamination at nucellar tissues by bacteria.

The use of Ascorbic acid (400mg/L) during the dissection step allows the reduction of an oxidative effect during procedure. Ovaries presented a high percentage of oxidation (58.4%) followed by stamens and tepals. In spite of the oxidation process, a yellow-white callus area was induced in all the media tested. Stamen did not show any change, however tepals and ovaries showed changes in size and morphology following callus formation.

Media supplemented with 0.45uM TDZ plus 2,26 uM2-4D and 9.0uM TDZ plus 8.2uM Picloram shown the best callus induction for all tissue tested. Medium supplemented with TDZ and Picloram gave us more callus frequency than other media. Ovary tissues have better induction on medium with 4.52uM 2-4D and stamens with 0.9uM TDZ plus 2.26uM 2-4D. Petals have shown the lesser callus induction. Callus recovered was transferred to DKW hormone free solid media for embryo induction.

Table 1:Response of explant type on Percentage of callus induced after 3 months of culture using different media

Media supplement (uM)			% of callus induces by explants type		
TDZ	2-4D	Picloram	Ovary	Staminoids	Tepals
		0.41	0	0	0
0.9	4.52		53	27	2.1
	4.52		71	39	1.7
0.45	2.26		63	41	5.4
0.45	4.52		55	34.6	1.25
0.9	2.26		66.3	41.7	0
0.9			16.3	8.33	0
9.0		8.2	63.8	27.1	6.67

Callus formation was evident only after 15 to 30 days of subculture on media without TDZ and supplemented with BAP (4.43uM) and 2-4D (2.26-4,52uM). Appearance of induced callus was wide-ranging in color from white to yellow, but in other cases it was brown.

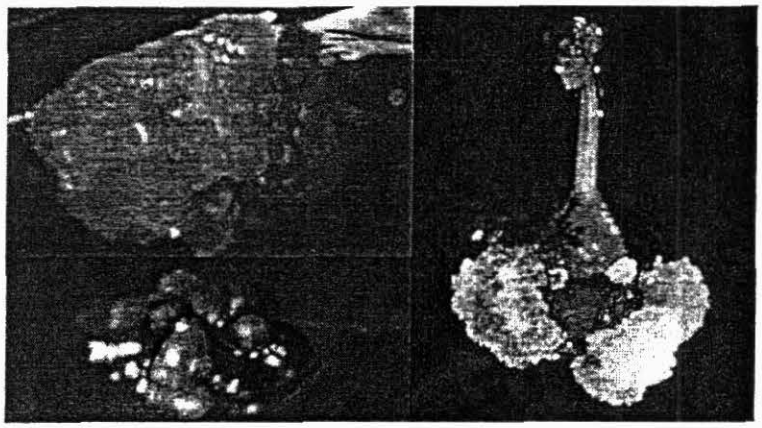

Figure 1. Details of avocado callus formation on different tissues: (a) nucellar tissue (b) tepals tissue and (c) ovaries

Conclusions

High oxidation rate was observed in all tissue tested; probably it substance create a sub-optimal growth conditions.

Preliminary observation with Lula and Reed materials has shown that callus initiation is clone dependent.

Induction media supplemented with high doses of Picloram (8,2 μM) and TDZ (9.0 μM) allows callus initiation on floral and nucellar structures.

Combinations of TDZ and 2-4D on media will be better for callus induction than simple use.
Low doses of Picloram do not induce callus formation on tissue tested.

The quality and aspect of callus induced depends of the kind of tissue used as explants. Callus coming from staminoids does not seem to be embryogenic, in contrast to ovary and tepals induce callus.

Future activities

- Determine the embryogenic potential of induced callus to promote embryo formation.
- Evaluate the effect of the basal salt and carbohydrate type on callus and embryo induction media.
- Preliminary evaluation of an alternative shoot propagation schema for *in vitro* conservation uses.

References

Márquez-Martín B.; Sánchez-Romero C.; Perán-Quesada R.; Barceló-Muñoz A.; Pliego-Alfaro F. Factores que
afectan la obtención de embriones somáticos blanco-opacos de aguacate. In Proceedings V World Avocado
Congress 2003. pp. 97-102.

Vidales-Fernández I.; Salgado-Garciglia R.; Gómez-Lim M.A.;. Ángel-Palomares E. and Guillén-Andrade YH.
Embriogénesis Somática de Aguacate (Persea Americana Mill. Cv. Hass). In Proceedings VWorld Avocado Congress 2003. Pp. 89-95.

Witjaksono S.R., Efendi D., Suarez I., Gasca I., Vazquez M., Gomez L.and Litz R. Genetic Transformation of Avocado. In Proceedings V World Avocado Congress 2003. pp. 115-118

Young, A.; Miller, C.; Antunez de Mayolo, G.; Swanson, J.D.; Pishak, S.; Maximova, S.; Guiltinan, M.Cacao tissue culture protocol book version 1.4, 2003

CONTENT

OUTPUT 3 Increased efficiency of NARS breeding programs using biotechnological tools

Activity 3.1 New collaborative arrangements and networks

3.1.1 Strengthening farmer seed systems in stress

L. Sperling
CIAT

Introduction

Within the last 20 years, disaster situations—drought, civil strife, floods, crop plagues, or combinations of these—coupled with systemic poverty, have become the norm for most countries of eastern, central, and southern Africa. Humanitarian relief practitioners, although skilled in quickly delivering short-term food aid, usually do not understand the technical complexities of the agricultural context. Even though seed aid began in the early 1990s, the long-term effectiveness of such activities remains disappointing. Both food and seed aid are still being delivered to many countries year after year.

Because they base their diagnoses on food assessments, relief practitioners are typically ignorant of or misunderstand stress situations as they apply to agriculture. For example, they commonly assume farmer seed systems to have collapsed or to have been inadequate in the first place and ignore issues promoting system stability, including, varietal diversity. Yet field results show that seed systems are resilient. For example, in Rwanda, even after its genocidal war, local seed markets continued functioning, and crop diversity profiles remained stable.

Even research institutions tend to view disasters as opportunities to expose farmers to "improved" varieties of current crops or to alternative crops. But evidence shows that system resilience, not only productivity, is also key to recovery and sustaining household food security after disasters.

CIAT, in partnership with relief and developmental agencies, facilitates the Seed Systems Under Stress Program, which concentrates on:

- Helping to shape emergency relief, particularly in terms of seed and germplasm
- Analyzing the effects of different types of disaster (war, drought, flood, or crop plague) on the functioning of a seed system (including its crop and variety diversity)
- Evaluating emergency operations to further refine practices of seed system maintenance and strengthening
- Working with policy makers to institutionalize "best practices"

- Developing robust assessment tools for use during and after disasters to diagnose the strengths and weaknesses of surviving systems, and thus target response

3.1.2 Seed Systems Under Stress Program: Projects and Results

L. Sperling
CIAT

The Program currently executes two main projects:

Project 1: Assisting disaster-affected and chronically stressed communities in eastern and central Africa: small-farmer seed systems

USAID-funded. Implemented jointly by CIAT, CRS, and CARE/Norway. Goals are to develop diagnostic tools (SSSA= Seed System Security Assessments) to determine the effects of a stress, either natural or man-made, on agricultural and seed systems (including on crop and variety diversity) and to analyze the effectiveness of various support strategies in reducing constraints. Action-oriented fieldwork evaluates on-the-ground implementation in Ethiopia, Zimbabwe, Burundi, Kenya, Uganda, Malawi, and Mozambique. Case studies and Project Briefs determine interventions appropriate to acute versus chronic seed-stress situations. They also address the various challenges to achieving seed security in terms of availability, access, and use.

All eight case studies were completed in 2004 (and are being prepared for formal publication).

Assisting disaster-affected and chronically stressed communities in eastern and central Africa: small-farmer seed systems: Case Studies Completed 2004

- **Burundi** Drought, civil strife, and seed voucedrs and fairs: the role of the trader in the local seed system
- **Kenya (west)** The use of informal seed producer groups for moving root-rot resistant varieties during periods of acute stress
- Kenya (East) **Comparison of Seed Voucher and Fairs (SV&F) and Direct Seed Distribution (DSD): Lessons Learned in Eastern Kenya and Critical Next Steps**
- Mozambique **Crisis management when the staple crop is destroyed by disease The case of Cassava Brown Streak Disease in coastal areas of northern Mozambique**
- **Uganda:**Seed vouchers & fair and agro-biodiversity in western Uganda
- **Zimbabwe:** Relief seed assistance in Zimbabwe
- **Ethiopia** Relief seed assistance in Ethiopia
- **Malawi** A review of seed security strategies in Malawi

Select Results

- Relief organizations are using an 'acute' response- seed aid--' to treat what are more often "chronic problems (and poverty-based)
- Seed aid has become repetitive in many stress sites (10-20 seasons) and tends to undermine commercial seed program development(although local seed markets continue to function—and be key).
- Some approaches, such as seed vouchers and fairs, better strengthen the local agriculture including seed systems. However, crop diversity, per se, is not maintained in a seed fair unless specifically built in as a program strategy.
- Seed systems even in stress are remarkably durable and rarely experience problems with lack of seed or loss of varieties. Rather than such problems of availability (lack/loss), the major farmer problem during crises is access (breakdown of social networks or little money to buy)
- In terms of crop and variety diversity, the major 'stress problems occur in cases of disease and virus build up-- NOT in the more dramatic contexts of civil war or drought.

Future Directions:

The program has focused till now on documenting what actually happens to farmer systems in emergency; and in evaluating the effectiveness of varied responses. It will now expand its focus in two ways:

- Concentrate on the development of diagnostic and seed system security assessment (SSSA) tools—so that the correct kind of support can be planned from the initial stages of the emergency.
- Help guide implementation of interventions—which bolster economies, seed markets—and diversity-- in stress situations

Major Collaborators: (beyond CIAT)
Catholic Relief Services (CRS)
Care/Norway (CN)
International Plant Genetic Resource Institute (IPGRI)

Project 2: Seed aid and germplasm restoration in disaster situations: synthesizing lessons learned and promoting more effective practices

IDRC-funded. This project analyzes trends in seed aid and germplasm restoration practice, and their possible interconnections, particularly in Africa. It reviews c.25 classic cases—developed through documentation, interviews, and selected field visits—to analyze current situations and provide a basis for improving practices over the next decade. More than 15 organizations are involved in synthesizing current practices, and raising global awareness of options for making progress. Recommendations and strategies for expanding and reinforcing crop diversity—in crisis contexts—will be a major project output. The Box below highlights the germplasm restoration cases being analyzed.

Key cases : germplasm restoration (possible restoration)	
Intervention	**CG Center/Other leading effort**
1. Somalia –early 1990s-drought/war	IPGRI
2. Rwanda- genocide.war	Seeds of Hope (CIAT lead)
3. Philippines,Cagayan Valley floods/drought, MVs, storage..)	IRRI/PhilRice
Liberia, Sierra Leone, Côte d'Ivoire, Guinea, Guinea Bissau (civil strife)	WARDA
4. Native Tubers in the Andes (disease/virus build up)	CIP
5. Eritrea millet (war)	ICRISAT
6. India finger millet (intensification)	ICRISAT
7. Afghanistan (war/drought)	ICARDA?
8. Mozambique flood	(studied by Noragric/ICRISAT)
9. Ethiopia drought	IPGRI/ Biodiversity Institute

Initial (Select) Results

- There are but a handful of cases, worldwide, where IARCS or NARs have restored germplasm to the farmer-level
- There are few (nil?) cases of a rigorous diagnostic showing that germplasm has been truly 'lost' (versus evolved)
- Concepts of 'germplasm restoration' are poorly developed. Research institutes often focus on the physical entity, the variety/seed, that is, giving germplasm back to communities from genebanks. What might be more crucial (in all contexts analyzed) is a focus on skill building--- to maintain the exisiting germplasm more adequately—and to expand the actual diversity base in use . (Genebanks per se, may have a narrow function in germplasm restoration)

Future Directions

This project looks at the interface between conventional seed aid—and germplasm aid per se. The two are being coupled so as a) to encourage a greater diversity perspective in mainstream seed system relief and b) to broaden which might be embraced by germplasm restoration—to give it a more systems perspective. The first next step will be to extend the findings very widely— to several donors are suggesting an explicit 'outreach' phase.

Major Contributors (beyond CIAT)

- IARCs
  International Plant Genetic Resource Institute (IPGRI)
  International Center for Research in the Semi-Arid Tropics (ICRISAT)
  West Africa Rice Development Association (WARDA)

- NGOs
  Catholic Relief Services (CRS)

World Vision International (WVI)
Save the Children (SC)
Action Aid
VECO (Zimbabwe)

- African Regional Networks/Institutes
  SADC Seed Security Network
  SADC Germplasm Resource Center

3.1.3 Use of massive propagation system to support Clayuca's research agenda: clone CM 507-37

R.H. Escobar[1], L. Muñoz[1], A. Alzate[2], B. Ospina[3] and J. Tohme[1].
[1] Agro biodiversity and Biòtechnology Project-SB-2 CIAT; [2] Cassava Improvement-IP3 program; [3] CLAYUCA

Introduction

The SB2 project has been adjusting and implementing the RITA® system developed by CIRAD (Alvard 1993) as a massive cassava propagation method The propagation rate shown is clone dependant. Previously we tested this system with 20 commercial clones and observed increases of up to 6-12 times in the propagation rate in contrast to 3-4 times on conventional solid media (Escobar *et al* 2001).
Clayuca needed to respond to a particular request from Mexican partner, and it consulted SB2 about how RITA could help to fulfill its requirements in a short period. The clone CM 507-37 had never been tested with RITA by the project.

Material and methods

We received 4 tubes with 5 plants in each one of CM 507-37 from the IP-3-Clayuca project; previously they needed to apply to MTA to gain access to this clone. The material was propagated. Four RITA containers were prepared with 10 buds in each one. The rest of the tissue was grown on 4E medium. Just three RITA were used for analysis because the other was lost, due to bacteria contamination.

Results

Starting from the initial tubes it was possible to recover a total of 69 explants (19 shoots and 50 buds). Material maintained in 4E medium (29 explants) gives us 147 explants during two propagation cycles, this material showed a low propagation rate, near 1:2.2 per cycle. In some cases it was necessary to transfer the explants without cuts to a new media.
Based on 30 buds coming from 3 RITA containers, it was possible to recover, in an initial propagation cycle, 132 structures. These were transferred onto media with activated charcoal for propagation. After that, it was possible to obtain 321 explants.

The time duration of each conventional cycle on solid media was 1-1.5 months. For RITA we needed 2 months per cycle. The propagation rate obtained with RITA was up to 5 times that of the conventional system (1:10.7 vs 1:2.2).
When both systems were put together we observed an average propagation rate of around 1:8

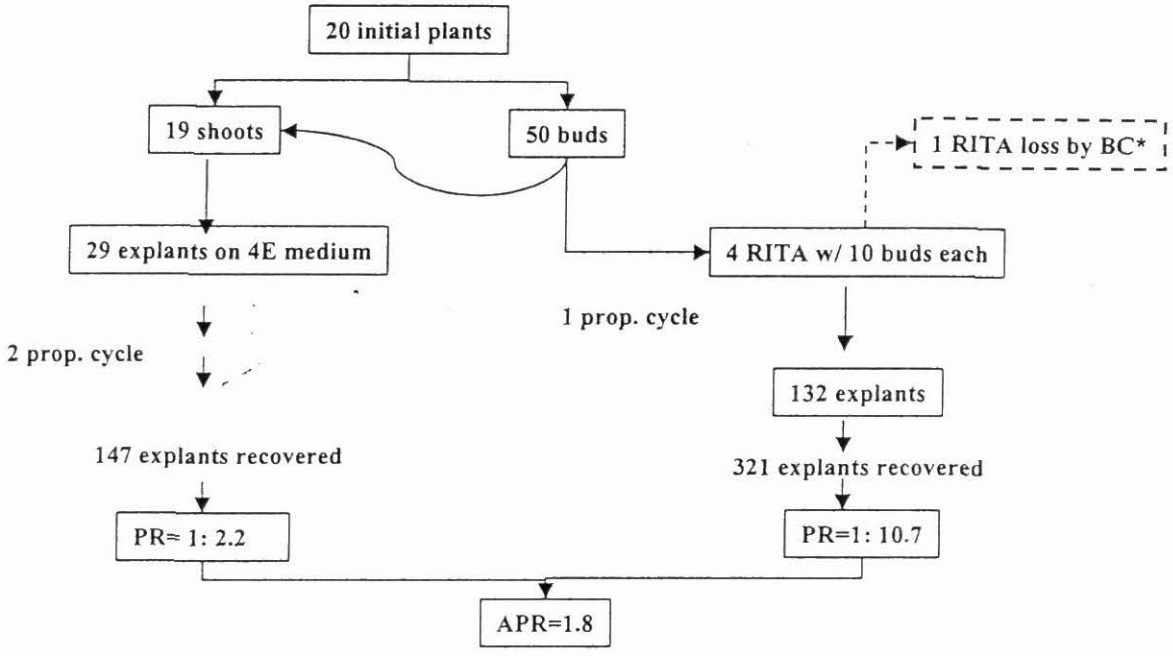

Diagram 1: Tissue culture methodology implemented by SB-2 project for CM 507-37 propagation. (*BC= Bacteria contamination)

Conclusions

- The combination of propagation methods (solid media and RITA systems) allows us to consider a solid part as a security back up for possible contamination occurrence.
- CM 507-37 shows a low propagation rate under normal 4E conditions. Material has a weak appearance and it did not respond quickly to culture conditions.
- RITA system shows a better propagation rate than conventional methods, up to 5 times higher than solid conditions.
- When it is necessary RITA could supply high volumes of planting material, however it is necessary to know the propagation rate before beginning in order to establish an appropriated propagation scheme.
- Propagation using RITA is clone dependent, for that reasons a propagation scheme could not be established with reference to others clone.
- We could obtain this propagation rate after 72 days of culture.

References

Alvard, D. et al. 1993. Plant Cell Tiss. Org. Cult. 32: 55-60

Escobar, R.H.; Muñoz, L.; Tohme, J.; Roca, W. 2001. In: Ann. Rep., Project SB-02, CIAT (Centro Internacional de Agricultura Tropical), Cali, CO. p. 241-243.

Roca W.M.1984. Cassava: In: Sharp, W.R.; Evans, D.A.; Ammirato, P.; Yamada, Y. (eds). Handbook of plant cell culture. V.2. Crops species. MacMillan Publishing Co. New York. P. 269-301

3.1.4 A new interactive tool for making biotechnology accessible to the Classroom

Escobar R.[1], Escobar F.[1], Gallego G.[1], Cortes D.[1], Chavez A.L.[1], Bohórquez A.[1], Caicedo A.L.[2], Soto M.[1], Narváez A.S.[3], Lozano E.[3], Lugo J.[3] and Tohme J.[1]

[1] Agrobiodiversity and Biotechnology Project, CIAT. [2] North Carolina State University, Department of Genetics, Raleigh, NC, USA, [3]Centro Auxiliar de Servicios Docentes-C.A.S.D., Sede Cali-Colombia

Introduction

One of a researcher's responsibilities is to try to diffuse his or her research to end-users. In attempting to do this for science education, many teachers from the public sector commented that some public schools have a lot of students per course and, in other cases it is not easy to find financial support to visit CIAT facilities. For this reason, in collaboration with science teachers from C.A.S.D., we developed interactive tools in a CD format to cover their requirements. These CDs include selected topics that provide teachers with up-date knowledge about new technologies. At the same time they facilitate the laboratory activities of students because they make available a building plan for some low-cost equipment.
The manual is written in simple terms to allow use by students as well as teachers.

Material and methods

A previous meeting with closer schools subscribing to C.A.S.D., allowed us to know how many biology teachers knew about new technologies, and state of the art in biotechnology. We asked at the same time if they had a problem with papers or manual written in English. Based on this, the CIAT team wrote a guidebook that included some topics such as tissue culture, molecular markers, bioinformatics and evolution, among other topics. Each chapter includes laboratory exercises and instructions on building low-cost equipment and how they could find cheaper chemical inputs or reagents. Later, some teachers were invited to review the manual and to attend a practical section at CIAT. After reaching consensus on terms and topics the team decided to design a CD written in a Macromedia Flash Player.

Results

After 2 years of work, a CD that includes 9 theoretical topics and 10 laboratory exercises will be release next October. Other CIAT researchers are writing a new topic to be included in a second version. In addition teachers are writing a proposal about how they could innovate the teaching of sciences courses.

The sharing of these experiences with other schools is through by CASD to consolidate and to promote the group among others members of the educational system.

Based on this collaborative effort between CASD and CIAT, teachers decided to include in its curriculum more emphasis on biotechnology and they established an "*Emphasis in Applied Natural Science*" that included more than 190 students during 2003-2004 period.
When CASD placed a new emphasis on the sciences, students were motivated and the students population enrolled in science programs increased from 40 students during 2002-2003 academic year to 190 students in 2003-2004 academic year.

Figure 1: (A) Teacher group from CASD adscript public schools involved in the process (B) Brochure to make known the Emphasis on Natural Science (C) Opening screen of the application: Biotechnology in the Classroom: Working document for high school teachers, Version 1.

However, many teachers are interested in knowing how they could access and adjust this topic in their schools, during a meeting in Bogotá personal from various Faculties of Education inquired about how could we articulate this project towards training a new generation of science teachers.

Conclusions

- This project allows CIAT to increase its scientific outreach activities within Colombia by promoting the spread of state of the art scientific knowledge to a huge number of students from the public sector.
- Some teachers are updating their concepts of certain biotechnological topics. As material is written in Spanish, it allows rapid adaptation to science courses.
- Students could discuss biotech-topics in their classes. They would promote general discussion of science with their classmates.

Future activities

- Include others topics to improve version1.
- If we find financial support, schedule a meeting with different actors that are interested in science teaching resources.
- Disseminate our experiences to other regions of Colombia and/or Latin American.

Activity 3.2 Cassava Biotechnology Network's activities

Project:

3.2.1 The Cassava Biotechnology Network in Latin America: Strategies for Integrating Small-Scale End-Users in Research Agenda-Setting, Testing and Evaluation

Alfredo A. C. Alves
CBN–LAC Coordinator

Introduction

Since 2001 the Cassava Biotechnology Network (CBN) have been involved in a regional framework for Latin America and the Caribbean (LAC) with 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). 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.

In the fourth year (2004), CBN-LAC have worked mainly in the following activities:

1) Implementing, monitoring and guidance of 11 projects (pilot sites and small grants programs) in Colombia, Brazil, Cuba, and Ecuador.
2) Scholarships for postgraduate studies in biodiversity under the Ginés-Mera Memorial Fellowship Fund, provided by IDRC.
3) Organizing the Sixth International CBN meeting (CBN-VI), held at CIAT, March 2004, and other information exchanges.

Report of the Projects

Project title

3.2.1 Use of *in vitro* technology by small farmers to clean and preserve native cassava varieties in Southern Colombia's Andean Region

Institution: Fundación para la Investigación y Desarrollo Agrícola (FIDAR)
Address: Calle 6A n.61-109, AA 25074, Cali, COLOMBIA
Email: fidar@colombianet.net
Collaborating institution:
Centro International de Agricultura Tropical (CIAT), Colombia

Staff directly involved
José M. Restrepo M. (Coordinator), FIDAR
Gloria Ospina, FIDAR
Roosevelt Escobar, CIAT
Joe Tohme, CIAT
Carlos Hernández, Farmer

Results

Cleaning of native materials by thermoterapy and in vitro cultivation

All the materials collected in the field, at Cauca Department, were planted in greenhouse and in laboratory at CIAT for seed cleaning and to give them again to the communities for establishing *in situ* Conservation Banks and to evaluate these materials in field to produce seed and to increase the agrobiodiversidad. The protocol used for this cleaning was the following: 1) growth of selected stakes under greenhouse conditions; 2) extraction of meristems; 3) *in vitro* growth; 4) *in vitro* thermoterapy (2 cycles); 5) implementation of the propagation scheme; 6) induction of roots; 7) hardening; and 8) transfer to greenhouse conditions. The main results for this objective were:

- 37 collected genotypes and used in *in vitro* propagation system.
- 2,064 plants in propagation, from the 37 genotypes (4E cultivation medium).
- 1,074 plants under root establishment, from 32 genotypes (Table 1).
- Five accessions in propagation systems to provide materials for rooting.
- 21 plants of 'rojita' (new material suggested by J. Restrepo).
- One plant, per accession, established in greenhouse for frog skin disease (FSD) certification.
- A propagation system for 'Bajuna' (to attend farmer's request, Oscar Vivas).

An in vitro seed production scheme adapted by the farmers

As showed in the previous phase of this project, the cost of the seed produced from *in vitro* plants is high, and cannot be assumed by the small and medium farmers. So, a procedure that facilitates the implementation of this technology was developed. The outline of this procedure is the following: 1) a local institution, such as an ONG or a regional school, multiply the seed from *in vitro* plants; 2) a farmer association, along with local institution assistance, produces vegetative material; 3) the local institution multiply this vegetative material by convencional rapid propagation (CRP) and distribute the seeds to other farmers' groups, who multiply the seeds by direct rapid propagation (DRP).

Seed production from in vitro plants and multiplied by two systems of rapid propagation

The main results for this objective were:

- Five improved varieties and one local produced from *in vitro* plants and multiplied by rapid propagation.
- 21,790 seeds multiplied *in vitro* by conventional rapid propagation (CRP) and 9,300 seeds by direct rapid propagation (DRP).
- 8 farmer organizations with cleaned material and able to continue increasing the seed production by DRP.
- Two farmer groups trained and able to multiply seeds by DRP.

Difficulties in the work plan

The low prices of roots and starch during 2002 and 2003 did not estimulate the cassava farmers and a low seed demand was evident. From December 2003 the price improved and the demand for high quality planting material is increasing so much. But, only a small amount of seeds have been produced due to lack of funds for greenhouse enlargement and for establishment of a drop irrigation system (Tables 2 and 3).

Another factor that affected the project is the high incidence of white fly (*Bemicia tuberculata*) and FSD, increasing the costs of production of the plots for commercial cultivation and for seed production, especially in the low areas (900 to 1300 m).

The above constraints, especially those related to pests and disease problems, didn't allow a wide participation of the farmers. To overcome this situation a small farmers' group was stimulated to accomplish the activities of collection, cleaning and propagation of *in vitro* seeds.

Communication and information dissemination

The divulgation of the project's outcomes was performed by meetings, lectures or posters in international, national and local events related to biotechnology tools for improving seed production:

Sixth Internacional Scientific Meeting of the Cassava Biotechnology Network. Marzo 2004. CIAT. Conferencia "El futuro de la Biotecnología en los países en desarrollo". J. Restrepo, C. Hernández, R. Escobar, J. Tohme.

Red Bio 2004. Implementación de técnicas de Cultivo de Tejidos con Agricultores. R. H. Escobar., L. Muñoz, C. M. Hernández, E. Caicedo, J. Restrepo y J. Tohme.

Métodos modernos para la siembra del cultivo de yuca. Febrero 2004. CIAT- C. Cafeteros de Risaralda-FIDAR. D. Villada, G. Jaramillo, J. Restrepo, R. Escobar.

Utilización de la semilla de yuca *in vitro* para mejorar la calidad sanitaria de la semilla de yuca. Noviembre del 2003. Santander de Quilichao. Taller para Agricultores del Norte del Cauca. J. Restrepo, C. Gallego, C. Hernández, R. Escobar.

Future activities

To establish, in the field, the 32 local varieties cleaned by thermoterapy and *in vitro* systems, in order to multiply these materials and to evaluate their field performance. Up to date 1,074 plants are available for field multiplication.

To establish the cleaned local varieties in three *in situ* banks, according to IPGRI methodology for conservation of Andean tuber and roots.

To assist three farmer organizations for producing cleaned seeds from *in vitro* plants, using the multiplication system evaluated in this project, and aiming the seed production for 200 hectares. All these groups are located in the high region Caldono, Morales and Piendamó.

To support and train two organizations in 'Norte del Cauca' and in 'Virginia' (Risaralda) on planting material production for low regions, in order to start a seed production program with collaboration of the coffee and cassava starch producers commitees in Risaralda.

Table 1 – Local collected accessions under in vitro condition

#	Accession Name	# of plants	
		Propagation	For rooting
1	Algodona gigante	190	45
2	Algonona rápida	34	30
3	Chiroza	108	45
4	Verde	100	45
5	Algodona Grande	14	-
6	Varita	186	45
7	Patepava	73	45
8	Vajuna Pequena	96	45
9	Correita	53	45
10	Independencia mejoradada	52	45
11	Vajuna Armenia	49	45
12	Totoqueña	15	-
13	Falsa Chiroza	52	45
14	Algodona Grande	40	22
15	Sata	7	-
16	Chiroza Roja	75	24
17	Blanquita	40	22
18	Algodona Rosada	51	25
19	Cascara Roja	33	45
20	Parroquiana	89	45
21	Amarilla	31	20
22	Algodona	1	-
23	Algodona Grande	34	-
24	Algodona Pequena	62	30
25	Bajuna	22	10
26	Bajuna Grande	52	20
27	Chiroza	9	-
28	Mejorada Independencia	39	45
29	Panameña	33	-
30	Parroquiana	54	15
31	Patepava	28	45
32	Sata	33	45
33	Sauce	26	25
34	Regional Morada	40	36
35	Varita	113	45
36	SM 5080-1	93	45
37	Yuca Blanca	37	30
	Total	**2,064**	**1,074**

Table 2 – Seeds provided to the farmers from *in vitro* plants and multiplied by rapid propagationsystem (Semester I of 2004)

Organization	Farmer	Varieties	# of Seeds
Grupo Santa Ana	Noemí Larrahondo Mariela Larrahondo Genis Banguero	MPER – 183 MBRA – 383 CM 6740 – 7	1,800
Grupo Pescador	Oscar Vivas Juan Bautista	MCOL 1522	500
Grupo Arrobleda	Jaime Cuenca Gustavo Jaramillo (Ciat)	HMC – 1 CM 523 – 7 MPER – 183	2,000
Grupo Alegrías	Alfaro Mina	MPER – 183 HMC – 1	240
Grupo San Rafael	Otilia Hurtado Ramiro Viafara	CM 6740 – 7 CM 523 – 7 HMC – 1	4,000
Grupo Asoyotoco	German Moreno	HMC – 1 MPER - 183	2,600
Cabildo La Concepción	William	MCOL 1522	650
Grupo FIDAR	Carlos Hernández	CM 6740 – 7 CM 523 – 7 MPER – 183 MBRA – 383 HMC - 1	10,000
TOTAL			21,790

Table 3 – Available seeds, from *in vitro plants*, to be planted in September 2004

Variedad	# Semillas
HMC – 1	3,000
MPER – 183	900
MBRA – 383	950
CM 523 – 7	3,000
MCOL 1522	1,450
TOTAL	**9,300**

Project title

3.2.3 Farmer participatory *in vitro* cleaning and multiplication of local and improved cassava varieties

Institution: Embrapa Cassava and Fruits (Embrapa/CNPMF)
Address: Caixa Postal 007, 44380-000, Cruz das Almas, Bahia, BRAZIL
Email: cfukuda@cnpmf.embrapa.br
Collaborating institution:
Empresa Baiana de Desarrollo Agricola (EBDA)
Farming communities of Caetité (Southeast of Bahia, Brazil)

Staff directly involved
Chigeru Fukuda (Coordinator), Embrapa
Wania Fukuda, Embrapa
Osvaldo Pereira da Paz, Embrapa
Osório Vasconcelos, EBDA
Josué Cerqueira, EBDA

Introduction

This project is being carried out in Maniaçu region, Caetité municipality, Southwest Bahia, Brazil, aiming to introduce and implement participatory biotechnology methodologies with small-scale cassava farmers for cleaning and multipling cassava varieties by a low-cost rapid *in vitro* multiplication techniques developed at CIAT.

Accomplished activities and highlights

- Completion of the field laboratory for low-cost micropropagação of in vitro cassava plants and production of clean planting material for farmers at 'Lagoa de Fora' community, Caetité city (Figure 1).

- Production of 2,000 *in vitro* plants at CNPMF of the 7 selected cassava varieties (003, 005, 1318, 1389, 1393, Aipim Cachorro-local, and Lazã-local), which were transfered to Caetité lab for micropropagation and multiplication.

- A field area of 250 m^2 was isolated by fence, for multiplication of cleaned planting material.

- Two hundred cleaned plants, from recommended variety 'Formosa', were acclimated at CNPMF and field established in the multiplication field, close to Caetite's field lab.

- Construction of a field plastic greenhouse for hardening and chambers for rapid propagation (Figure 2).

- Training for 18 persons (farmers from different cassava growing communities, leaders from farmers associations, and EBDA's technicians), focusing all the lab and field steps for producing cleaned cassava planting material. This training was scheduled to attend the request of many cassava farmers from other communities, who have been highly enthusiastic by the project's implementation and by the activities accomplished after field lab establishment.

- The field laboratory, established in 'Caetité' have been very well accepted by the cassava farmers communities and by the local institutions, which are willing to assist its sustainable operation. A large article was published in the most popular Bahia's newspaper ('A Tarde' newspaper of 16/Feb/2004) reporting the achievements and importance of this project to the region.

Figure 1 - Field laboratory for low-cost micropropagação of *in vitro* cassava plants and production of cleaned planting material for farmers at 'Lagoa de Fora' community, Caetité, Bahia, Brazil.

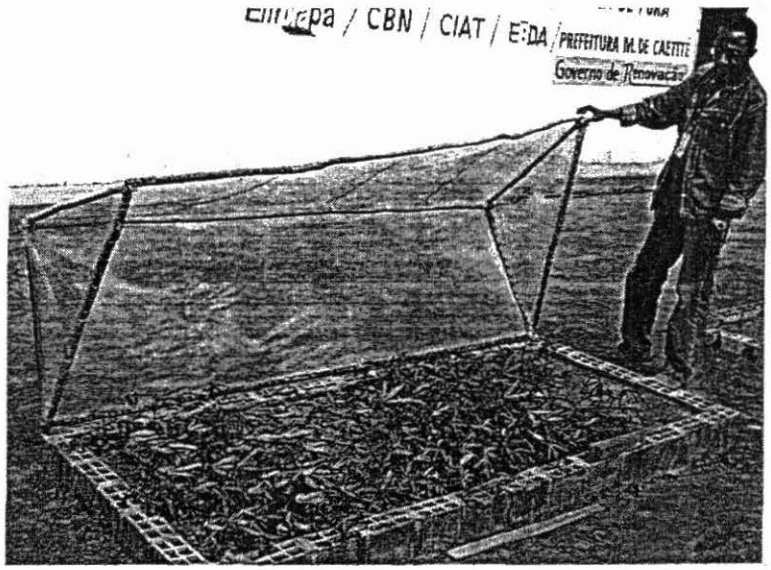

Figure 2 –Field chamber for rapid propagation of cleaned cassava planting material. Caetité, Bahia, Brazil.

Project title

3.2.4 Diagnosis of the use and production of cassava in Manabí province, Ecuador

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.

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.

Cassava zones in Manabí province

Zone #1

- Tropical semihumid
- Cassava production especially for self-consumption at farm level and for home.
- Incipient organization
- Woman's participation is high but not visible
- Cassava commercialization is not described
- North area of Ecuador which is adjacent with Esmeralda's county, from San Viciente up to Cojimies
- ***Provinces: Perdenales, Jama, Sucre, San Vicente (no survey)***

Zone #2

- Dry tropic
- Area of great cassava commercialization, especially for processed forms
- Woman's participation is high but not visible
- Low cassava production
- South area of Manabí, from Jipijapa up to division with Guayas
- Provinces: Jipijapa, Puerto López, 24 de Mayo, Olmedo, Paján

Zone #3

- Dry tropic
- High cassava production
- High organization of farmers
- Portoviejo and their surroundings, central area of Manabí.

- Dry area: Miguelillo
- Semihumid area: Jaboncillo
- Woman's participation is high but not visible
- Provinces: Manta, Montecristi, Jaramijó (no survey), Rocafuerte, Calceta, Pichincha, Santa Ana, Portoviejo, Bolívar, Junín

Zone #4

- Semihumid tropic
- Great commercial expansion and land use for cassava production
- High quality of processed forms
- High dependence of the cassava and its processed starches
- Very poor communities. High levels of malnutrition. High emigration.
- Low level of life quality, organization and participation.
- Low participation of woman
- From Junín to Chone
- Provinces: Tosagua, Chone, Flavio Alfaro, El Carmen

Additional information

- Cassava cultivated area in Ecuador: 20,000 ha, 60% concentrated in Manabí
- *According to an agricultural production census (performed in 2000), there are 20,577 UPAs (Unidades de Producción Agropecuaria), from which 17,846 have only cassava. The biggest cassava production is found in UPAs from 20 to 50 ha.*
- *In Santo Domingo there are 1,200 ha in plots from 5 to 10 ha.*
- *The intercropped cassava is found in 8,320 UPAs, which occupy 8,408 ha. This cultivation belongs to small and medium producers.*
- *The farm average size is from 5 to 10 ha. The associated crops are corn, beans, and peanut.*
- *There is no treatment for planting materials and no pest and disease control.*
- Classification of the plot size:

Province	*Small*	*Medium*	*Large*
Santo Domingo	up to 10 ha	10-100 ha	> 100 ha
Manabí	< 1 ha	1-5 ha	> 5 ha

Project title

3.2.5 Genetic manipulation of proline biosynthesis in cassava aiming to increased tolerance to water stress

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* The laboratories of Plant Biotechnology from both institutions involved in the present project are licensed by The Brazilian Ministry of Agriculture to conduct research on genetically modified organisms (CNPMF CQB 075/98 and IAPAR CQB 059/98).

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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.

Materials and Methods

Plant material

Cuttings of cassava genotypes MCol 22, Aramaris, Aipim-Brasil and three genotypes selected for their high β-carotene content (BGM 1153, BGM 1692 and BGM 1728) 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 (Figure 1). All cultures were maintained at 27±1°C under 16-hour photoperiod.

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 with 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.

Tissue Culture and Embryogenesis induction

Young leaves from in vitro grown plants were used as primary explants. Approximately 3 mm long leaves were excised and transferred to embryogenesis medium (Figure 2). Embryogenesis induction medium is according to Mathews et al. (1993).

Conclusions

The present research project is according to the proposed time table presented in the grant application.

Future directions

In order to ensure the continuity of the project on genetic transformation of cassava, a research proposal was submitted to the Brazilian Northeastern Bank (BNB). Such project which is a joined project with Universidade Federal do Ceará (UFC) was recently approved for a three-year grant. The total of the approved budget is R$62,000.00 (approximately US$20,000.00). The main objective of the proposed project is to establish genetic transformation protocols using cassava varieties adapted to Northeast Brazil. The gene of the antibacterial peptide sarcotoxin IA will be used aiming at resistance to bacterial blight caused by *Xanthomonas axonopodis* pv. *manihotis*. The sarcotoxin gene construct will also be used as a model for the establishment of genetic transformation protocol of five cassava varieties of both economical

and social importance. Such information is complementary and will be of great value to the present project supported by CBN.

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.

Table 1. Macronutrient, micronutrient, vitamins and growth regulators composition of the culture media used in the establishment of in vitro explant donor plants.

Macronutrient ($mg.L^{-1}$)	**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 ($mg.L^{-1}$)		
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 $g.l^{-1}$	20 $g.l^{-1}$
Agar	7 $g.l^{-1}$	7 $g.l^{-1}$
Phytagel	1.8 $g.l^{-1}$	1.8 $g.l^{-1}$

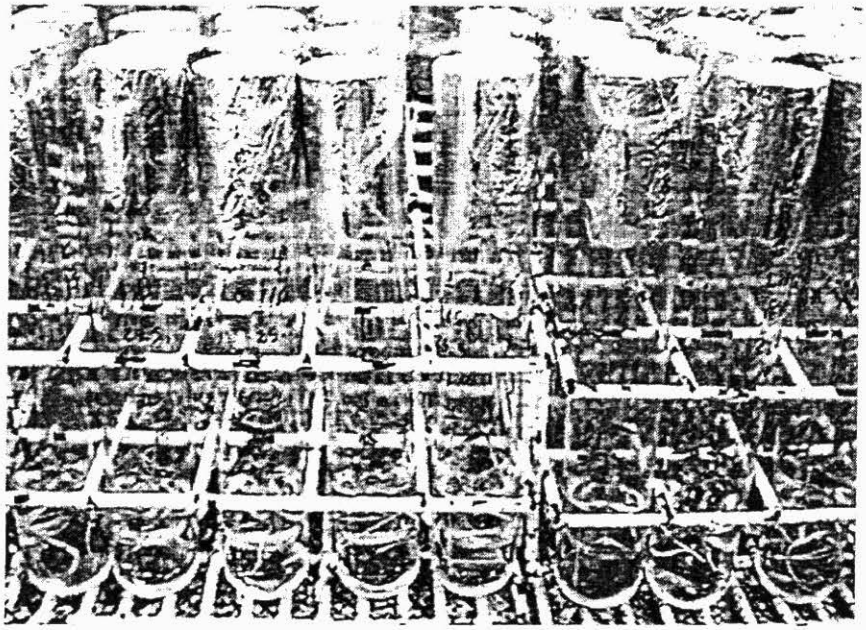

Figure 1. Explant donor plants of the six genotypes on rooting medium

Figure 2. In vitro grown explant donor plants (A). Explants on embryogenesis induction medium (B).

References

Groll, J.; Mycock, D.J.; Gray, V.M.; Laminski, S. (2001). Secondary somatic embryogenesis of cassava on picloram supplemented media. Plant Cell Tissue and Organ Culture 65, 201-210.

Mathews, H.; Schöpke, C.; Carcamo, R.; Chavarriaga, P.; Fauquet, C.; Beachy, R.N. (1993). Improvement of somatic embryogenesis and plant recovery in cassava. Plant Cell Rep. 15, 328-333.

Roca, W.M.; Rodrígues, J.A.; Mafla, G.; Roa, J. (1984). Procedures for recovering cassava clones distributed in vitro. CIAT, Colombia.

Sambrock, J.; Fritsch, E.F.; Maniatis, T. (1989). Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sarria, R.; Torres, E.; Angel, F.; Chavarriaga, P.; Roca, W.M. (2000). Transgenic plants of cassava (*Manihot esculenta*) with resistance to Basta obtained by *Agrobacterium*-mediated transformation. Plant Cell Reports 19: 339-344.

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.

Project title

3.2.6 Development of protocols for genetic transformation of cassava genotypes from Northeast Brazil

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Introduction

Genetic transformation is a reality for cassava and promises to speed up the improvement of the crop. The technology is limited, however, to few cultivars, mainly due to genotypic differences. So far there are no reports on transformation of Brazilian cultivars, albeit Brazil is the largest cassava producer and consumer in America. It was decided to select few Brazilian clones that were more suitable for genetic transformation by testing their *in vitro* regeneration capacity after transformation. CBN funded a small project to bring transformable tissues, mostly somatic embryos, from eight farmer-preferred cultivars from Brazil, as part of collaboration between CIAT, the Federal University of Ceará (Brazil) and Embrapa

Agroindústria Tropical (Brazil).

Materials and Methods

Somatic embryos from the following clones were induced in Brazil and brought to CIAT: Água Morna (BGM 365), Amansa Burro (BGM 549), Aparecida (BGM 123), Bujá Preta (BGM 1467), Milagrosa (BGM 004), Rosa (BGM 260), Rosinha (BGM 394) and Tapicina (BGM 1063). Embryos matured and produced green cotyledons that were used as explants, together with Friable Embryogenic Callus (FEC; Taylor et al) that was induced in CIAT in some of the clones.

Transformation tests were performed with *Agrobacterium* (Agl1-pCAMBIA1305.2; LBA4404-pGV1040). After cocultivation and elimination of bacteria, the explants were set up on media for organogenesis to produce shoots, under selection with antibiotics (hygromaycin or geneticin, depending on the plasmid). Shoots were regenerated and tested for the presence and expression of the markes genes *gus* or *gus*-Plus.

Results and Discussion

It was possible to regenerate shoots and plants from two varieties, Tapicina and Bujá Preta, which survived selection with hygromycin, after transformation with pCAMBIA1305.2. However, they were negative for the gus test (Figures 1). The test with real-Time PCR also showed the absence of the *hpt* gene in the regenerated plants (Figure 2). The most likely explanation of these results is that regenerated plants were escapes from selection. It is common when selecting shoots that regenerate from cotyledons, through organogenesis (Zhang et al 2000). Extended exposure to antibiotic will make shoots fail to elongate and produce plants, even if they are transgenic, while early removal of selection and induction of multiple shoots will enhance the proliferation of escapes. Zhang et al (2000) recommend increasing the selection up to15 mg/l of hygromycin, for up to two weeks, and maintaining it at 10 mg/l until shoot primordia develop.

Clone Bujá Preta was able to produce FEC, at low frequency, which was used for transformation with pGV1040. Unfortunately, putatively transformed FEC did not pass selection with geneticin for one week, indicating that it was probably too rough for cells to regenerate plants.

Recommendations

Although the results of the tests were not as expected, there were no transgenics, they should not preclude the research on transformation of Brazilian cultivars. As mentioned above, getting transgenics with these cultivars is probably a matter of fine-tuning the selection regime to avoid escapes. On the other hand, the fact that one cultivar produced FEC encourages the search for better ways to induce this easily transformable tissue in more clones.

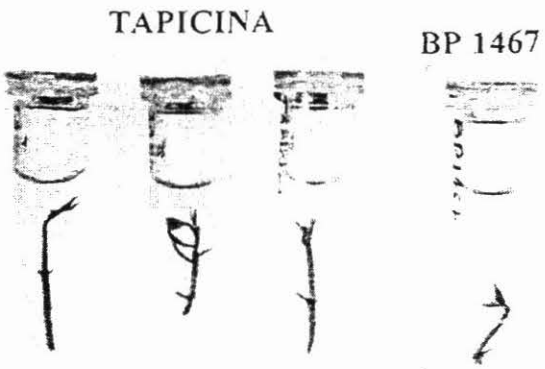

Figure 1. Gus tests for shoots regenerated from Tapicina and Bujá Preta (BGM 1467) after transformation of somatic cotyledons with Agrobacterium Agl1-pCAMBIA1305.2. None of the tissues showed the typical blue color.

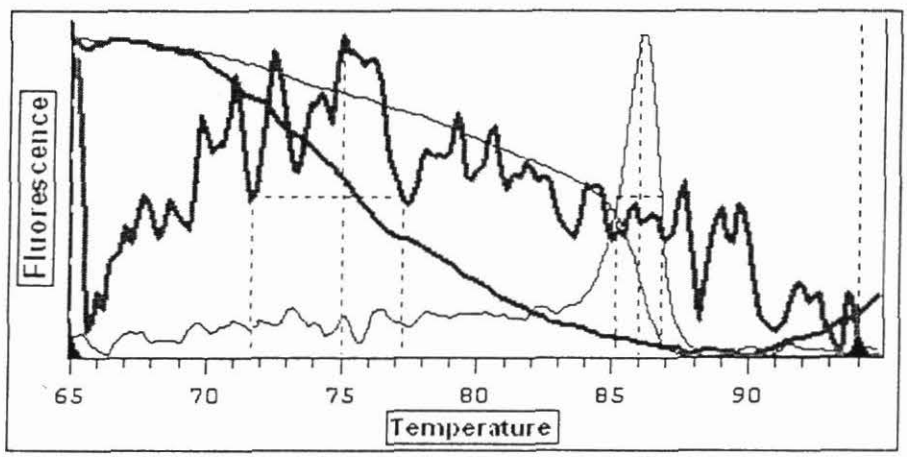

Figure 2. RealTime PCR to detect the *hpt* gene in Tapicina. The red peak indicates the presence of the gene in a positive control, the plasmid pCAMBIA1305.2, while the green lines show no evidence of overlapping peaks for the same gene in the DNA of clones Tapicina or Bujá Preta (not shown).

References

Zhang et al (2000) Plant Cell reports 19:939-945

Project title

3.2.7 Isolation of genes involved in the sugary phenotype in the storage root of cassava (*Manihot esculenta* Crantz)

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Introduction

In this study was explored the variability of free sugar and novel starch accumulated in the storage root of identified wild clones of cassava 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.

Outputs

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.

Conclusions Remarks

- The new sugary phenotype of cassava storage root showed to be related to several cellular processes as revealed by the differential gene expressed and identified by the subtractive cDNA libraries and macroarray analyses.
- The gene coding for the branching enzyme is not expressed in the storage root of the sugary clone CAS36.1.
- About six proteins are not present in the waxy locus of the amylase-free starch in the sugary clone CAS36.0.
- A new technology to produce a concentrate of Glucose has developed that do not involve either chemical or enzymatic hydrolysis of starch.

The outcomes of this study have been accepted for publication in the following article:

Carvalho, LJCB, de Souza, CRB, Cascardo, JCM, Junior, CB, Campos, L. 2003 Identification and characterization of a novel cassava (*Manihot esculenta* Crantz) clone with high free sugar content and novel starch. Plant Molecular Biology (Accepted)

Project title

3.2.8 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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Introduction

This project aims 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 in Cuba. 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. The project's activities is being carried out in municipalities of 'Santo Domingo', province of 'Villa Clara' (homogeneous environment), and in 'La Palma', community of 'San Andrés', province 'Pinar de Rio' (heterogeneous environment).

Accomplished activities and highlights

- Survey application to know how the flow of cassava and cocoyam seeds was 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. The main outputs from this survey was the relationship evaluation among farmers' groups and their roles within the process of acquisition, distribution and use of planting material. Also, this survey allowed the selection of the cassava varieties to used in this project (Blanca, CMC- 40, Prieta, Papa, Especial, Amarilla, and Señorita).

- 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 was to divulgate the project, discuss the general problems related to cassava and cocoyam cultivation, and establish the priorities of future actions.

- Accomplishment of the 2nd project's workshop aiming to establish the characteristics of *in vitro* plants and to arrange local conditions for *in vitro* plants of cocoyam, which have been distributed to the actors. Also, an adjusted timetable of activities and agreements were established. This event complemented the first workshop, when many farmers 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.

- Production of the first group of in vitro plants of the selected cultivares, in the laboratories of the participants research institutions. 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 have been cultivated in farmers' experimentation fields.

- Training at INIVIT for two actors from INCA, about cassava and cocoyam micropropagation process.

- Workshop on experimental methods and on development of facilities for *in vitro* plants climatization

- Facilities installation for *in vitro* plants climatization, by farmers from 'La Palma'.

Future directions

- Development of facilities for *in vitro* plants climatization in each community, according to the actors' initiative.

- Project's monitoring by visits and and participatory evaluation of the implemented experimental variants.

- Final workshop to evaluate the achieved outcomes and the project's constraints, aiming the planning of a 2nd phase's project.

- Preparation of a farmer's guide and a monograph on the project's results in order to divulgate project's outcomes to farmers, research centers, universities and scientists interested in the topic.

Project title

3.2.9 Application of molecular techniques for genotypes differentiation of Manihot spp.

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Introduction

The microsatellite technique is attractive to study due to the SSR (Simple Sequence Repeats) abundance in the plant genome and the high polimorphy and adaptability to the automation, and in turn, it has been appropriate for the cassava germplasm (Dixon *et al.*, 2002; Azudia *et al.*, 2002), for what can contribute to a better knowledge of the present genetic diversity in the cassava Cuban collection, and to the establishment of relationships between the accessions and their wild or cultivated relatives.

This study was carried out to evaluate the Cuban cassava genetic diversity and its phylogenetic relationships with cultivated relatives of Africa, South and Central America, in order to establish a sustainable management of the Cuban genetic resources.

Methodology

A total of 94 cultivars, according to their economic and/or genetic importance, were collected in the cassava Cuban collection held at 'Instituto de Investigaciones en Viandas Tropicales' (INIVIT, Cuba). Also, 54 clones coming from Africa and America were incorporated (12 from Nigeria, 10 from Tanzania, 12 from Guatemala and 20 from South America) and other 13 genotypes of genetic interest recommended by the International Center of Tropical Agriculture (CIAT, Colombia).

Thirty six SSR markers were evaluated. These markers were chosen by their clear bands pattern and robustness showed in several cassava diversity studies (Fregene *et al*, 1997; Mba *et al,* 2001). The analyses of diversity and genetic differentiation were developed from the data of 34 SSR markers, selected by their diallelic nature, very well defined patterns, and wide cassava genomic coverage.

Results and Discussion

From a total of 36 SSR markers evaluated, two were monomorphic (SSRY-127 and SSRY-132), while the rest of the primers presented a high level of amplification and polimorphism for the 142 cassava genotypes (*Manihot esculenta* Crantz) studied in five groups of different geographical origin: Cuba, Guatemala, Colombia, Nigeria, and Tanzania.

Genetic diversity

The genetic diversity indexes (Table 1) shows the high polimorphism observed in the evaluated microsatellites. Cuba presented the biggest average number of alleles per locus (5.8), and 100% of the loci polimorphics (the same as Guatemala). Cuba and Tanzania presented the highest indexes of medium observed heterozygosity (Ho). The medium expected heterozygosity (He) was 0.6292 ± 0.0120 (corrected for small sample sizes according to Nei (1978)).

The average proportion of individuals heterozigous (Ho) was high (0.5918 ± 0.0351). The estimates of diversity genetic He and Ho of the cassava from Latin America were not significantly different to Africa's, which reveals a comparable polimorphism level in Cuba, in relation to other studied population. It is interesting that although the total heterozygosity (Ht) is high (0.6538 ± 0.1770), only 7.4% of this is due to differentiation among accessions, the rest is due to variations among countries (Hs).

Genetic relationship among cassava accessions by geographical origin

Figure 1 shows the accessions distribution based on calculation of the genetic distances of the proportion of shared alleles (PAC) and estimates as 1-PAC, which is the most appropriate estimator for microsatellite data, assuming the pattern of infinite alelos (MAI) (Kimura and Crow, 1964) and when the relationships are narrow.

ACP represents the graphic relationships among accessions of different geographical origin with the particularity that the biggest number of accessions corresponds to Cuba, the rest of the genotypes represents an specific structure previously studied by other authors in their corresponding ACP.

There was a tendency of the Cuban clones to form two groups and to present genotypes in particular positions and far from those groups. There is a narrow relationship of some Cuban clones with two genotypes of Guatemala and Tanzania in the fourth quadrant.

Genetic variation within cassava Cuban germplasm

Although there are two very defined groups of Cuban germplasm, there is a wide variability among the accessions. Some clones move away from that structure due to very peculiar characteristics within Cuban cassava germplasm. The results found in Cuba correspond to the peculiar characteristic of the cultivated cassava germoplasma for many years (from aboriginals) in the island (Borda, 1975; Rodríguez et al., 2000), which has generated a differentiation of this population in relation to that found in the continent.

Conclusions

The cassava Cuban clones studied by SSR show a high genetic differentiation among them and although their diversity is high, there is evidence of their relationship with the cultivated genotypes of Africa, Central and South America.

Table 1. Genetic diversity within groups of cultivated cassava, classified by country. Standart deviation estimated by *jackknifing* on loci (200 reps). H_t, H_s, D_{st}, y G_{st} are given on loci and groups (countries).

Population	n	#loc	#loc_P	PLP	K	K_P	HO_p	HE_p	HEc_p
CUBA	86	34	34	100.0	5.8	5.8	0.6016	0.6314	0.6351
GUATEMALA	10	34	34	100.0	4.2	4.2	0.5556	0.6063	0.6385
COLOMBIA	11	34	33	97.1	4.5	4.6	0.5675	0.6087	0.6396
NIGERIA	16	34	33	97.1	4.5	4.6	0.5885	0.5949	0.6136
TANZANIA	10	34	31	91.2	4.2	4.5	0.6459	0.5869	0.6190
Mean	5 Popul.			97.06	4.64	4.72	0.5918	0.6057	0.6292
Stand Dev.				3.60	0.65	0.61	0.0351	0.0169	0.0120

	Ht	Hs	Dst	Gst
Mean	0.6538	0.6057	0.0482	0.0740
Stand Dev.	0.1770	0.1682	0.0253	0.0377
95% CI	0.5780	0.5341	0.0383	0.0618
95% CI	0.7137	0.6663	0.0585	0.0878

Leyenda:

n: Number of individuals
#loc: Number of loci
#loc_P: Number of polimorphic loci
PLP: Percentage of polimorphic loci
K: Number of alleles per locus
K_P: Number of alleles per locus polimorphic
HO_p: Observed heterozygosity
HE_p: Expected heterozygosity

Hec_p: Expected heterozygosity corrected for small size samples (Nei, 1978)
Ht: Total genetic diversity
Hs: Average genetic diversity within population
Dst: Average genetic diversity between population
Gst: Coefficient of genetic differentiation.

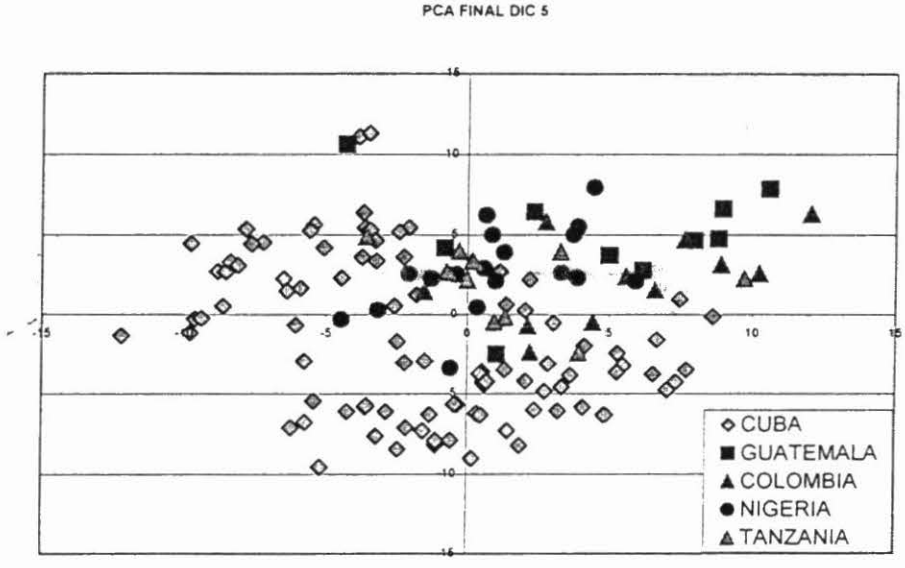

Figure 1. Analysis of Main Components (ACP) based on the distance 1-proportion of shared alleles (1-PAC), among cassava accessions of Cuba, Colombia, Guatemala, Nigeria, and Tanzania

References

Azudia C, Monte L, Debouck D, Fregene M (2002) Simple Sequence Repeat (SSR) Marker Assesment of Genetic Diversity of Cassava Land Races from Guatemala. Annual Report-2002, Project IP3: Improved cassava for the developing world. CIAT-2002, Output 8-17.

Dixon A, Raji A, Marin J , Fregene MA (2002) Simple Sequence Repeat (SSR) Marker Assesment of Genetic Diversity of Cassava Land Races from Nigeria. Annual Report-2002, Project IP3: Improved cassava for the developing world. CIAT-2002, Output 8-12.

Fregene M, Angel F, Gomez R, Rodríguez F, Chavarriaga P, Roca W, Tohme J, Bonierbale M (1997) A molecular genetic map of cassava. Theor Appl Genet 95: 431-441.

Mba REC, Stephenson P, Edwards K, Melzer S, Mkumbira J, Gullberg U, Apel K, Gale M, Tohme J, Fregene MA (2001) Simple Sequence Repeat (SSR) Markers Survey of the cassava (Manihot esculenta Crantz) Genome: Towards an SSSR-Bassed Molecular Genetic Map of Cassava. Theoretical and Applied Genetics. 102: 21-31.

Fregene MA, Bernal A, Duque MC, Dixon AGO, Thome J (2000) AFLP analysis of African cassava (Manihot esculenta Crantz) germplasm resistant to the cassava mosaic disease (CMD). Theor. Appl. Genet. 100:678-685.

Kimura M, Crow JT (1964) The number of alleles that can be maintained in a finite population. Genetics 49:725738.

Nei M (1978) Estimation of average heterozygosity and genetics distances from a small number of individuals. Genetics 89:583-590.

Project title

3.2.10 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*. 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.

Methodology

Plant samples

A selection of samples for SSR analysis was made considering geographical location and ecological characteristics. Final data sets include wild *M. leptophylla* designated by **Ant** and cultivated cassava from these different origins as listed in Table 1.

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. *Manihot esculenta* samples were collected in traditional fields in the western provinces of Manabí and Esmeraldas, coded collection number and site are listed in Table 1. Bitter *Manihot esculenta* samples were provided by Dr. Gerard Second from two native communities in French Guiana while in cooperation with PUCE.

Two groups of data were established given that during the course of this research samples of bitter cassava from French Guiana were depleted and had to be replaced by similar samples in the second data set. Coded sample names are given in Table 1.

Primers

Two groups of data sets were established. 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. The second data set used primers (Mba, et al 2001) selected based on information from M. Fregene (CIAT, com. pers.) in the attempt to produce data sets that could be compatible with similar diversity studies. The total number of primers used is 37 (Mba, et al 2001), which include the following: SSRY3, SSRY90, SSRY30, SSRY31, SSRY38, SSRY40, SSRY68, SSRY88, SSRY108, SSRY135, SSRY169, SSRY179, SSRY12, SSRY19, SSRY21, SSRY45, SSRY49, SSRY47, SRY52, SSRY61, SSRY59, SSRY79, SSRY63, SSRY106, SSRY110, SSRY82, SSRY175, SSRY34, SSRY102, SSRY171, SSRY51, SSRY182, SSRY5, SSRY177, SSRY69, SSRY181, SSRY164.

PCR conditions were based on Mba et al (2001). Visualization of polymorphisms were performed manually with a BioRad GT Sequencing System; silver staining was performed according to Promega®.

Data analysis included principal coordinate analysis using NTSYS v. 2.1. Possible introgressions were interpreted by the genotype allocation program STRUCTURE.

Results and Discussion

Results were obtained for each of the data sets. Multivariate analysis (Figure 1 & 2) shows a clear genetic differentiation of *M. leptophylla* from cultivated cassava, this supports once again that this wild species is genetically very distant from cassava and therefore cannot be considered a synonym to *M. esculenta* ssp. *flabellifolia*, as suggested by Allem (2002).

Among cassava varieties this study shows a genetic differentiation of bitter and sweet types (Figure 1 & 2), as has been documented previously (Elias & Santos et al, 2004; Chiwona-Karltun et al, 2003; Chiwona-Karltun, 1998; Narváez-Trujilllo et al., 2002), however this differentiation is of less importance than a geographic differentiation of the samples. Specifically, bitter cultivars are more genetically related to sweet cultivars from the Ecuadorian Amazonian Basin than the sweet cultivars between themselves. This points out that sweet cultivars from coastal Ecuador (western side of the Andes) have a different genetic pool than the cultivars from the eastern side of the Andes regardless of their sweet and bitter differentiation, at the same time, on the second axis of variation the sweet cultivars form the western side of the Andes are similar to *M. leptophylla* samples (Figure 1). These results suggest the existence of genepools in the cultivars from the western side of the Andes that are not explained by a one ancestor scenario, as has been suggested up to the moment (Olsen & Schaal, 1999) and may be suggestive of ancient gene flow from the local wild species once the domesticate form was wide spread giving way to a differentiated genepool; however conclusive evidence to support this idea must still be generated. It may also be indicative of on-going present day gene flow through introgression events. Regarding the second scenario, the results of this study show the existence of three individuals that support this process. Analysis through a random population allocation based on allelic frequencies software consistently supported gene flow in these individuals regardless the data set (results not shown).

Further statistical analysis is needed to confirm and expand these results, which will be adequately published.

References

Allem, A. 2002. The origins and taxonomy of cassava. In: L.J. Hillocks, J.M. Thresh and A.C. Belloti, eds. CAB International 2002 Cassava: Biology, Production and Utilization.

Mba, REC, P. Stephenson, K. Edwards, S. Melzer, J. Nkumbira, U. Gullberg, K. Apel, M. Gale, J. Tohme, M. Fregene. 2001 Simple sequence repeat (SSR) marlers survey of the cassava (*Manihot esculenta* CRANTZ) genome: towards an SSR-based molecular genetic map of cassava. TAG 102. 21-31.

Morillo, E., F. Fuenmayor, P. Cesar de Carvalho and G. Second. 2001 AFLP and SSR Polymorphism: Evidence of Significant Levels of Introgression from Manihot glaziovii and M. carthaginensis into Traditional Varieties of Cassava in their Area of Origin. In: Fauquet, CM y Taylor, NJ, eds. Cassava: An ancient crop for modern times. [Compact disc] Proceedings 5th International Meeting of the Cassava Biotechnology Network. 2001 November 4-9, St. Louis Mo. USA.

Narváez-Trujillo, A. & G. Second. 2002. *Manihot leptophylla* (Euphorbiaceae), una especie restringida a la costa pacífica de Colombia y Ecuador y su relación con otras especies del género. En: Rangel-Ch., J.O., J Aguirre-C & M.G. Andrade-C (eds.) Libro de Resúmenes VIII Congreso Latinoamericano de Ciencias y Segundo Colombiano de Botánica. Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá.

Table 1. List of samples, including scientific names, type of variety and geographical location, used in the molecular characterization of genetic diversity using 37 SSR primers (Mba, et al, 2000).

DATA SET 1

Sample Name	Species	Type	Geographical origin
Bit12	*Manihot esculenta*	Bitter	French Guiana
Bit13	*Manihot esculenta*	Bitter	French Guiana
Bit14	*Manihot esculenta*	Bitter	French Guiana
Bit15	*Manihot esculenta*	Bitter	French Guiana
Bit10	*Manihot esculenta*	Bitter	French Guiana
Bit11	*Manihot esculenta*	Bitter	French Guiana
Bit16	*Manihot esculenta*	Bitter	French Guiana
Est1	*Manihot esculenta*	Sweet	Napo, Ecuador
Est2	*Manihot esculenta*	Sweet	Napo, Ecuador
Est3	*Manihot esculenta*	Sweet	Napo, Ecuador
Est4	*Manihot esculenta*	Sweet	Napo, Ecuador
Est5	*Manihot esculenta*	Sweet	Napo, Ecuador
Est11	*Manihot esculenta*	Sweet	Napo, Ecuador
Est6	*Manihot esculenta*	Sweet	Napo, Ecuador
Est7	*Manihot esculenta*	Sweet	Napo, Ecuador
Est8	*Manihot esculenta*	Sweet	Napo, Ecuador
Est9	*Manihot esculenta*	Sweet	Napo, Ecuador
Est10	*Manihot esculenta*	Sweet	Napo, Ecuador
Wst1	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst2	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst3	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst4	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst5	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst6	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst7	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst8	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst17	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst9	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst10	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst11	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst12	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst13	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst14	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador
Wst15	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador

Wst18	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador
Wst16	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador
Lep1	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep2	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep3	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep4	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep5	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep6	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep7	*Manihot leptophylla*	Wild	Esmeraldas, Ecuador
Lep8	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep9	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep10	*Manihot leptophylla*	Wild	Esmeraldas, Ecuador
Lep11	*Manihot leptophylla*	Wild	Esmeraldas, Ecuador
Lep12	*Manihot esculenta*	not cultivated	Pichincha, Ecuador
Lep13	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep14	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep15	*Manihot leptophylla*	Wild	Los Ríos, Ecuador
Lep16	*Manihot leptophylla*	Wild	Los Ríos, Ecuador
Lep50	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep17	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep49	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep18	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep19	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep20	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep21	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep22	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep23	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep24	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep25	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep26	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep27	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep28	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep29	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep30	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep31	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep32	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep33	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep34	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep35	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep36	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep37	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep38	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep39	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep40	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep41	*Manihot leptophylla*	Wild	Manabí, Ecuador

Lep42	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep43	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep44	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep45	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep46	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep47	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep48	*Manihot leptophylla*	Wild	Manabí, Ecuador

DATA SET 2

Sample Name	Species	Type	Geographical origin
Bit1	*Manihot esculenta*	Bitter	French Guiana
Bit2	*Manihot esculenta*	Bitter	French Guiana
Bit3	*Manihot esculenta*	Bitter	French Guiana
Bit4	*Manihot esculenta*	Bitter	French Guiana
Bit5	*Manihot esculenta*	Bitter	French Guiana
Bit6	*Manihot esculenta*	Bitter	French Guiana
Bit7	*Manihot esculenta*	Bitter	French Guiana
Bit8	*Manihot esculenta*	Bitter	French Guiana
Bit9	*Manihot esculenta*	Bitter	French Guiana
Bit10	*Manihot esculenta*	Bitter	French Guiana
Bit11	*Manihot esculenta*	Bitter	French Guiana
Est1	*Manihot esculenta*	Sweet	Napo, Ecuador
Est2	*Manihot esculenta*	Sweet	Napo, Ecuador
Est3	*Manihot esculenta*	Sweet	Napo, Ecuador
Est4	*Manihot esculenta*	Sweet	Napo, Ecuador
Est5	*Manihot esculenta*	Sweet	Napo, Ecuador
Est6	*Manihot esculenta*	Sweet	Napo, Ecuador
Est7	*Manihot esculenta*	Sweet	Napo, Ecuador
Est8	*Manihot esculenta*	Sweet	Napo, Ecuador
Est9	*Manihot esculenta*	Sweet	Napo, Ecuador
Est10	*Manihot esculenta*	Sweet	Napo, Ecuador
Wst1	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst2	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst3	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst4	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst5	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst6	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst7	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst8	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst9	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst10	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst11	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst12	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst13	*Manihot esculenta*	Sweet	Manabí, Ecuador
Wst14	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador

Wst15	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador
Wst16	*Manihot esculenta*	Sweet	Esmeraldas, Ecuador
Lep1	*Manihot leptophylla*	Wild	Manabí, Ecuador
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Lep4	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep5	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep6	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep7	*Manihot leptophylla*	Wild	Esmeraldas, Ecuador
Lep8	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep9	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep10	*Manihot leptophylla*	Wild	Esmeraldas, Ecuador
Lep11	*Manihot leptophylla*	Wild	Esmeraldas, Ecuador
Lep12	*Manihot esculenta*	not cultivated	Pichincha, Ecuador
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Lep16	*Manihot leptophylla*	Wild	Los Ríos, Ecuador
Lep17	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep18	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep19	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep20	*Manihot esculenta*	not cultivated	Esmeraldas, Ecuador
Lep21	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep22	*Manihot leptophylla*	Wild	Guayas, Ecuador
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Lep24	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep25	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep26	*Manihot leptophylla*	Wild	Guayas, Ecuador
Lep27	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep28	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep29	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep30	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep31	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep32	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep33	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep34	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep35	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep36	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep37	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep38	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep39	*Manihot leptophylla*	Wild	Manabí, Ecuador
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Lep41	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep42	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep43	*Manihot leptophylla*	Wild	Manabí, Ecuador

Lep44	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep45	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep46	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep47	*Manihot leptophylla*	Wild	Manabí, Ecuador
Lep48	*Manihot leptophylla*	Wild	Manabí, Ecuador

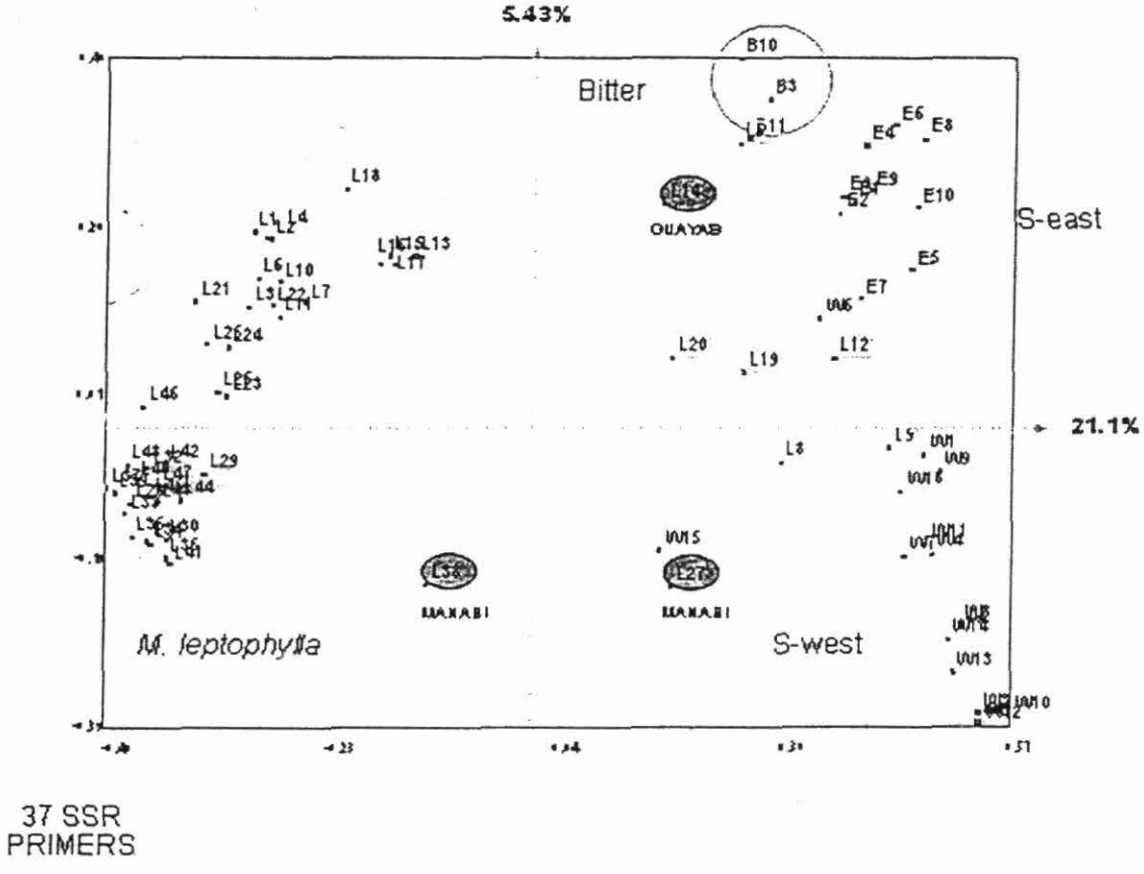

Figure 1. Principal coordinate analysis based on Dice genetic distance of the total sample set. Putatively introgressed individuals are L27 and L38. S-west = sweet cultivars from western Ecuador, S-east = sweet cultivars from Ecuadorian Amazon Basin, Bitter = bitter cultivars from French Guiana. *M. leptophylla* is the local wild species

Project title

3.2.11 *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.

This project started in March 2004. The following initial activities were accomplished: workshops about socialization and gender aspects, and collection of information of the 20 target communities.

Expected 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.

Project title

3.2.12 Cleaning of released varieties and recovery of cassava genetic resources in production area of Ecuador

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Staff directly involved

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Elizabeth Caicedo, CBN-CIAT
Roosevelt Escobar, CIAT

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.

This project started in July 2004. The participant's team have worked on the organization of the groups of communities that will participate in this project.

3.2.13 Other CBN Activities

Organization of the Sixth International CBN meeting (CBN-VI)

From 8 to 14 March 2004, CIAT hosted the Sixth International Scientific Meeting of the Cassava Biotechnology Network (CBN-VI) with 148 participants from 28 countries from all continents. The theme of the meeting was "***Adding value to a small-farmer crop***" to discuss how biotechnology tools can assist small farmers by developing a range of technologies to add value and income to cassava farmers.

Several topics were discussed in the 40 plenary tematic lectures and 125 posters presentations, from which can be mention the following scientific highlights:

Transgenics are a reality and are moving rapidly from the lab to the field.
Genome mapping with greater saturation of markers, nearing identification of CMV (cassava mosaic virus) resistance gene, and extensive EST libraries.
Biodiversity and population genetics with variations in pigments, architecture, germination, cyanogens, photosynthesis, drought tolerance, etc.
ACMV (African cassava mosaic virus) with new variants and satellite genome
Post harvest deterioration: functional genomics moving forward
Double haploids: identification of recessive traits to avoid genetic load
Expression profiles of pathogens in cassava: bacterial blight
IPM and biological control of insect pests.
Improved industrial varieties in SE Asia.
In vitro plants: successful propagation of disease-free plants for small farmer's groups in Colombia.

The plenary presentations, posters, and others CBN-VI's documents can be found at CBN website:
http://www.ciat.cgiar.org/biotechnology/cbn/sixth_international_meeting/index.htm

Strengthening participatory process and gender analysis in CBN projects

The CBN's social scientist, Elizabeth Caicedo, has provided a consistent contribution to the CBN's projects carried out in Colombia, North of Cauca Department. This contribution aims the evaluation of the participatory processes generated in the on going projects, stimulating activities with farmer participation, gender analysis, and biotech tools. Several meetings, involving regional cassava growing communities, as well as workshops, were accomplished in order to establish this colaboration. Some events and their objectives are highlighted below:

Meeting with representatives of the AMUC (Municipal Association of the Cauca's User Farmer) (Santander de Quilichao, 28/Jan/04) – Discussion of alternative methods for seed production and characteristics of *in vitro* propagation.

Workshop on elaboration of projects with gender perspectives for participants of the cassava development poles (CIAT, 16-19/Mar/04) – Stimulate the elaboration of project's proposal focusing gender analysis. Some proposals were drafted aiming the CBN small grants program.

Workshop on cassava pathology (CIAT, 01/Jul/04) – General recommendations to achieve a free disease cassava cultivation (with collaboration of John Loke, CIAT's cassava virology unit).

Workshop about new cassava varieties and management and control of white fly and frog skin disease (Santander de Quilichao, 25/Aug/04) – Coordinated by Roosevelt Escobar, Ginés-Mera fellowship.

3.2.14 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. The fund aims, mainly, to provide opportunities and support to female and male master and doctorate'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.

A total of 7 MSc fellowships, for Andean Region, were approved and started in October/2003. For the 2004 second round will be offered 5 MSc fellowship for Uganda and Rwanda and 1 doctorate award for Andean Region (Colombia, Ecuador or Peru).

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 updated. 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.

In addition, CBN has established a great number of informal linkages with other networks and organizations to inform them about activities and exchange information.

Activity 3.3 Databases and Libraries

Bio Informatics Support at CIAT Annual Report

3.3.1 Bioinformatics

"The collection, classification, storage, and analysis of biochemical and biological information using computers, especially as applied in molecular genetics and genomic".

This Report brings information about the main activities involved in the Bio Informatics Support at CIAT; and will be focused in the 2 main topics:

- Maintenance and Support of the Hardware and Software Tools for BioInformatics for this year
- The participation of CIAT in the Generation Challenge Program(GCP) – SP4 Subprogram.

Fernando Rojas ;Joe Tohme; Mathias Lorieux; GCP Participant Institutes

Introduction

CIAT has established a platform of hardware and Software for the support of it's research activities in the Biotechnology Area, and the activities of maintenance and support of that platform for this year are reflected in this report.

CIAT is one of the Institutes participating in the Generation Challenge Program (http://www.generationcp.org/) this challenge Program involve the capture, storage, integration, analysis, and dynamic dissemination of substantial volumes of diverse and dispersed genomic, genetic resource, and crop improvement information.

The challenge of linking and integrating these information components into a coherent information gateway will therefore play a central role within the Challenge Program and forms the basis of SP4 (BioInformatics Subprogram of the GCP)

Methodology

- Research Activities about Software Tools, Databases and Gathering of Data Locally.
- Downloading, implementation, test and Deployment of the distinct Software Tools
- Interaction with scientists for the effective use of the tools
- Participate in the SP4 subprogram of the GCP

Results:

- **Hardware Tools:**

Actually at CIAT we have established this Hardware Tools:

GENE3	GENE4
Servidor LINUX (Red Hat Linux 8.0.3-27) Procesador PEntium III Intel a 1400MHZ Memoria: 2 GB Disco: 65 GB ModePerl 1.29 and Mode Python Installed PHP 4.3.4; Apache 1.3.24 MySql 3.23.49	Servidor LINUX (Red Hat Linux 8.0.3-27) Procesador PEntium III Intel a 1400MHZ Memoria: 1 GB Disco: 32 GB PHP 4.3.4; Apache 1.3.24 PostgreSQL 7.3.4

- **Software Tools**

This year the software tools installed and deployed at CIAT are :

EMBOSS is "The European Molecular Biology Open Software Suite ".

EMBOSS is a free Open Source software analysis package specially developed for the needs of the molecular biology (e.g. EMBnet) user community. The software automatically copes with data in a variety of formats and even allows transparent retrieval of sequence data from the web. Also, as extensive libraries are provided with the package, it is a platform to allow other scientists to develop and release software in true open source spirit. EMBOSS also integrates a range of currently available packages and tools for sequence analysis into a seamless whole. EMBOSS breaks the historical trend towards commercial software packages.

GUI:

W2H is a free WWW interface to sequence analysis software tools like the GCG-Package (Genetic Computer Group), EMBOSS (European Molecular Biology Open Software Suite) or to derived services (such as HUSAR, Heidelberg Unix Sequence Analysis Resources).

It tries to cover as much functionality as possible, and to do it as user friendly as we could. It gives you the opportunity to access more than hundred programs from any computer platform with a JavaScript enabled web browser. The development of W2H started in 1996 by Martin Senger at the DKFZ (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and is maintained since 1997 in a collaborative project between DKFZ and EMBL-EBI (European Bioinformatics Institute, Hinxton, UK) by Peter Ernst and Martin Senger..

At ciat: http://gene3.ciat.cgiar.org/w2h or through the Bio Informatics site:

http://gene3.ciat.cgiar.org/blast/inicio.htm

STACKPACK with D2_CLUSTER and CRAW:

The stackPACK clustering system allows rapid clustering, alignment and analysis of ESTs as well as full-length sequences by providing sophisticated gene indexing capabilities that go well beyond other clustering and assembly tools.
The stackPACK system accepts input in GenBank flat file format or FASTA format. In addition to these, stackPACK also accepts PHRED quality score file formats.

Clustering Data The clustering step of stackPACK uses **d2_cluster**, a high-performance comparison algorithm that rapidly determines the relative similarity of large datasets of genetic sequences.

CRAW (CRAWview: for viewing splicing variation, gene families, and polymorphism in clusters of ESTs and full-length sequences ; Chou A., Burke J.; Bioinformatics 15 (5) 376-381) is thus employed to analyze alignments, partition sub-assemblies and provide a simple means to view clusters. After CRAW processing, stackPACK further analyzes clusters to refine consensus sequences, maximize consensus sequence length, create final alignments and to select the best consensus sequence

http://gene3.ciat.cgiar.org/stackpack

http://gene3.ciat.cgiar.org/stackpack/support/commandline.html

3.3.1 A database of phenotypic data collected on 10,000 rice T-DNA insertion lines

M. Lorieux – J. Lozano – E. Robayo
Partners: IRD
Cirad
Génoplante
Project funded by IRD and 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 (Sallaud et al 2003). Rice was chosen as a model species because of its small genome and because of all the genomic resources available for this species (ESTs, genetic maps, complete sequence, etc.). The lines were 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. We focus here on the first topic.

Materials and Methods

Screenhouse
Ten thousand T_0 plants were produced at Cirad and grown in Cirad and IRD glasshouses in Montpellier, France. Twenty-five T_1 seeds per T_0 plant were received at CIAT and were sown in a screenhouse. Sowing was carried out in eight batches of 1,250 lines, with about three weeks delay between the batches. Seeds were pre-treated by heat for three days at 50 °C to break dormancy, and planted in plastic trays with a mixture of CIAT (67 %) and Santander de Quilichao (33 %) soils. Germination was determined at ten days after sowing (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 four-hectares field was set up following the requirements of the ICA (Instituto Colombiano Agropecuario). The entire field was covered by nets to avoid damage and seed dissemination by birds. Plantlets were transplanted at 25 DAS. A basic fertilization composed of Mono-Ammonium Phosphate, Iron Sulfate, Potassium Chloride and micro-elements was applied. The field was irrigated two times a week. Control lines of Nipponbare cv. were planted for 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 done when the plants were approximately 45 days old. A second evaluation was done at flowering, while the ultimate observation was done at maturity. This maximized the chances to detect phenotypic variations, as various traits could be observed at only one of these stages. Moreover, this permitted to follow the evolution of a suspected phenotype at early stage and possibly confirm or invalidate it.

Results

Mutant Phenotypes

In the screen house, 8.5 % of the lines showed phenotype variation in comparison to the wild type. In the field, the rate was of 13.6%. The overall mutant phenotypes percentage was 16.7 %.

Numerous lines showed chlorotic or albino plantlets, with associated deficiency in leaf development. General abnormal development was also frequently observed. The most common phenotypes included several types of albinism, sterility, dwarfism more or less pronounced, chlorotic leaves, rolled leaves, awning, modified leaf shape, white streaks, lesion mimics, general abnormal development, late flowering, round hull, modified tillering.

Redundancy of phenotypes was frequently observed between two or more lines. This is probably due to the fact that these lines proceed from the same transformation event. We thus applied a correction to the calculation of the percentage of observed mutant phenotypes. If several lines proceed from the same callus and share at least one trait, only the first entry is retained. This leads to a corrected estimation of 12.2 % of mutant phenotypes (screen house + field).

Database Set Up

A local database of all data relative to growth conditions, germination, flowering, and phenotypic observations was set up. This database is mainly used as a working tool to facilitate data entry and compilation. However, it also can be used for data browsing, as it permits the display of information by mutant bar code number or CIAT number. Several

options for searching for lines or traits according different criteria are available. Moreover, the database offers tools for computing basic statistics over traits and lines.
This database also displays photographs of the mutant phenotypes (see screenshots for details). More than 18,000 photos are available.
A flat data file is regularly extracted form the database in order to fill the Génoplante Oryza Tag Line database available online (http://genoplante-info.infobiogen.fr/OryzaTagLine).
Discussion
The overall mutation rate was higher than it is currently observed in other mutant collections, where visual phenotypic screening typically identifies about 3 to 5% of mutants. A part of that excessive mutation rate could be eliminated by clone redundancy analysis. 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 Retarded Growth (RG), tillering, height and delayed flowering. Also, in some cases we chose to include some doubtful data, as it is preferable to eliminate false-positive data after more detailed analyses for a specific trait than to miss real data.

Conclusion

The overall process of seed multiplication and phenotypic analysis worked very well. The timetable was respected, and valuable phenotypic data were produced. The phenotypic database will constitute a precious tool for selecting lines for functional genomics studies. We plan to extend the phenotypic analysis and seed multiplication to the entire collection (35,000 lines).

References

C Sallaud, D Meynard, J van Boxtel, C Gay, M Bès, J P Brizard, P Larmande, D Ortega, M Raynal, M Portefaix, P B F Ouwerkerk, S Rueb, M Delseny, E Guiderdoni. 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theoretical and Applied Genetics 106:1396-1408.

Figure 1. Screenshots of the rice T-DNA insertion lines phenotypic database
Figure 1a. Main menu

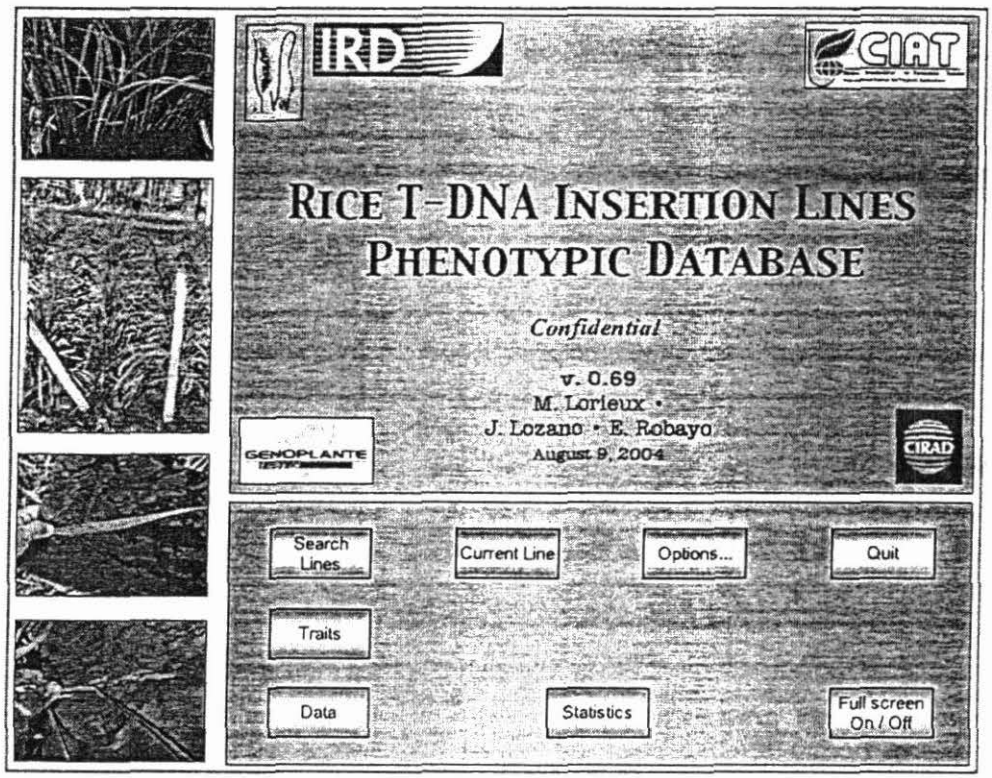


Figure 1b. Trait browsing

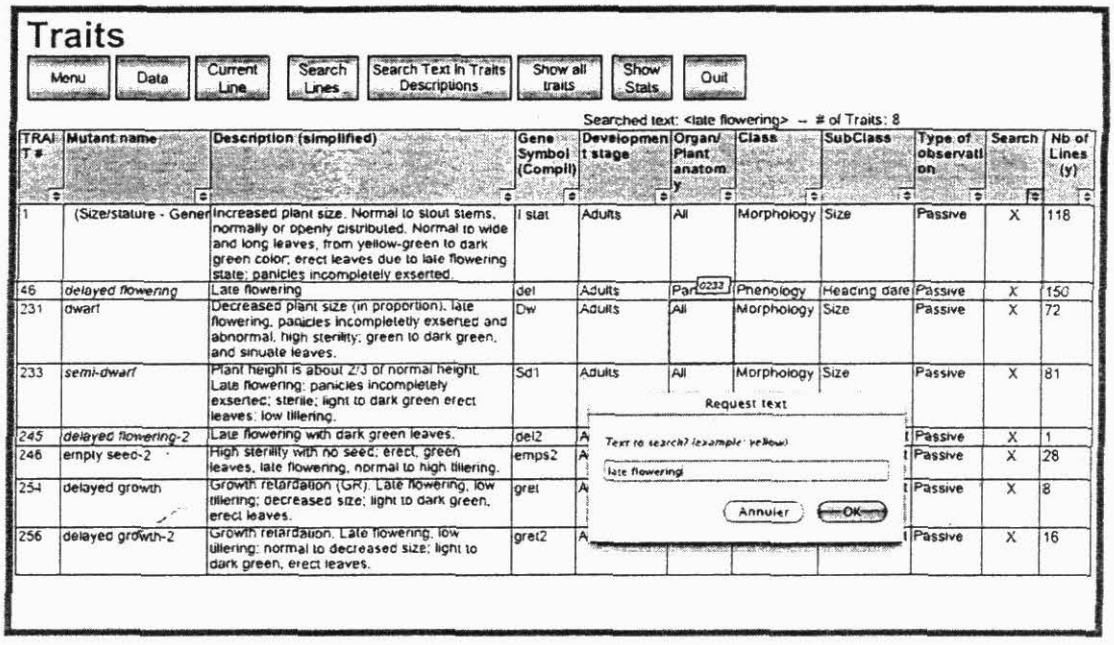


Figure 1c. Search for lines by trait code or full text

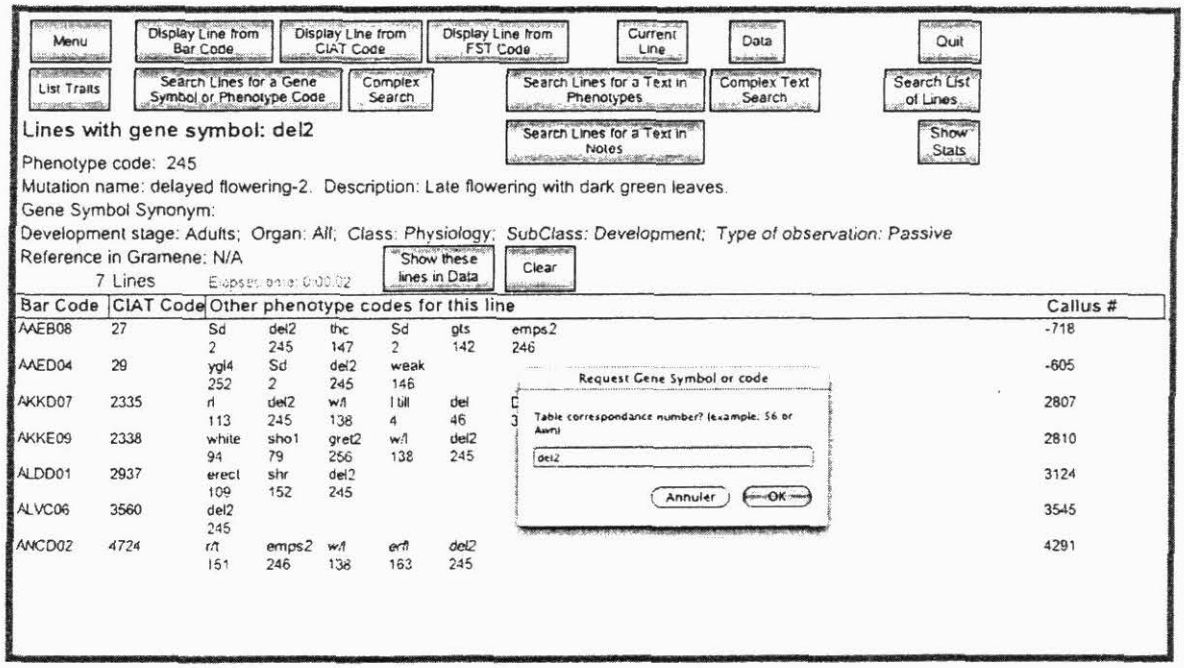


Figure 1d. Statistics on traits and lines

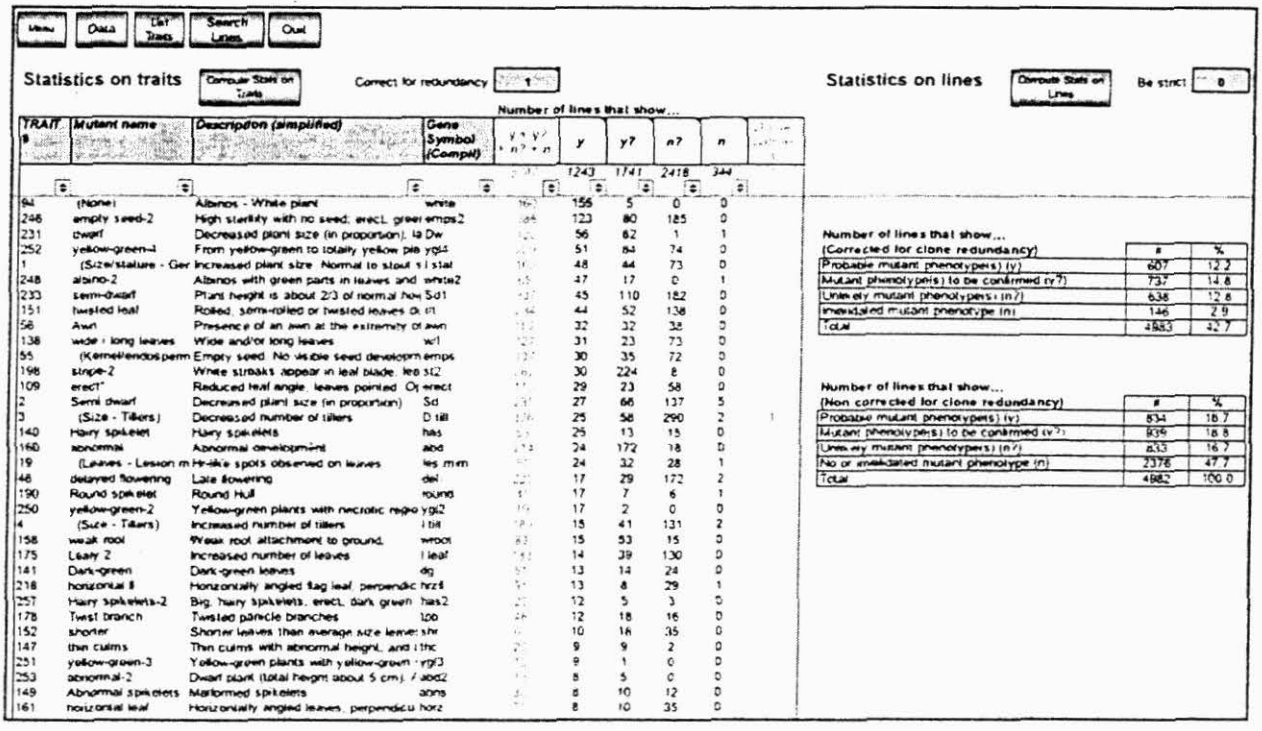
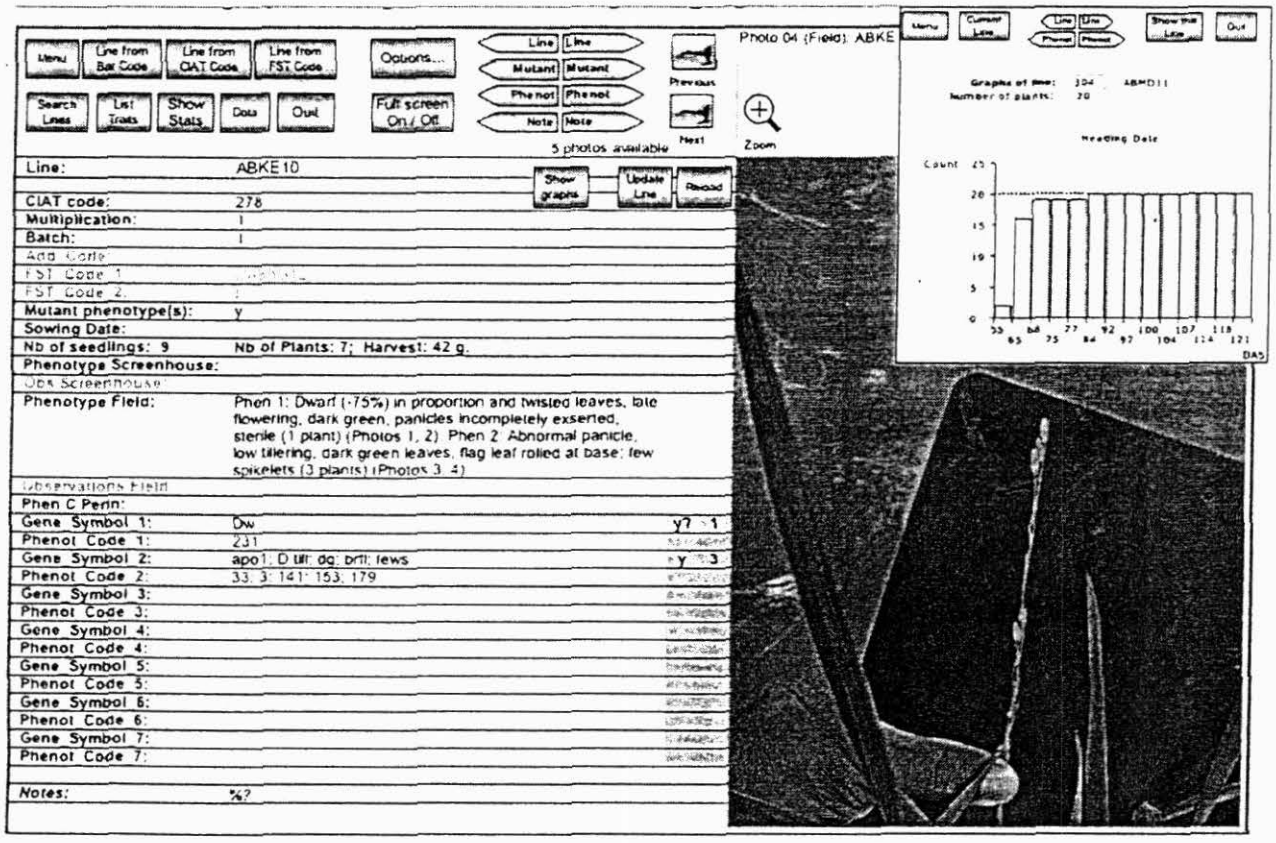


Figure 1e. Display of phenotype, codes, statistics, heading date graphics and photographs for each line



3.3.2 The Generation Challenge Program – SP4 at CIAT

The Generation Challenge Program (GCP) Platform & Network established (Fig 1)

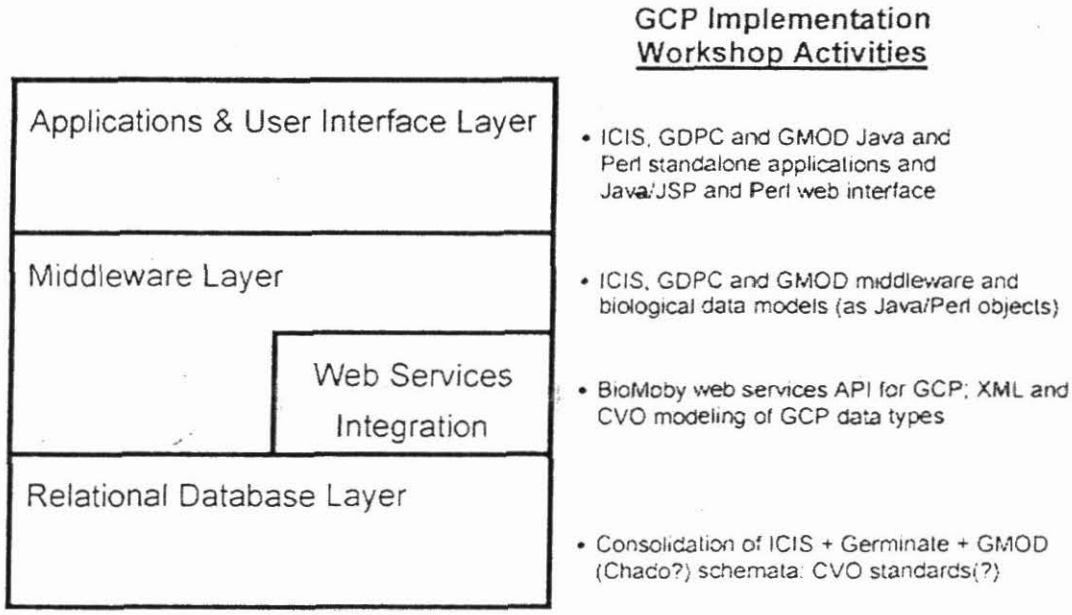
Web Services Technology

A web service is a WWW based Interface to a program running on a host computer that allows that program to be run, and the results returned, from anywhere on the Internet.Web services typically use a messaging protocol called "Simple Object Access Protocol" (SOAP), which defines a message structure that contains both data, and the information the host computer needs to map the incoming data to the appropriate program. SOAP Interfaces are described by a machine readable document written in a form of XML Called Web Service Description Language (WSDL).

In the web service paradigm, a third party "registry" holds metadata about web services, and acts as a broker between a client who is looking for a data service, and the service provider that can accomplish this request. Human intervention is required at several steps of the process: Creation of the metadata query to discover services of interest, selection of that service, and (often) manipulation of data prior to service execution such that it conforms to the service providers interface.

The reference implementation of the GCP information platform will be based on the International Crop Information System (ICIS; http://www.icis.cgiar.org/), in particular, the new Java language based implementation.
To meet the genotyping needs of the GCP, ICIS will be extended to handle molecular data using the basic design of the "Germinate" schema (http://bioinf.scri.sari.ac.uk/cgi-bin/germinate/germinate.cgi) and the GDPC middleware (http://www.maizegenetics.net/gdpc/index.html).
The reference implementation of the GCP information network will be based on both Java and Perl language bindings of the BioMoby web services technology (http://www.biomoby.org/). The resulting systems will also incorporate technology relating to controlled vocabulary and ontology inspired by community standards such as the Gene Ontology Consortium (http://www.geneontology.org/) and the Open Biological Ontology (http://obo.sourceforge.net/). We will also focus on high level (UML) data modeling of GCP relevant biological concepts and entities, captured as a first draft XML DTD specification. The reference implementation may be represented graphically (Figure 1).

Figure 1 – GCP Platform & Network Architecture



Why BioMOBY – Executive Summary:

• The more simplistic of two extant Web Services systems (the other being myGrid (http://www.mygrid.org.uk/) capable of mapping biological datatypes to their applicable services
• Implementation within minutes!
• Strong support from the Plant genomic community (for historical reasons)
• Community driven, needs based development philosophy
• Open source, freely available
• Strong, responsive distributed online support community
• Uses existing standards as much as possible
• Arbitrarily extensible to new areas of biological and/or non biological

knowledge and data

• Increasing support in existing browsers and client side tools and libraries.

Conclusion

Stackpack with d2_cluster and craw is a key software in the process of the clustering pipeline for CIAT

BioMOBY support will soon be integrated into the Rat Genome Database, and is currently being built into standalone applications such as the workflow management

systems Taverna (http://taverna.sourceforge.net/) from the European Bioinformatics Institute, and Pegasys (http://www.bioinformatics.ubc.ca/pegasys/) from the Bioinformatics Centre of the University of British Columbia

Future Plans

- **Implement of the Clustering Pipeline**
- **Implement of Web-Services Technology Based in *BioMoby***
- **Research and Install New Software tools as:**

  G-pipe: Graphical Pipeline Generator; PISE: Web Interfaces for G-Pipe

- CIAT Will develop (BioMoby web services based) adaptor modules in (Java/Perl) to CIAT's non-ICIS public germplasm, genomic and related databases
- CIAT will Expose SINGER(cassava, Bean, Forrages) data-banks with WEB-SERVICES technology
- *Continue working in the tasks 10-11 SP4 in GCP (Integrated Germplasm* Information System) and in task 13 (LIMSYS TASK)

3.3.3 CIAT Bioinformatics Platform

Rojas, Fernando; Soto-Suárez, Mauricio; Galindo, Leonardo Miguel and Joe Tohme. Biotechnology Research Unit, Centro Internacional de Agricultura Tropical (CIAT), AA 6713, Cali, Colombia.

Introduction

At CIAT we are working on research of the central crops in the national economy (cassava, bean, rice). We have developed molecular biology technologies aiming to the improvement of crop productivity. The application of these technologies at different levels such as DNA sequencing, mapping and gene expression analysis have generated a high amount of biological data. Additionally, there is a high amount of biological information in multiple levels (phenotypical, genotypical, physiological) that should be linked through computational biology.

Currently, the available information generated by CIAT is scattered and not standardized (different operating systems, different hardware platforms, different programming languages). Furthermore, the on-line bioinformatics databases are heterogeneous and not completely known (Gramene, TAIR, AcDB, NCBI), and the platforms to work at a high throughput level are not well implemented. Finally, the necessary tools for the storage, manipulation, analysis and sharing of the information is scarce or non-existent. For these

reasons the development and application of bioinformatic resources is necessary to make an efficient and in-depth analysis of the information generated by CIAT.

We are developing a Bioinformatics Platform to manipulate and analyze a high amount of information. Initially, we have focused on a Bioinformatics Platform in sequence analysis. However, in the near future the platform will connect all this sequence information with Microarray analysis, SNPs (Single Nucleotide Polymorphism), phenotypic and field information.

Methodology

For the implementation process we performed the following steps:

- Biological Questions: Identify the main biological questions present in the different projects.
- Databases filtering: Using the information obtained with the biological question, we selected all on-line databases that can help us to solve such questions.
- Software filtering: We selected also all on-line software coupled to databases that can help us to solve biological questions.
- Local Installation: We are installing databases and software locally at CIAT
- Pipeline development: We have implemented in the biotechnology research unit the Pipeline system developed by Benoit Piegu and Richard Cooke (Perpignan University, France).
- Relational connection: We are aiming to establish a connection between the pipeline, LIMSys and the local platform in order to automate the information flow.

Results

Pipeline implementation. We have implemented in collaboration with Perpignan University a pipeline system for primary sequence analysis and filtering. Briefly: the pipeline platform has a set of software tools (Phred, Vecscreen, Blast, StackPack, D2_Cluster, Craw, Phrap, Cross_Match) that process a sequenced DNA fragment, clean it from vector, perform redundancy analysis, clustering of the sequences, assembling of cluster data, analyses of contigs data and assign putative functions using the BLAST algorithm.

The pipeline will be connected with a local platform that contains different databases and software to perform sequence analysis at different levels (figure 1): ORF searching, promoter search, gene prediction, metabolic pathways, protein motifs, *in silico* mapping and microarray analysis. Furthermore the pipeline will also have direct connections to the LIMsys for data analysis and retrieval to projects (refer to the CIAT Laboratory Information Management System annual report on this same issue).

Databases and Software Locally Installed. We have performed a rigorous informatics process to test and locally install different databases and Software. This local platform includes:

Databases:

- International Collaboration of nucleotides and sequences databases (NCBI, SWISS-Prot and DDBJ).
- Organism specific databases: *Arabidopsis,* sorghum, cassava, barley, rice, maize, bean and *Brachiaria* from distinct sources (EST, BAC,NUC,PEP).
- AceDB: Bean genes, rice genes and cassava genes.
- Phenotypical Unique Database: Currently in process of data curation for Beans, and Cassava (Local Information in CIAT).

Software and tools:

- PHRAP, PHRED, CONSED, CROSS_MATCH, POLYBAYES (for SNPs analysis), STACKPACK, D2_CLUSTER, CRAW, VECSCREEN, EMBOSS (with W2H Interface), RASMOL, BAMBUS, PHD2FASTA, TIGR Assembler, LUCY, SEQUENCE VIEWER, BLAST, WWW_BLAST_SERVER, BLASTBATCH, PRIMER3. Currently, these programs can be used individually under command line orders, but a user-friendly interface will be implemented.
- Perl utilities

Filter for new Databases and Software. We have selected the most important databases and software according to the project's needs from paper published by Galperin, M.Y. 2004, and we are in the local installation process. Likewise we have selected additional analysis tools (Pfam, Prosite, Uniprot, Interpro) to implement them at a local level and use them along with the databases.

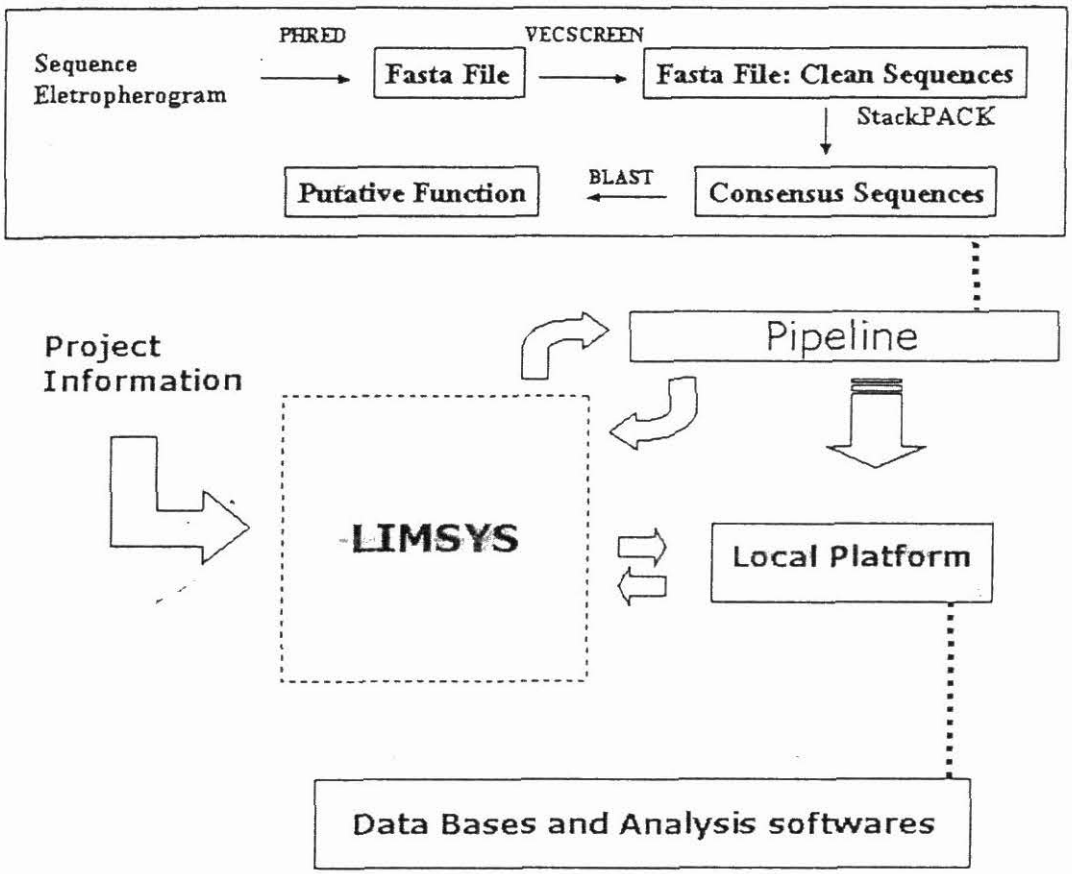


Figure 1. This diagram shows the information flow in the Bioinformatics platform. The Pipeline system can process raw sequences "in batch" and obtain clean sequences with a putative function assigned. This information sequence can come from the LIMSys or other local database and after pipeline processing the clean sequence can return to LIMSys or be submitted to further analysis through the local analysis tools and databases. The user can also perform different individual bioinformatics analysis using the local platform. This process will be perfectly flexible and customizable.

Future Plans

- In the near future we will develop a relational connection between the pipeline, LIMSys and the local platform in order to automate the tracking, sort, analysis and retrieval of sequence information and thus reach high-throughput processing of the information sequence.
- Design and implement user WEB interfaces for the work with the PIPELINE CLUSTERING.

- Design and implement other PIPELINES; and integration of all the PIPELINES into the platform.

References

- Galperin, M.Y. The Molecular Biology Database Collection: 2004 update. Nucleic Acids Research. 2004. 1;32 Database issue:D3-22.

3.3.4 Databases about distribution of wild relatives of crops

D.G. Debouck (CIAT, GRU)

We have continued with the establishment of databases about the geographic distribution of wild relatives for the so-called mandate crops. The objectives of that work are:

i. correct identification of materials collected and kept in *ex situ* conservation facilities (namely CIAT genebank, and other collaborating institutions). An output of this work is the taking of digital images of vouchers and to make them available through our web site (a service acclaimed by the Botanical Society of Colombia).
ii. geographic distribution of wild relatives of direct interest in breeding activities (namely acquisition of germplasm useful to the breeders).
iii. distribution of wild relatives genetically compatible with the crop, in view of introduction and management of transgenical crops.
iv. monitoring of modification/ destruction of natural habitats and disappearance of populations.

This year we have collated information in the following herbaria: CHAPA, CR, ENCB, F, INB, and P. These data will help us to build up the pilot for the component of threat analysis for a regional project in preparation with the Natural Resources Division of the World Bank. This pilot was agreed upon with the six partners of the project (CONABIO of Mexico, I. von Humboldt of Colombia, INBio of Costa Rica, CIAT, Cornell University and Smithsonian Institute) at first meeting in February 2004. Agreements to 'repatriate' that information to CONABIO of Mexico and INBio of Costa Rica were also made.

Activity 3.4 Training and Workshops Agrobiodiversity and Biotechnology Principal Staff

3.4.1 National and International Collaboration

Joe Tohme, Biofortification and Agronomic Meeting.Washington. Atended PAC., Oct/03

Joe Tohme, Reunión Dr. Nadim Khourly (World Bank). Bogotá. Nov/03

Joe Tohme, Ministerio de Agricultura, Bogotá. January, 04.

Joe Tohme, Meeting Harvestplus, Washington, January, 04.

Joe Tohme, Asistencia lanzamiento del Proyecto: Desarrollo de capacidades para implementar en Colombia el protocolo de Cartagena en Bioseguridad, Instituto Humboldt. January, 04

Mathew Blair - M. Blair – January 8-15, 2004 – Plant & Animal Genome Conference – San Diego, California -USA - co-organized Generation Challenge Program markers meeting.

Martín Fregene. January 2004. Attendance of the Plant and Animal Genome Meeting, San Diego and the choice of marker system meeting of sub programme 1 of the Generation Challenge Program (GCP).

César Martínez. Plant and Animal Genome Meeting XII. Attend the CP Generation Group meeting and get up to date on developments related to plant genomics. A poster was presented San Diego,Ca, USA.January 10-14/04.

Zaida Lentini. Biosafety Meeting. Bogotá. January, 2004

Mathias Lorieux. Attendance to Plant ans Animal. Genome Conference. Cornell. USA. January, 2004

Mathias Lorieux, January13-18: Participation to the Plant and Animal Genome Conference (San Diego, CA)

Mathias Lorieux, February14-20: Participation to the Bioinformatics SP4 workshop of the Generation Challenge Program (Rome, Italy)

Joe Tohme, Sustentación Diana Bernal. Universidad de los Andes. February, 2004

César Martínez. Rice Technical Working Group Meeting. Present a paper entitled Performance of Interspecific Rice Lines from the cross Bg90-2/*O.rufipogon* in Eleven Locations in Latin America". New Orleans.L.,USA. Feb29- March3, 2004.

Joe Tohme, Sustentación Tesis Rosa González. Universidad Nacional. March, 04

Joe Tohme, Meeting Colciencias. March, 04

Joe Tohme, Meeting, Ministerio de Agricultura, March, 04

Mathew Blair – March 14-18, 2004 – South African Plant Breeding Association Meeting Durban, South Africa - presented a poster on marker assisted selection and met with colleagues from Eastern and Southern Africa regions.

Martín Fregene. March/April 2004. Visit to ARI-Mikocheni, Tanzania, on the RF MAS cassava breeding project

Mathew Blair – April 24-29, 2004 – Universidad Autónoma de México – Cuernavaca, Mexico - seminar presentation and coordination with UNAM team on generation of EST sequences for common bean.

Mathias Lorieux. Visitó ensayo Challenge Program. Villavicencio. Marzo, 2004

Manabu Ishitani. Visited JIRCA. Japón Embassy. Bogotá March, 2004

Louise Sperling, Bridging the gap between relief and development: Best practices in seed stress situations. March 2-3

Louse Sperling, Seed Aid and Germplasm Restoration in Disaster Situations: Synthesis of Lessons: launch meeting. March 8-12

Louise Sperling. Participatory Plant Breeding:Country-specific Workplan Formulation and Basic Skill-building. April 18-24

Joe Tohme, Participation Harvestplus Workshop, Roma – IFPRI. April, 04

Zaida Lentini, University of Wageningen, The Netherlands. April 21-23. Laboratory of Jan Custer. To initiate collaboration for the development of haploid technology for cassava funded by CIAT – Rockefeller Foundation project. Official collaboration agreed.

Zaida Lentini, ILRI, Nairobi- Kenya, April 25-30. To attend and give a presentation on behalf of CIAT at the Workshop: CAGT – Crops with Appropriate Gene Technologies. Activity part of the SP3 sub-program component of the Generation Challenge Progra. This workshop was coordinated jointly between CIAT and CIP. The outcome was the

CIAT participation in two research proposals submitted to the Challenge Program in May 2004.

Zaida Lentini, Bellagio Rockefeller Center. Bellagio, Italy. May 24-29. To contribute with a book chapter, give a talk, and attend the Workshop on Crop Ferality and Volunteerism: A Threat to Food Security in the Transgenic Era, organized by Jonathan Gressel, Plant Sciences Weizmann Institute of Science, Rehovot, Israel and financed by the Rockefeller Foundation. A book with a total of 24 chapters is currently in press by CRC, Boca Raton, Florida.

Zaida Lentini, Dominican Republic. June 21-26, 2004. To attend REDBIO 2004. V Latin American and Caribbean Meeting on Agricultural Biotechnology. Dominican Republic. Works presented: two keynote talks and 5 posters.

Joe Tohme, AID Conference. USAID. USA. May,04

Louise Sperling, Reaching EndUsers in HarvestPlus: Coordination and Workplan Meeting. (May 5-7)

Martín Fregene. May 2004. Visit to the Donald Danforth Center on the development of a proposal for the Gates Foundation challenges in global health program

Joe Tohme, Sustentación Adriana Arango, Dirección Escuela de Postgrados, Universidad Nacional, Bogotá. June, 04

Joe Tohme, Roma. Reunión . IP Meeting HarvestPlus: Three Challenge Program. IPRI June, 2004.

Mathew Blair – June 8-10, 2004 – University of California – Davis – Sacramento, California, USA -USAID-Linkage program conference and coordination with bean research colleagues at UC-Davis.

Mathew Blair – June 12-18, 2004 – University of Geneva – Geneva, Switzerland – presented paper at the Phaseomics III conference and met with colleagues to discuss progress on TILLING / mutagenesis.

Mathew Blair – June 20-25, 2004 – Instituto Agronómico del Mediteraneo – Zaragosa, Spain – attend the Genotyping Workshop of the Generation Challenge Program.

César Martínez. V *Encuentro Latinoamericano* y del Caribe de Biotecnología Agrícola. Boca Chica, República Dominicana, 21-25 Junio, 2004. Present a Poster y participe in work groups related to biotecnology and rice.

Mathias Lorieux, June 20-25: Participation to the RedBio meeting (Boca Chica, Dominican Republic)

Mathias Lorieux, July 2-9: Participation to the Phenotyping workshop of the Generation Challenge Program. July 6: Talk at the Annual Génoplante Technical Committee meeting. Presentation of the results obtained for the T-DNA mutants phenotyping and multiplication. (Montpellier, France).

Mathias Lorieux, July 13-23: Cornell University. Preparation of a proposal for the competitive grant call of the Generation Challenge Program (Ithaca, NY)

Louise Sperling, Seed Aid and Germplasm Restoration in Disaster Situations: Synthesis of Lessons: case análisis. June 27-July2

César Martínez. Visit to RiceTec, Texas A&M University, and Lousiana State University. Assess progress made in marker assisted selection in rice and explore opportunities for collaboration. 26-31 July, 2004.

Mathias Lorieux. Attended the Phenotyping Protocols Workshop. Montpellier, France. July, 04

Joe Tohme, Visita Universidad de Yale y Rockefeller Foundation. July, 04

Mathias Lorieux. Proposal CP Proyect. Cornell University. USA. July, 04

Manabu Ishitani. Presentación Tesis Mauricio Quimbaya. Universidad Nacional. Bogotá. July, 04

Manabu Ishitani. Phenotyping Workshop and water déficit Agropolis International Montpellier. France. July, 04

Manabu Ishitani. Workshop on phenotyping and water deficit for Generation CP
The purpose of the workshop is to develop standards for phenotyping drought tolerance and other stress tolerance across crops. July 5-9 at Agropolis, Montpellier, France

Martín Fregene. July 2004 Visit to ARI-Mikocheni, Tanzania, on the RF MAS cassava breeding project

Zaida Lentini. Workshop on Genebanks and GMOs. IPGRI. Rome. August, 04

Joe Tohme, visited Illinois University USA. August, 04

Manabu Ishitani. Japan Embassy. August, 04

Zaida Lentini, IPGRI, Rome. August 28-Sep 1. To attend the Workshop on genebanks and GMOs. Biosafety Guidelines to handle CG genebanks were discussed and agreed in the meeting.

Joe Tohme, Attended Pre-Congreso de Biotecnología. Universidad Nacional, Bogotá. Agosto 30 – Sept 3, 04

Mathias Lorieux, August 16 -24: IRD. Preparation of a proposal for the competitive grant call of the Generation Challenge Program (Montpellier, France)

Mathias Lorieux, September 13-20: Tsukuba Research Center. Visit to different laboratories of the Rice Research Center to envisage the possibilities of collaboration (Tsukuba, Japan)

Mathias Lorieux, September 22-25: Participation to the Annual meeting of the Generation Challenge Program (Brisbane, Australia)

Martínez, César. Plant Animal Genome Conference XII.Cornell University. September, 04

Mathew Blair – September 12-17 – EMBRAPA – Centro Nacional de Pesquisa Arroz e Feijão – coordination with plant breeding and genetics units – presented two seminars on the Generation and Harvest Plus challenge programs.

Mathew Blair – September 21-24 – University of Queensland – attend Generation Challenge Program annual meeting –present – Brisbane, Australia – September 21-25, 2004.

Manabu Ishitani. Generation CP Annual Research Meeting
The purpose of the meeting is to review progress of each subproject in the CP. September 22-24 at Brisbane, Australia

Manabu Ishitani. Visiting MAFF of Japan, Japanese research institutions (RIKEN, NIAS, RITE and JIRCAS) and universities (Nagoya University and NAIST).
The purpose of the visit is to develop collaborative research projects, particularly for cassava, forages and rice. September 13 to 17, September 27, Japan

Martín Fregene. September 2004. CIAT-CLAYUCA technical back-stopping meeting to the Nigerian Starch Mill, Ahila

Martín Fregene. September 2004. Attendance of the Annual Colombian Biotech meeting Bogota, Colombia (Jaime Marin, Wilson Castelblanco, and Yina Puentes).

Martín Fregene. September 2004. Attendance of the Generation Challenge Program (GCP) annual meeting in Brisbane Australia (Paula Hurtado) .

Martín Fregene. October 2004 Visit to ARI-Mikocheni, Tanzania on the RF MAS cassava breeding project

Martín Fregene. October 2004. Attendance of a conference on biotechnology, Ibague,

Colombia (Anna Maria Correa,)

Martín Fregene. November 2004. Attendance of the ISTRC-AB Meeting Mombasa

Martín Fregene. November 2004. Attendance of a consultants meeting at the International Atomic Energy Agency, Vienna, on development of a research contract for vegetatively propagated crops.

Mathias Lorieux, November 15-17: Participation to the Rice Functional Genomics Conference (Tucson, Arizona)

Alvaro Amaya, Santa Fe community College, Gainesville, Florida USA.

Catalina Astrid Chaparro Pulido. Universidad de Malaga. Universidad Internacional de Andalucía. España.

3.4.2 Training, Visiting, Workshops, International and National Conferences for Agrobiodiversity and Biotechnology Personnel

Eliana Gaitán, Conferencia Ministerio de Agricultura, Oct, 2003

Juan Jairo Ruíz, Asistencia a curso sobre manejo de cultivo de lulo. Universidad Jorge Tadeo Lozano. Bogotá. Nov/03

SB-2 personnel, Taller Grupo Comunidades. Santa Ana. Dic, 2003

Fernando Rojas. Asistencia al Challenge Program Workshop. IFPRI. Enero, 2004

Paul Chavarriaga. Training in Richard Sayre's Lab at Ohio State University. Follow up on USAID funded project "Expanding the range of uses of cassava starch: A source of income generation", OH, USA. February, 2004

Z. Lentini, Gene Flow Analysis for Assessing the Safety of Bio-Engineered Crops in the Tropics. CIAT, CIBCM and FBES of University of Costa Rica, Hannover University and BBA, Germany, February 21-28, 2004. Total of 20 participants. Coordinated by Z. Lentini.

Paul Chavarriaga. Interview for the TV Channel Caracol, program "Abriendo Campo", on genetic modification of crops at CIAT, perspectives and applications. March 2004.

SB-2 personnel attended Sixth International Scientific meeting of the Cassava Biotechnology Network (CBN-VI), CIAT. Cali – Colombia, March 8-14, 2004

Paul Chavarriaga. Workshop "Biotecnología Básica y Plantas Modificadas Genéticamente", offered to Syngenta, CIAT, March 26 2004

Eliana Gaitán, Visita laboratorio del Dr. Perry Cregan del Dpto de Agricultura, USA. Marzo, 2004

Gerardo Gallego, Seminario Nueva Visión y Políticas de Desarrollo Rural. Hotel Tequendama, Bogotá. Marzo, 2004

Elizabeth Caicedo. Asistencia al Taller sobre Sistematización. Bogotá. Marzo, 2004-09-30

Echeverry Morgan. Entrenamiento en PCR, Institución BIOMOL. Bogotá. April, 2004

Gerardo Gallego, Conversatorio sobre creación de Centros de Excelencia en COLCIENCIAS. Bogotá. Abril, 2004

Paul Chavarriaga. Workshop CAGT-Crops with appropriate gene technologies: drought tolerance for increasing performance under marginal conditions, April 26 to 30 April 2004. ILRI campus. Nairobi, Kenya

Paul Chavarriaga. Seminar "Estatus de la Yuca Transgénica en Colombia y el Mundo", Universidad Tecnológica de Pereira, Pereira, May 2004.

Lucia Chavez, . HarvestPlus Training Course on Carotenoid Analysis held on May 3-14, 2004 at the Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, Brazil. This participation was fully sponsored by HarvestPlus Program.

Lucia Chavez, Taller Sistema de Gestión de Calidad de Laboratorios Conforme a la Norma ISO/IEC 17025. CIAT, Colombia 22-25 de junio, 2004

Myriam Cristina Duque. Summer Institute in Statistical Genetics at North Carolina State University 1. 2004. Introduction to Genomic Science : May 26 y 28, 04. Introduction to Bioinformatics: June 2 - 4, 04 . Microarray Analysis: June 7 - 9 , 04

SB-2 personnel attended V Latin American and Caribbean Meeting on Agricultural Biotechnology (REDBIO). Presentation. June 21-25. 2004. Dominican Republic.

Catalina Romero. Attended Advance Techniques Plant Science Course. Cold Spring Harbor. Visita Instituto de Massachussets, Harvard, Cornell and Standfort University. USA. June, 2004

Eliana Gaitán, Entrenamiento SNPs – Arroz. Cornell University, USA. July, 2004

Paul Chavarriaga. Interview for the TV Channel Telepacífico, program "El Agora", on genetically modified plants and their impact in society. August 2003.

SB-2 personnel attended II National Biotechnology Congress. Bogotá. 1-2 September 2004. Outstanding Presentation in the agricultural session.

Soto, M. NSF Rice Oligonucleotide Array Workshop. The Institute for Genomic Research (TIGR), Rockville, MD. September 20-22, 2004.

Paul Chavarriaga. Seminar "Current Developments in Genetic Modification of Cassava at CIAT", Biofortification Meeting, Uganda, Sept 15-19. 2003

Paul Chavarriaga. In collaboration with Agrobio and the Colombian Association of Scientific Journalism (ACPC) offered workshops on Genetically Modified Plants and Scientific Journalism in Monteria, Villavicencia, Pereira and Bucaramanga, since 2002. The workshops are attended by journalism students, of local universities, and professional journalists, most of the latters associated with radio and newspapers. The average number of attendants was 50 per workshop.

Paul Chavarriaga and Catalina Romero. Seminar "La Genómica y su Relación con la Transformación Genética", Universidad Nacional de Colombia-Palmira, specialization on biotechnology, September 2004.

Paul Chavarriaga. Interview for "El Relator", a virtual newspaper, sponsored by Universidad Autónoma de Occidente-Cali, available in the net of official and private schools of Cali.

Paul Chavarriaga. Organization and participation in the workshop "Biotecnología Agrícola con Énfasis en Modificación Genética de Cultivos", offered to Senators of Colombia, CIAT, October 2004.

Paul Chavarriaga. Two talks for the "Taller de Bioseguridad Agropecuario" offered to members of the Colombina Technical Committee on Biosafety (CTN), celebretad in CIAT, October 2004.

Z. Lentini, Biosafety Workshop for the Colombian Inter-Institutional Group responsible for implementing the Cartagena Protocol. Miniterys of Agriculture, Health, Enviroment, Commerce, Foreign Affairs, Invima, ICA, InstituteVon Humboldt , CVC, National Department of Planning, Colciencias, Agriculture and Animal National Technical Biosafety Committees. GEF-World Bank Project. October 4-6, 2004. Total of 31 participants. Coordinated by Z. Lentini

Z. Lentini, Workshop on Gene Flow Analysis and Environmental Biosafety. CIAT, CIBCM and FBES of University (Costa Rica), Hannover University, BBA, and University of Freiburg (Germany), Costa Rican National Biosafety Committee, Costa Rican National Academy of Sciences, ICA, Instiutte von Humboldt, and Ministery of Envrionment (Colombia), CENRAGEN (Brasil), CAN (Peru), CIBIOGEM (Mexico). October 7-9, 2004. Total of 41 participants. Coordinated by Z. Lentini

Lucia Chavez, 6th Graduate Course on the Production and Use of Food Composition Data in Nutrition, Wageningen, held in The Netherlands 5-24, October 2003. This participation was partially financed by HarvestPlus Program.

Roosevelt Escobar, Asistencia y Sustentación Proyecto Crioconservación en papa . Universidad del Tolima, Ibagué.

Roosevelt Escobar visiting Richard Litz' Laboratory. Sacramento, USA. Nov.17,04

Staff SB-2, Asistencia V Encuentro Latinoamericano y del Caribe en Biotecnología Agrícola. REDBIO.

Myriam Cristina Duque. Curso métodos estadísticos aplicados al análisis de datos moleculares dictado en INIAF- Santo Domingo, República Dominicana

Fernando Rojas, Participation in the Consultation Workshop for Subprogram 4- informatics in the Challenge Program unlocking Genetic Diversity in Crops for the Resource Poor. IPGRI – Rome.

Fernando Rojas, Participation in the Workshop for Subprogram 4 – Generation Challenge Program Information Systems Platform & Network Design. CIMMYT – México

Fernando Rojas, Participation in the Generation Challenge Program (GCP) Information Systems Platform and Network Implementation Workshop. IRRI - Phillipines

Isabel Moreno Cabrera. BSc. Student Molecular Genetics. Universidad del Valle, Cali - Colombia

Lina Maria Quesada. BSc. Student Microbiology and Biology. Universidad de los Andes. Bogotá-Colombia

Gisela Beldarrín Martínez. Instituto Nacional de Investigaciones Agropecuaria. INIA.

Lucy Milena Díaz. BSc. Student Biology. Universidad Nacional de Colombia - UNAP

Juan Manuel Díaz Soto. Msc. Recursos Filogenéticos. Universidad Nacional de Colombia. UNAP

Jorge Luis Fuentes Lorenzo. PhD. Genética Molecular. Jefe Dpto de Radiobiología – CEADEN, Cuba.

Camilo López. Visiting Research. Desarrollo de microarreglos para genes de resistencia a la bacteriosis de la yuca

Sergio Prieto. Training on: Bioensayos con Gusano Cachón y Transgenics

Ernesto Robayo. Visiting Research. Desarrollo de una Base de Datos de Fenotipos de Mutantes de Arroz. Training on Biotechnology.

Robert Zeigler. Director Genetic Resources Challenge Program, USA, March 8-9, 04

Ivonne García. Training: Análisis de la variabilidad genética de accesiones de Cauchao usando microsatélites de yuca. Universidad Nacional, Bogotá (OXG??)

Edwin Edgar Iquize. IBTA-Chapare, Bolivia. Training: Muestreo de Insectos. (MCD)

Valerie Verdier. IRD- Perpignan University. Microarray Cassava

Victor Manuel Núñez, Corpoica. Training on : Uso de microarreglos de DNA en la investigación agrícola.

Alejandro Chaparro. Universidad Nacional de Colombia. Training on: Uso de los microarreglos de DNA en la investigación Agrícola.

Catalina Oviedo. Eafit Medellín. Process Engineering. Developing of a process for sterilizing Phaseolus sedes with CL_2 vapors

César Ocampo. Recursos Genéticos Training on Microsatélites de yuca, evaluación colección colombiana *in vitro*

Universidad de Amazonía, V .Semestre Programa Ingeniería Agrícola, visited Agrobiodiversity and Biotechnology Project on April 2004

Jim Kelly, Michigan State University visited SB-2 on July 16, 04

Balasubramanian Ramani (bala), Botany Institute. University of Hannover, Germany

Alexander García Castro, Queensland University, Australia

Jonh Beeching, Department of Biology & Biochemistry, University of Bath

Ryo Akashi, Asóciate Profesor, Department of Biological Production, and Environmental Science, Faculty of Agriculture, Miyazaki University, Japan

Morris Levy and Maria Mercedes Maya. Purdue University. USA

Ibonne Aydee García. Universidad Nacional de Colombia, Bogotá. Training on Análisis de la Variabilidad Genética de Accesiones de caucho usando microsatélites de Yuca y AFLP.

Johanna Arango, MSc. Universidad Gottinham

Vivian Tatiana Villaba. Universidad de Cartagena. Training on. Obtención de la Biblioteca de cDNA de la piña y entrenamiento en genómica vegetal. .

Tahúra Ghneimth . IVIC. capacitación en uso de marcadores moleculares,

Reinaldo Pastor Tovar, Universidad Nacional Experimental de Guayana – UNEG, Venezuela. Training on: Tissue Culture, Breeding, Agronomy of Cassava crops.

Cristina Patrice Holmes. Dalhonsie University, Department of Biothics. Training on: Biofortificacion and Biotechnology.

Juan Carlos Florez, Universidad del Tolima, Colombia

Sandra Niño Aguirre, Colegio Alemán. Cali

Lady Carolina Rosero Ortegón, Universidad Javeriana, Dpto de Biología

Claudia Florez, Cenicafé. Training on construcción de librerías de CDNA.

Maria Eugenia Buitrago, Training on Técnicas Analíticas para determinación de compuestos Carotenoides.

Carlos Arturo Díaz Dagua, Universidad del Valle. Training on : Molecular Evolution en el Subribu *Espeletiiane* (*Asteraceae*) y sus relaciones: El aislamiento y análisis de genes de desarrollo y fisiología.

Lisa David. Genome and Plant Development. Conducting research work in the area of Looking for genes involved in he Weedy Rice Syndrom.

Adriana Almeida, research work for your MSc Thesis: Secuenciación automatica, cultivo de tejidos y transformación genética.

Josette Lewis and Penny Nestel, USAID visited Agrobiodiversity and Biotechnology Project, Feb. 8-12, 04

Andrea Davila – Centro Fitogenético Pairumani / Univ. San Simon – Cochabamba, Bolivia (August – December 2004). – evaluation of genetic diversity in Bolivian accessions of common bean.

Carlos Cesar Caula – Cuban Institute of Biotechnology – (to March 2004) training in microsatellite mapping, marker assisted selection and gene tagging.

Dennis Flores – Instituto Peruano de Leguminosas / PROMENESTRAS / PROMPEX – Chiclayo, Peru (July – Sept 2004) – training in Andean bean breeding.

Hernan Campos – Univ. San Simon – Cochabamba, Bolivia (to April 2004) – training in population evaluation.

Kattia Delgado – Instituto Peruano de Leguminosas / PROMENESTRAS / PROMPEX – Chiclayo, Peru (April - May 2004) – training in Andean bean breeding and marker assisted selection.

Orlando Chaveco – Cuban Ministry of Agriculture (October 2004) – training in Andean bean breeding.

Paul Kimani – CIAT-Kenya/ University of Nairobi (August 2004) – specialization in marker assisted selection

Rowland Chirwa – CIAT-Malawi (August 2004) – specialization in marker assisted selection

Gloria Iriarte - CENICAFE (short visits) – preparation of a publication on Advanced backcross method in common bean.

Gloria Santana – CORPOICA – Rionegro, Antioquia, Colombia (Sept 2003) – training in molecular marker techniques and indirect selection for BCMV resistance, at CIAT.

Tereza Cristina Olivieras Borba – EMBRAPA-CNPAF, Univ. Federal Goias (Sept – Nov 2004) – development of fluorescent microsatellites for common bean.

Dr. Thaura Ghneim. IVIC, Venezuela. One-month training in molecular tools applied to rice breeding. February .2004.

José Leonardo Bocanegra. BS student. Universidad del Tolima. Thesis project" Identification of molecular markers associated with resistance to Rhizoctonia solani in interspesific rice lines". January-December 2004.

Andrés Gonzalo Gutiérrez. BS student. Universidad del Tolima. Nine month's training in molecular techniques applied to rice breeding. March-December,2004.

Ms Elizabeth Okai (Ghana) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date January 2007)

Henry Ojulong (Uganda) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date January 2006)

Olalekan Akinbo (Nigeria) Ph.D. student University of the Free State, Bloemfontein, South Africa (expected finish date January 2007)

Martha Isabel Moreno (Colombia) Universidad de Valle, Cali, Colombia (expected finish date October 2004)

Ms Fanny Cruz (Mexico), Agropecuaria Santa Genoveva, Mexico

Paola Alfonso (Colombia) Universidad Javeriana, Bogota, Colombia (Pasantia September - December 2004)

Jixin Zhou, (China) Chinese Academy for Tropical Agricultural Sciences, (CATAS), Guangxi, China

Ms Claudia Ferreira (Brazil), Nacional Fruits and Cassava Research Institute (CNPMF), Cruz das Almas, Brazil

Z. Lentini, ICESI University

Z. Lentini, Rice Growers Committee – Saldaña, Tolima

Z. Lentini, Nacional University - Medellin

Z. Lentini, National University of Education and Technology – Duitama

Z. Lentini, Nacional Univeristy - Bogotá
Z. Lentini, Professors from the Quito University (Ecuador)

Z. Lentini, SENA - Buga

María Fernanda Alvarez. Institution: Los andes University, Bogotá. Training type: Bsc Thesis. Duration: one year

Ernesto Robayo. Institution: Javeriana University, Bogotá. Training type: visiting scientist. Duration: one year

Leonardo A Daza Lopera. Institution: Instituto Tecnico Agropecuario (ITA). Buga, Valle del Cauca. Training type: specialization as technician in agronomy. Duration : six months

Luis F Scarpeta Salomen. Institution: Instituto Tecnico Agropecuario (ITA). Buga, Valle del Cauca Training type: specialization as technician in agronomy. Duration : six months

John Janer Manzano. Institution: Instituto Tecnico Agropecuario (ITA). Buga, Valle del Cauca. Training type: specialization as technician in agronomy. Duration : six months

Napoleón Reyes. FLAR. Rice Anther Culture

Juan Figueroa. FLAR. Rice Anther Culture

Campo E. Manrique. FLAR. Rice Anther Culture

Yubiri Mujica. FLAR. Rice Anther Culture

Angela Mina. Ángela Mina. B.Sc. El Valle University. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Yamid Sanabria. B.Sc. Universidad del Tolima. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Julie Orjuela. B.Sc. Universidad del Tolima. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Maria A Loaiza Torres. Institution: Instituto Tecnico Agropecuario (ITA). Buga, Valle del Cauca. Training type: specialization as technician in agronomy. Duration : six months

Frida Bengtsson, MSc. Agricultural University of Norway. Seed Relief, HIV/AIDs and agrobiodiversity; a case study from Mbere District, Kenya

Jennifer Joy West. MSc. Agricultural University of Norway. Seed Relief, HIV/AIDs and agrobiodiversity; a case study from Mbere District, Kenya

3.5 Publications

3.5.1 Refereed Journals, Books

Aluko, G.; Martinez, C.;Tohme,J.; Castano,C. Bergman,C. and Oard,JH. QTL mapping of grain quality traits from the interspecific cross *Oryza sativa* x *O.glaberrima*. Apr. 2004. Theor Appl. Genet 109:630-639.

Blair, M.W.; Pedraza,F.; Buendia, HF.; Gaitán-solis, E.; Beebe,S.E.; Gepts, P. and Tohme,J. Development of a genome-wide anchored microsatellite map for common bean. Nov. 2003. Theor.Appl.Genet. 107(8):1362-1374.

Borrero, J., César P. Martínez, M.C.Duque, S.J.Carabali y J.Silva. 2004. Análisis de la adaptación regional de líneas interspecíficas de arroz en Colombia. Fitoctenia Colombiana Vol.1:36-43.

Camilo Lopez, Mauricio Soto, Silvia Restrepo, Benoît Piégu, Richard Cooke, Michel Delseny, Joe Tohme and Valérie Verdier. Cassava Gene expression profile in response to *Xanthomonas axonopodis* pv.*manihotis* infection using a cDNA microarray. Molecular Plant Microbe Interaction (MPMI). Submitted. (M.Soto)

Camilo Lopez, Véronique Jorge, Benoît Piégu, Chickelu Mba, Diego Cortes, Silvia Restrepo, Mauricio Soto, Michèle Laudié, Christel Berger, Richard Cooke, Michel Delseny, Joe Tohme and Valérie Verdier. A unigene catalogue of 5,700

expressed genes in cassava (*Manihot esculenta*): identification of genes implicated in cassava bacterial blight resistance and starch biosynthesis. Plant Molecular Biology. Accepted.

Cach, N.T., J.I. Lenis, J.C. Perez, N. Morante, F. Calle and H. Ceballos. 2004. Inheritance of relevant traits in cassava (Manihot esculenta Crantz) for sub-humid conditions. (Submitted to Plant Breeding).

Calle, F., J.C. Perez, W. Gaitán, N. Morante, H. Ceballos, G.Llano & E.Alvarez. Genetics of relevant traits in cassava (Manihot esculenta Crantz) adapted to acid-soil savannas. (Submitted to Euphytica).

Ceballos, H., C.A. Iglesias, J. C. Pérez, & A.G.O. Dixon, 2004. Cassava breeding: opportunities and challenges. Plant Molecular Biology (in press).

Chávez, A.L., T. Sánchez, G. Jaramillo, J. M.l Bedoya, J. Echeverry, E. A. Bolaños , H. Ceballos, & C.A. Iglesias 2004. Variation of quality traits in cassava roots evaluated in landraces and improved clones. (Submitted to Euphytica and accepted for publication after minor changes).

Durán LA, Blair MW, Giraldo MC, Machiavelli R, Prophete E, Nin JC, Beaver JS (2004) Morphological and Molecular Characterization of Common Bean (Phaseolus Vulgaris L) Landraces From The Caribbean. Crop Science (accepted)

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.

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. , Okogbenin E., Marin J., Moreno I., Ariyo O., Akinwale O., Barrera E., Ceballos H., and Dixon A. (2004). Molecular Marker Assisted Selection (MAS) of Resistance to the Cassava Mosaic Disease (CMD). Molecular Breeding (in press)

Frei A, Blair MW, Cardona C, Beebe SE, Gu H, Dorn S (2004). Identification of Quantitative Trait Loci for Resistance to *Thrips palmi* Karny in Common Bean (Phaseolus vulgaris L.). Crop Science (accepted)

Galindo, LM; Gaitan-Solis, E; Baccam,P; Tohme, J. Isolation and characterization of Rnase LTR sequences of Ty1 - copia retrotransposons in common bean. Feb. 2004. Genome 47 (1): 84-95

Gomez OJ, Blair MW, Frankow-Lindberg BE, Gullberg U. (2004) Molecular and Phenotypic Diversity of Common Bean Landraces from Nicaragua. Crop Sci. 44: 1412-1418

González-Torres R.I., R. Araya Villalobos, E. Gaitán Solís & D.G. Debouck. 2004. Wild common bean in the Central Valley of Costa Rica: ecological distribution and molecular characterization. Agron. Mesoam. 15(2): 145-153.

Ishitani M, Rao I, Wenzl P., Beebe S and Tohme J. (2004) Integration of genomics approach with traditional breeding towards improving abiotic stress adaptation: drought and aluminum toxicity as case studies. Field Crops Research 90: 35-45

Jaramillo, G., N. Morante, J.C. Perez, F. Calle, H. Ceballos*, B. Arias and A.C. Bellotti. 2004. Diallel analysis in cassava (Manihot esculenta Crantz) adapted to the mid-altitude valleys environment. **Crop Science** (in press)

Kelemu, S., Mahuku, G., Fregene, M., Pachico, P., Johnson, N., Calvert, L., Rao, I., Buruchara, R., Amede, T., Kimani, P., Kirkby, P., Kaaria, S., and Ampofo, K. (2004). Harmonizing the agricultural biotechnology debate for the benefit of African farmers. African Journal of Biotechnology 2(11):394-416.

Liao H, Yan X, Rubio G, Beebe SE, Blair MW, Lynch JP (2004) Basal root gravitropism and phosphorus acquisition efficiency in common bean. Functional Plant Biology (accepted)

Lenis, J.I., F. Calle, G. Jaramillo, J.C. Perez, H.Ceballos, and J.H. Cock. 2004. The effect of leaf retention in cassava productivity. (Submitted to **Field Crops Research** and accepted for publication after minor changes).

Lentini Z. and Espinoza A.M. 2004. Coexistence of Weedy Rice and Rice in Tropical America: Gene Flow and Genetic Diversity. In: J. Gressel (Ed.). "Crop Ferality and Volunteerism: A Treta to Food Security in the Transgenic Era?. Chapters XXIV. CRC Press. Boca Raton, FL (*In press*). 25 pp.

Lopez, C.E.; Zuluaga, A.P.; Cooke,R.; Delseny, M. Tohme, J. ;Verdier, V. solation of resistance gene candidates (RGCs) and characterization of an RGC cluster in cassava. Aug.2003.Mol. Gen. Genomics 268(5):658-671

Martínez, César P., Jaime Borrero, Fernando Correa, Myriam C. Duque. En: Arrocero Moderno.G.Robayo(ed) pag.136-141. Bogota, Colombia. 244p.

Martínez, CP., Borrero J., Almeida A., Duque MC, Carabali J, Silva J, Tohme J. 2004. Performance of Interspecific Rice Lines from the Cross BG90-2 /*O.rufipogon* in Eleven Locations in Latin America. Paper. 30th Rice Technical Working Group Meeting. New Orleans, L, USA. Feb29-March 3, 2004.

Morante, N., X. Moreno, J.C. Perez, F. Calle, J.I. Lenis, E. Ortega, G.Jaramillo and H. Ceballos. 2004. Precision of selection in early stages of cassava genetic improvement. (Submitted to Crop Science).

Muñoz,L.C.;Blair,M.W.;Duque,M.C.;Tohme,J.;Roca,W. Introgression in common bean x Tepary Bean interespecific congruity-backcross lines as measured by AFLP markers. Mar. 2004. Crop Science 44 (2):637-645

Muñoz LC, Blair MW, Duque MC, Roca W, Tohme J (2004) Level of introgression in inter-specific (Phaseolus vulgaris x P. acutifolius) congruity-backcross lines. Crop Science 44: 637-645

Nguyen Thi Cach, JC Perez, JI Lenis, F Calle, N Morante and H. Ceballos. 2004. Epistasis in the expression of relevant traits in cassava (Manihot esculenta Crantz) for sub-humid conditions (Submitted to Journal of Heredity).

Okogbenin E., Marin J., and Fregene M. (2004). An SSR-based molecular genetic map of cassava and QTL analysis for early yield in a Pseudo F_2 population Journal of Agronomy and Crop Science (In Press)

O Reilly, K.; Gómez-Vásquez, R.; Buschmann, H.'Tohme, J.; Beeching,R. Oxidative stress responses during cassava post-harvest physiological deterioration. Nov. 2003. Plant Molecular Biology 53(5):669-685

Perez, J.C., H. Ceballos, F.Calle, W. Gaitán, N. Morante, G.Llano & E.Alvarez. Additive, dominance and epistatic effects of relevant traits in cassava (Manihot esculenta Crantz) adapted to acid-soil savannas. (Submitted to Theoretical and Applied Genetics).

Perez, J.C., H. Ceballos, Jaramillo, G., N. Morante, F. Calle, B. Arias and A.C. Bellotti. 2004. Analysis of the relative importance of epistasis in cassava (Manihot esculenta Crantz) adapted to the mid-altitude valleys environment. (Submitted to Crop Science).

Perez Velázquez, J.C. C.L. Souza Jr. L.A. Narro, S. Pandey, and C. De León. Genetic effects for maize traits under acid and non-acid soils. (Submitted to Euphytica)

Reddy, BVS, AF Rangel, B. Ramaiah and R. Ortiz. A research amd network strategy for sustainable sorghum production systems for Latin America. 2004. In MCS Bantilan, UK Deb, CLL Gowda, BVS Reddy, AB Obilana and RE Evenson (Eds.) Sorghum Genetic Enhancement: research process, diseemination and impacts. ICRISAT, Patancheru 502 324, Andhra Pradesh, India pp. 139-148.

Restrepo, S.. Velez, C.; Duque, MC and Verdier, V. Genetic Structure and population dynamics of *Xanthomonas axonopodis* pv *manihotis* in Colombia from 1995 to 1999.Jan. 2004 Applied and Environmental Microbiology 70(1):255-261.

Santana GE, Blair MW, Morales F, Mahuku G, Jara C, Castaño C (2004) Uso de tecnicas clasicas y avanzadas para identificar genotipos de frijol resistentes a antracnosis y mosaico común. Fitotecnia Colombiana (accepted).

Sánchez, T., H. Ceballos, A.L. Chávez, C. Iglesias, D.Debouck, G. Mafla, and J. Tohme. 2004. Variation in crude protein content in cassava (Manihot esculenta Crantz) roots. (Submitted to Journal of Food Composition and Analysis).

Sánchez, T., A.L. Chávez, H. Ceballos, D. Ropdriguez, and P. Nestel. 2004. Reduction or delay of post-harvest physiological deterioration in high-carotene cassava roots. (Submitted to Journal of the Science of Food and Agriculture).

Sperling L.. Anon: "En cas de catastrophe, les semences locales sont disponibles, mais souvent inaccessibles". La Revue Durable, 12: Septembre-Octobre 2004.26-27.

Segovia V., Z. Lentini, JJ Ruiz , I. Sanchez and W. Roca *.In Vitro* Vegetative Propagation of Solanum quitoense (Lulo). I) Factors affecting the development of vigorous in vitro plants. Plant Cell Reports (submitted)

Segovia V., Z. Lentini, A. Mejía, I. Sanchez and W. Roca. *In Vitro* Vegetative Propagation of Solanum quitoense (Lulo).II) Plant regeneration of elite commercial farmer-selected clones. Plant Cell Reports (submitted)

Taylor N, Chavarriaga P, Raemakers K, Siritunga D and Peng Z (2004) Development and application of transgenic technologies in cassava. Plant Molecular Biology (In Press).

Toquica, SP.; Rodriguez, F. ; Martinez, E.; Duque, MC; Tohme,J Molecular characterization by AFLPs of Capsicum germplasm from Amazon Department in Colombia. Sep. 2003. Genetic Resources and Crop Evolution 50(6):639-647

Xia L., Peng K., Yang S., Wenzl P., de Vicente M.C., Fregene M., and Kilian A. (2004) DArT for high-throughput genotyping of cassava (Manihot esculenta) and its wild relatives Theoretical and Applied Genetics (in Press)

Yan X, Liao H, Beebe SE, Blair MW, Lynch JP (2004) Molecular mapping of QTLs associated with root hairs and acid exudation as related to phosphorus uptake in common bean. Plant and Soil (accepted)

3.5.2 Proceedings, Abstracts and Others

Almeida, A., O.Giraldo, CPMartínez, J.Borrero, MC Duque, J. López, J.Tohme.2004. Identification of QTLs for Yield and Yield Components in a Backcross Population between *Oryza barthii* and Lemont. Poster. RedBio Santo Domingo, Dominican Republic. June 22-25,2004.

Aluko,G., CP Martinez, C.Castaño, J. Tohme, J H Oard. 2004.QTL, Multivariate and Population Structure Analysis of Agronomic Traits in the interspecific cross *O.sativa* x *O.glaberrima*. Poster. Plant and Animal Genome Conference XII. San Diego, California USA. January 10-14, 2004.

Berkeley, California, USA, 24 October 2003, invited seminar: "Observations about *Phaseolus carteri*: endemisms, phylogeography, and breeding" (in honour to the late Dr Annetta Carter).

Blair MW, Pedraza F, Buendia HF, Gaitán-Solís E, Beebe SE, Gepts P, Tohme J (2004) Development of a genome-wide anchored microsatellite map for common bean (*Phaseolus vulgaris* L.) Annual Report of the Bean Improvement Cooperative 47:71-73.

Blair MW, Checa O, Beebe SE (2004) Yield components measured in a climbing x bush bean RIL population. Annual Report of the Bean Improvement Cooperative 47:297-298.

Blair MW, Giraldo MC, Buendia HF, Tovar E, Guerrero AF. (2004) Evaluation of microsatellite diversity in common bean parental surveys. Annual Report of the Bean Improvement Cooperative 47:187-188.

Blair MW, Beebe S, Grusak M, Tohme J, Rao I (2003) Development of Genetic and Genomic Tools to Study Nutritional Quality and Aluminum Tolerance in Common Bean. In Summaries of Legume Genomics Projects from Around the Globe. Community Resources for Crops and Models. Legume Genomics. Special issue, ed. G. Stacey, K Vendenbosch. Plant Physiology 131: 862-863.

Blair MW. G. Iriarte (2003) Ampliación de la base genética del fríjol (*Phaseolus vulgaris*) cultivado – 1° Congreso Venezolano de Mejoramiento Genético y Biotecnología Agrícola - Universidad Central de Venezuela – Maracay, Venezuela. October, 15-17, 2003.

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.) Paper presented at Bean Improvement Cooperative Meeting, Sacramento, California. October 26-30, 2003.

Blair MW (2003) Options for Increasing Iron Content in Bean: USAID funded Bean Biofortification Project and CGIAR Harvest Plus Challenge Program. 3rd Annual

Conference: USAID-African Partnership in Biotechnology. Ibadan, Nigeria. November 17-19, 2003.

Blair MW, Buendia HF, Castaño M, Santana GE, Morales F (2004) Adaptation and use of SCAR markers for BCNMV resistance (*bc-3* and dominant *I* genes) in an Andean bean breeding program. Poster presented at the South African Plant Breeding Conference, Durban, South Africa. March 14-18, 2004.

Blair MW (2004) Progress in genetic mapping of microsatellite markers for common bean (*Phaseolus vulgaris* L.). Abstract presented at Phaseomics III, Geneva, Switzerland. June 13-15, 2004.

Blair MW, Tomkins J (2004) Bean Genomics. SLO / USAID linkage program conference, Davis, California, USA. June 8-10, 2004.

Broughton WJ, Aung YY, Blair M, Hernandez G, Pankhurst CE (2004) Variation in *Rhizobium* host-specificity in various *Phaseolus* accessions. Abstract presented at Phaseomics III, Geneva, Switzerland. June 13-15, 2004.

Pankhurst CE, Blair MW, Broughton WJ (2004) TILLING in beans. Abstract presented at Phaseomics III, Geneva, Switzerland. June 13-15, 2004.

Broughton WJ, Hernandez G, Blair MW, Gepts P, Vanderleyden J (2003) The Phaseomics International Consortium. In Summaries of Legume Genomics Projects from Around the Globe. Community Resources for Crops and Models. Legume Genomics. Special issue, ed. G. Stacey, K Vendenbosch. Plant Physiology 131: 860-862

B. Pickersgill, M.I. Chacón Sánchez & D.G. Debouck. 2003. Multiple domestications and their taxonomic consequences: the example of Phaseolus vulgaris. In: Knüpffer H. and J Ochsmann (eds.), "Rudolf Mansfeld and Plant Genetic Resources". Proceedings of a symposium dedicated to the 100th birthday of Rudolf Mansfeld, Gatersleben, Germany, 8-9 October 2001. Schriften Genet. Ressourcen 22: 71-83.

CBN-VI. March 8-14, 2004 Several topics were discussed in the 40 plenary tematic lectures and 125 posters presentations.

Crouch JH, Buhariwalla HK, Blair MW, Mace E, Balaji J, Serraj R (2004) Biotechnology-based contributions to enhancing legume productivity in resource-poor areas. In: Serraj R (ed.) Symbiotic Nitrogen Fixation: Prospects for Enhanced Application in Tropical Agriculture. Oxford & IBH, New Delhi, India.

CIAT-TSBFI working group on CP-BNF : T Amede, E Amézquita, J Ashby, M Ayarza, E Barrios, A Bationo, S Beebe, A Bellotti, MW Blair, R Delve, S Fujisaka, R Howeler, N

Checa O, Blair MW, Beebe SE (2004) Genotypic variation in climbing ability traits in a common bean RIL population. Annual Report of the Bean Improvement Cooperative 47: 301-302.

Coulibaly, I., Revol, B., Noirot, M., Poncet, V., Lorieux, M., Carasco-Lacombe, C., Minier, J. & Hamon, P. (2003) AFLP and SSR polymorphism in a *Coffea* interspecific backcross progeny ((*C. Heterocalyx* x *C. canephora*) x *C. canephora*). In 7th International Congress of Plant Molecular Biology, Barcelona, June 23-28 2003), in press.

Congress on Biotechnology held in Bogotá. September 1-2, 04 4 oral presentations and presenting 14 posters. CIAT also participated in the Pre-Congress event, giving 13 lectures on issues related to genomics and transgenic organisms.

Daniel G. Debouck. 2004. Phylogeographic migrations of *Phaseolus* beans in the New World, and consequences for taxonomy, conservation and breeding. Annu. Rept. Bean Improvement Coop. (USA) 47: 29-30.

Daniel.G. Debouck, J. Engels & L. Guarino. 2004. Domestication and development of plant cultivars. Encyclopedia of Life Support Systems developed under the auspices of the UNESCO. V. Squires (ed.). EOLSS Publishers, http://www.eolss.net., Oxford United Kingdom. Pp. 1-18. Book Chapter

Jesús Alonso Beltrán, Morgan Echeverry, Paul Chavarriaga y Joe Tohme (2004) Análisis molecular de plantas transgénicas de yuca, *Manihot esculenta* (Crantz), mediante reacción en cadena de la polimerasa (PCR) en tiempo real. Presentación oral XXXIX Congreso ACCB, Ibagué, Octubre 2004.

Fory L.F, R. Pineda, E. González, A. Mina, J. C. Florez, K. Arcia, M. C. Duque, y Z. Lentini. 2004. Coexistencia del arroz maleza y el arroz en América tropical: Análisis de flujo de genes. Memorias del Segundo Congreso Colombiano de Bioctecnología. 1-3 Septiembre de 2004. Bogotá D.C. Colombia. 471 paginas

Fory L. F., R. Pineda, E. González, A. Mina, J. C. Florez, K. Arcia, M. C. Duque, and Z.Lentini. 2004. Coexistence of weedy rice and rice in tropical America: Gene flow analysis. REDBIO 2004. V Latin American and Caribbean Meeting on Agricultural Biotechnology. Dominican Republic June 21-25, 2004.

González-Torres R.I., E. Gaitán, R. Araya, O. Toro, J. Tohme & D.G. Debouck. 2004. Additional evidence on gene flow events in *Phaseolus vulgaris* in Costa Rica. Annu. Rept. Bean Improvement Coop. (USA) 47: 167-168.

González-Torres R.I., E. Gaitán, M.C. Duque, O. Toro, D.G. Debouck & J. Tohme. 2004. Estimation of gene flow in *Phaseolus vulgaris* L. using molecular markers: microsatellites and polymophisms of chloroplast DNA. Presented at the congress of RedBio, Santo Domingo. August.

González-Torres R.I., E. Gaitán, M.C. Duque, O. Toro, J. Tohme & D.G. Debouck. 2004. Medición del flujo de genes en frijol común mediante marcadores moleculares. Presented at the 2nd congress of biotechnology, Bogotá, Colombia. September.

González E., Luisa Fernanda Fory, Rosana Pineda, Paola Ruiz, Juan José Vásquez, Edgar Corredor, Myriam Cristina Duque y Zaida Lentini. 2004. Caracterización de la Diversidad Genética, Morfológica y Fenológica de Cuatro Poblaciones de Arroz Maleza (Arroz Rojo) Colectadas en las Regiones de Huila y Tolima. Memorias del Segundo Congreso Colombiano de Bioctecnología. 1-3 Septiembre de 2004. Bogotá D.C. Colombia. 471 paginas

González E., L. F. Fory, R. Pineda, P. Ruiz, J. J. Vásquez, E. Corredor, M. C. Duque, and Z. Lentini. 2004. Coexistence of weedy rice and rice in tropical America: Characterization of genetic diversity. REDBIO 2004. V Latin American and Caribbean Meeting on Agricultural Biotechnology. Dominican Republic June 21-25, 2004.

Guillaume Albini, F. C., Stéphane Rouillé, Fabrice Legeai, Bruce Thomas, Emmanuelle Karsenty, Delphine Samson, Mathieu Falque, Marie-Henriette Flament, Johann Joets, Philippe Leroy, Jean-Pierre Martinant, Denis Scala, Alain Murigneux, Laurent Decoussset, Dominique Brunel, Sebastien Aubourg, Brigitte Courtois, Mathias Lorieux, Boulos Chalhoub, Emmanuel Barillot, Aymeric Duclert1 (2003). Software development for genetic/physical map storage, visualization and analysis. Paper presented at the Genoplante conference, Poitiers, France, March 2003

Guiderdoni, E., Piffanelli, P., Sallaud, C., Gay, C., Larmande, P., Bès, M., Piégu, B., Bourgeois, E., Johnson, A., Lorieux, M., Périn, C., Droc, G., Morel, J., Barnola, I., Petit, C., Perez, P., Tester, M., Hibberd, J., Tohme, J., Ghesquière, A., Delseny, M. & Glaszmann, J. (2003) Generation, characterization and evaluation of the Genoplante rice insertion line library. In First Intl symposium on rice functional genomics - IRFGS 2003, Shanghai, China).

Grusak MA, Burgetti CL, Knewtson SJB, Lopéz-Milláni AF, Ellisi DR, Li CM, Musetti VM, Blair MW (2004) Novel approaches to improve legume seed mineral nutrition. Presented paper AEP/ICLGGII.

Hernandez G, Ramírez M, Blair MW, Lara M, Blanco L, Muñoz M, Barazesh S, Verdoorn E, Graham M, Vance CP (2003) Comparative analysis of common bean (Phaseolus vulgaris) nodule, root, pod and leaf expressed sequence tag (EST) libraries: a platform for "Phaseomics" research. Presented paper AEP/ICLGGII.

Johnson, S Kaaria, S Kelemu, P Kerridge, R Kirkby, C Lascano, R Lefroy, G Mahuku, H Murwira, T Oberthur, D Pachico, M Peters, J Ramisch, I Rao, M Rondon, P Sanginga, M Swift and B Vanlauwe. (2004) BNF: A key input for integrated soil fertility management in the tropics. In: Serraj R (ed.) Symbiotic Nitrogen Fixation: Prospects for Enhanced Application in Tropical Agriculture. Oxford & IBH, New Delhi, India.

Lentini Z and A.M. Espinoza. 2004. Coexistence of weedy rice and rice in tropical America: gene flow and genetic diversity – *In:* J. Gressel (Ed.). "Crop Ferality and Volunteerism: A Threat to Food Security in the Transgenic Era?". Chapters XXIV. CRC Press. Boca Raton, FL (*In press*). 25 pp.

Lentini Z., 2004. Gene technologies and biosafety at CIAT. Generation Challenge Program. Workshop: CAGT – Crops with Appropriate Gene Technologies. ILRI Campus.Nairobi, Kenya. April 26-30, 2004

Lentini Z., E. Tabares, L. Fory, T. Agrono, G. Delgado, C. Ordóñez, F. Correa, M. A. Santana, and N. Tumer. 2004. Sheath blight resistant transgenic rice. REDBIO 2004. V Latin American and Caribbean Meeting on Agricultural Biotechnology. Dominican Republic June 21-25, 2004.

Lentini Z., J.J. Ruiz, V. Segovia, E. Tabares, F. Hincapie, J. Cock, and F. Parra. 2004.*In vitro* vegetative propagation and regeneration of *Solanum quitoense* (lulo) plants and their use as elite clones by farmers. REDBIO 2004. V Latin American and Caribbean Meeting on Agricultural Biotechnology. Dominican Republic June 21-25, 2004.

Larmande, P., Gay, C., Sallaud, C., Perin, C., Perez, P., Lorieux, M., Morel, J., Johnson, A., Ruiz, M., Courtois, B. & Guiderdoni, E. (2004) Nucleic Acids Research 01697.

Lorieux, M. (2004) MapDisto, a tool for easy mapping of genetic markers. Poster presented at the RedBio conference, Boca Chica, Dominican Republic, June 2004).

Louise Sperling,. J. Lancon, and M, Loosvelt, eds. 2004 Participatory Plant Breeding and Participatory Plant Genetic Resource Enhancement An Africa-wide Exchange of Experiences (Sélection participative et gestion participative des ressources génétiques en Afrique :Échange d'expériences) Proceedings of a workshop held on M'Be, Ivory Coast. May 7-May 10, 2001 . Cali Colombia: PRGA (425 pages)

Louise. Sperling, T. Osborn and D. Cooper, eds. 2004.Towards More Effective and Sustainable Seed Relief. Rome; Food and Agriculture Organization of the United Nations (130 pages)

Louise, Sperling. Non-refereed: (focus on Sperling work: written anonymously)

Louise Sperling. Workshop: Seed Aid and Germplasm Restoration in Disaster Situations: Synthesis of Lessons Learned and Promotion of More Effective Practices: (Three presentations)

- Germplasm restoration in Rwanda? Analysis and Lessons Learned
- Germplasm restoration(?) in the Cagayan Valley, the Phillipines: IRRI/PhilRice
- Cassava Mosaic Disease and Seed Aid: NARO/Uganda

Louise Sperling. Bridging the gap between relief and development: Best practices in seed stress situations (Two presentations)
Seed aid: lessons in the making
Assessment of seed system and seed needs in emergencies

Louise Sperling. (Case studies prepared for USAID project: To be elaborated into book, L. Sperling editor see body of text for full list).

Mathias Lorieux, Jaime Lozano, Ernesto Robayo, Emmanuel Guiderdoni, Michel Delseny, Alain Ghesquière. 2004. Rice insertion T-DNA mutant collection: phenotypic data and seed multiplication. Poster presented at the PlantGems international conference, Lyon, 22-24 sptember 2004`.

Montecillos, México, México, 7 July 2004, invited conference at the Colegio de Postgraduados: "Es *Phaseolus* parte de la flora andina? – Nuevos aportes a un viejo debate".

N. Chaves, R. Araya Villalobos & D.G. Debouck. 2003. Polinización natural del frijol común en Costa Rica. Programa de Investigación y Transferencia en Tecnología Agropecuaria. VII Reunión Annual, Santo Domingo, Heredia, Costa Rica. Ministerio de Agricultura y Ganaderia de Costa Rica. San José, Costa Rica. pp. 35-40.

Palmira, Colombia, 12 March 2004, invited presentation at the 6th congress of the Cassava Biotechnology Network: "Biodiversity of cassava: challenges and innovations for its conservation".

Paris, France, 13 February 2004, invited seminar at the Muséum National d'Histoire Naturelle: "*Phaseolus*: un faux retour à la case départ . . . vers un grand genre?".

Palmira, Colombia, 11 August 2004, invited conference for the 25th anniversary of ASCOLFI: "De las curiosidades encontradas en el mercado de Tlatelolco con relación a una agenda de investigación en agricultura tropical".

Pasto, Colombia, 16 October 2003, invited conference on the World Food Day: "Recursos fitogenéticos andinos: construcción, unicidad, y desafíos".

PM Kimani, I Wagara, M Blair (2004) Selection of climbing bean lines tolerant to common bacterial blight, beans common mosaic virus and web blight. Annual Report of the Bean Improvement Cooperative 47: 309-311.

Quintero M., E. Tabares, R. Escobar, G. Delgado, and Z. Lentini.2004. Increased rice embryogenesis in microspore derived callus using temporary immersion system (RITA). REDBIO 2004. V Latin American and Caribbean Meeting on Agricultural Biotechnology. Dominican Republic June 21-25, 2004.

Quintero M., E. Tabares, R. Escobar, G. Delgado y Z. Lentini. 2004. Incremento de Embriogénenesis en Callos Derivados de Microsporas de Arroz Utilizando el Sistema de Inmersión Temporal (RITA®). Memorias del Segundo Congreso Colombiano de Bioctecnología. 1-3 Septiembre de 2004. Bogotá D.C. Colombia. 471 paginas

Reversat, G., Lorieux, M., Ghesquiere, A., Fernandez, L. & Brar, D. S. (2003) Status of rice research on nematode resistance in Asia and Africa: progress and potential. Paper presented at the 7th International Conference on Plant Disease, 3-4-5 December 2003, Tours, France.

Ruiz J.J., V. Segovia, E. Tabares, F. Hincapie, F. Parra, J. Cock, y Z. Lentini. 2004. Propagación vegetativa y regeneración *In vitro* de plantas de lulo (*Solanum quitoense*) y su evaluación como clones élite por agricultores. Memorias del Segundo Congreso Colombiano de Bioctecnología. 1-3 Septiembre de 2004. Bogotá D.C. Colombia. 471 paginas

REDBIO, Dominican Republic. June 21-25, 2004. Project staff had 16 presentations in symposium or workshop and presented 16 posters. Several project staff were part of the organizing committee or chairs of sessions.

Ramirez M, Hernandez G, Blair M, Lara M, Blanco L, Muñoz M, Barazesh S, Verdoorn E, Graham M, Vance CP (2004) Comparative analyses of common bean (*Phaseolus vulgaris*) expressed sequence tag libraries. Abstract presented at Phaseomics III, Geneva, Switzerland. June 13-15, 2004.

Rubyogo, J.C., L. Sperling and T. Remington: 2004 Seed Systems and Seed Relief: an Annotated Bibliography

R. Araya Villalobos & D.G. Debouck. 2003. Observaciones sobre poblaciones de frijol silvestre (*Phaseolus vulgaris* L.) en Costa Rica. Programa de Investigación y Transferencia en Tecnología Agropecuaria. VII Reunión Annual, Santo Domingo, Heredia, Costa Rica. Ministerio de Agricultura y Ganaderia de Costa Rica. San José, Costa Rica. pp. 29-34.

Toluca, México, 21 September 2004, invited presentation at the 20th national congress of phytogenetics: "Reflexiones y opciones para un sistema nacional de recursos genéticos vegetales".
Santo Domingo, Costa Rica, 13 January 2004, invited presentation in the IPGRI- INBio Regional Workshop on access and benefit-sharing in PGRFA: "A viewpoint from CIAT".

Sacramento, California, USA, 27 October 2003, invited conference to the 17th biennial meeting of the Bean Improvement Cooperative (USA): "Phylogeographic migrations of *Phaseolus* beans in the New World and consequences for taxonomy, conservation and breeding".

SB-2 Palmira, Colombia, 4 October 2004, invited seminar for the Biosafety GEF Colombia project: "Cultivos transgénicos y no transgénicos, biodiversidad y conservación de recursos genéticos: pueden co-existir?".

Santo Domingo, Costa Rica, 30 June 2004, invited conference for the 15th anniversary of INBio: "Parientes silvestres de plantas cultivadas del Neotrópico, oportunidades para agregar valor a la biodiversidad y multiplicar el esfuerzo de conservación".

Tabares E., L. Fory, T. Agrono, G. Delgado, C. Ordoñez, F. Correa, M.A. Santana, N. Tumer y Z. Lentini. 2004. Transformación Genética de Arroz para Conferir Resistencia al Añublo de la Vaina. Memorias del Segundo Congreso Colombiano de Bioctecnología. 1-3 Septiembre de 2004. Bogotá D.C. Colombia. 471 paginas

3.5.3 Thesis

Andrés Felipe Salcedo, BSc.Universidad del Valle. Clonaje y caracterización parcial de genes implicados en la biosíntesis de carotenos en raíces de yuca, Nov. 2003.

Adriana Arango MSc..National University Identification of candidate genes for aluminium resistance in *Brachiaria*. **Meritoria**

Diana Bernal: 2004. Contribución al estudio de la genómica Funcional del Fenómeno de Apomixis: Aislamiento de Secuencias de *Brachiaria* sp expresadas diferencialmente entre pistilos de individuos apomícticos y sexuales". Universidad de los Andes.

Duina Posso: Identificación de secuencias de ADN genómico polimórficas entre las variedades de yuca (*Manihot esculenta* Crantz) CM2177-2 y NGA-2 así como de MECU 72 y MCOL 2246 mediante la tecnología DarT (Diversity Arrays Technology) basada en microarreglos. Título Profesional: Bióloga con énfasis en genética. Universidad del Valle, Nov/03

Eliana Gaitán– Solis, PhD. Obtención y uso de secuencias microsatelitales GA/CA en estudios de diversidad genética en las especies de palmas colombianas *Attalea amygdalina, Ceroxylon alpinum* y *Ceroxylon sasaimae* , realizada en la Universidad Nacional de Colombia, Palmira. Nov, 2003. **Meritoria**

Gerardo Gallego: PhD. Estudios preliminares para el aislamiento y clonación de genes deresistencia a p*yricularia grisea* en arroz usando la estrategia de clonacion posicional. Universidad Nacional de Colombia, Sede Palmira. **Meritoria**

Mauricio Quimbaya. Pre-grado. Biología. Establecimiento de un exámen de selección fenotípica para la identificación de genes de resistencia al aluminio usando como modelo *arabidopsis thaliana*. Universidad Nacional de Bogotá. Sept/ 04

Rosa González. 2004. Stimation of gene flow on Phaseolus vulgaris using molecular markers: microsatellites and polymorphims of chloroplast. Universidad Nacional, Palmira Thesis awarded with **Merit recognition by Universidad Nacional de Colombia.**

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.

Development of strategies for better targeting of seed relief and linking relief and rehabilitation. Donor: FAO

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

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.

An integrated approach for genetic improvement of aluminium resístanse of crops on low fertility acid soils

Knowledge and tools for the modulation of post-harvest physiological deterioration in cassava

Seed aid and germplasm restoration in disaster situations: Síntesis of Lessons learned and promotion of more effective practices

Evaluation and multiplication of 5000 lines de T-DNA mutants

Model of food safety assessment of transgenic crops

Rice Functional genomics consortium

Bean genomics for improved drought tolerance in Africa and Latin America

Delivery of transgenic rice cultivars to seed producers and farmers in Tropical America

Following a multi-step approach involving biosafety assessment, mutritional testing and negotiations on intelle.

Ginés - Mera memorial fellowship fund for prostgraduate studies in biodiversity

Cassava biotechnology network IV

Gene flow análisis for assessing the safety of Bio-engineered crops in the tropics. BMZ Germany.

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.

"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.

BMZ-Germany - "Bean genomics for improved drought tolerance in Latin America", a. 750,000 USD (2003-2006).

Colciencias – "Obtención de nuevas variedades de fríjol común con atributos de rendimiento y potencial para nuevos mercados, utilizando selección convencional y asistida por marcadores moleculares" submitted by Universidad Nacional with CIAT - 22,000,000 Col (2004-2007).

Generation Challenge Program - "Genotyping, molecular marker development and QTL analysis of common bean" 266,000 USD (2004-2005)

Harvest Plus Challenge Program – "Biofortified crops for human nutrition" various donors a. 300,000 USD/yr (2003-2008).

USAID – "Breeding staple crops for improved micronutrient value", a. 400,000 USD (2002-2004)

Seed Aid and Germplasm Restoration in Disaster Situations: Synthesis of Lessons Learned and Promotion of More Effective Practices. IDRC. Overview and synthesis: Classic Seed Aid cases ; 'High-profile' germplasm restoration cases. June 2003-March 2005.

Assisting Disaster-Affected and Chronically-Stressed Communities in East and Central Africa: Focus on Small Farmer Seed Systems. USAID. Case Studies: Assessment Tools for 'disaster'. Policy Briefs. March 2002- October 2004

Consultancies:*

a) Towards More
Effective and Sustainable Seed Relief" Proceedings. FAO. (CIAT is co-editor here). August/Sept 2004

b) Agro-biodiversity and Seed Relief:leaflet. GTZ. (CIAT is co-editor here). Until completed

International Rice Functional Genomics Consortium (Yale/CIAT). USDA and CIAT core funds. US$67000.oo

High iron –zinc rice. CIDA,Canada. US$1,568.000/six years starting 2005.

Modification of Flowering in cassava and Mango using cloned flower gene from *Arabidopsis*. Rockefeller foundation US$280,000 for 4 years

Development of an *In Vitro* Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding. CIAT – ETH (Switzerland) - SCIB (China). Donor: ZIL, Switzerland. CHF 229,258. Approved October 2004.

Flowers, Fruits and Roots: Modification of Flowering to Improve Traits of Agricultural Importance. CIAT - Max Planck Institute, Germany. Donor: The Rockefeller Foundation. USD 410, 640. Approved October 2004.

Génoplante. Project for phenotypic and characterization of a series of T-DNA mutants, for a total of USD 61,000.

Challenge Program "Unlocking Genetic Resources in Crops for the Resource-Poor". Four projects for a total of USD 67,000.

USAID – Development of a BAC library of the African rice species, *O. glaberrima* – USD 14,000.

Génoplante. Project for phenotypic and characterization of a series of T-DNA mutants, for a total of USD 61,000.

Challenge Program "Unlocking Genetic Resources in Crops for the Resource-Poor". Four projects for a total of USD 67,000.

USAID – Development of a BAC library of the African rice species, *O. glaberrima* – USD 14,000.

3.6.2 Proposál and concept notes

Rice improvement for the Caribbean Region through molecular breeding

Understanding genetic diversity of cassava (Manihot esculenta and wild relatives for broadening the crops genetic base and sustainable agricultural development

Production of Quality planting material of tropical fruits through biotechnology to supplement small farmers income in the Colombian Llanos

The CIAT Regional Biotechnology Lab

Enhancing Cassava's potential for combating vitamin A deficiency

Producción entable y sostenible de guanábana mediante el desarrollo de tecnologías de post-cosecha y el aprovechamiento de subproductos

Combating hidden hunger in Latin America: Biofortified crops with improved vitamin A, essential minerals and quality protein

Gene discovery for Aluminum resístanse in common bean and Brachiaria

Light, water and stomata: gene targets for múltiple abiotic stress tolerance

Development of a common plant stress DNA chip and its application for crop imrpovent

Request to Syngenta for Bt genes, or other insecticidal genes available, to produce transgenic cassava plants that help control the hornworm Erinnyis ello in Colombia. Prepared by Paul Chavarriaga and Manabu Ishitani. February 2004.

Flowers, Fruits and Roots: Modification of Flowering to Improve Traits of Agricultural Importance. A Proposal Submitted to the Rockefeller Foundation. Prepared by The Department of Plant Developmental Biology, Max Planck Institute for Plant Breeding Research, Cologne 50829; and The International Center for Tropical Agriculture, AA 6713, Cali, Colombia. April 2004.

Engineering the Plastid Genome of Staple Crops for High Expression and Containment of Transgenes. A proposal submitted to the Generation Challenge Program. Prepared by Marc Ghislain, (CIP); Zaida Lentini, (CIAT); Paul Chavarriaga (CIAT); Alessandro Pellegrineshi, (CIMMYT); Leena Tripathi, (IITA); Swapan Datta (IRRI); Henry Daniell, (UCF USA); Jean-Vincent Escalant, (INIBAP); Isabel Cristina Bezerra, (EMBRAPA); Daphrose Gahakwa, (NARO/KARI, Uganda). May 2004.

Improvement of Iron, Zinc and Protein Levels in Crops Using Selected Transgenes. A proposal submitted to the Global Challenges in Global Helth initiative of the National Institute of Health. Prepared by Joe Tohme and Paul Chavarriaga at CIAT in collaboration with the laboratory of Doctor Ann M. Hirsch, Department of Molecular, Cell and Developmental Biology,University of California. June 2004.

Engineered chromosomes for delivering sets of genes to staple crops. Submitted to the Grand Challenge in Global Health Initiative of the National institute of Health. Prepared by Joe Tohme, Alvaro Mejía and Paul Chavarriaga in collaboration with Dr. Daphne Preuss of The University of Chicago. June 2004.

Cooperative Project to Enhance and Exchange Technologies for Producing High-Quality Seed of Staple Crops in Uganda, Kenya, Rwanda and Colombia. A concept note submmited to the Rockefeller Foundation. Prepared by Joe Tohme, Roosevelt Escobar and Paul Chavarriaga in collaboration with the PBA Foundation and CORPOICA from Colombia, the National Agricultural Research Organization (NARO) in Uganda, and the Institut des Sciences Agronomiques du Rwanda (ISAR). September 2004.

Interdisciplinary Center for Non-Conventional Breeding of Crops Relevant to Colombia (ICNCB). A proposal submitted to COLCIENCIAS seeking fuding for the creation of a Center of Excellence in Colombia. Prepared by Paul Chavarriaga from CIAT, in collaboration with CIB (Medellín), National University (Bogotá) and CENICANA (Cali). October 2004.

BioCassava Plus, a project that will develop new cassava cultivars designed to improve the nutritional status of sub-Saharan Africa. Gates Foundation, US$260,000 for 5 years

Development of Low-Cost Technologies for Pyramiding Useful Genes From Wild Relatives of Cassava into Elite Progenitors. GCP, US$894,420 for 3 years

Identifying the physiological and genetic traits that make cassava one of the most drought tolerant crops. GCP, US$78,806 for 3 years.

Validation of Diversity Arrays Technology (DArT) as a platform for efficient discovery and utilization of molecular marker – trait associations in orphan crops. GCP, US$860,405 for 3 years

Capacity Building in molecular breeding and transfer of technology to improve preferred Cassava Varieties for consolidation of Food Security and generation of income for small-scale farmers in Africa. FAO, US$332,000 for 3 years

'Express' Dissemination of Improved Cassava Varieties in Nigeria and Senegal Based on the Automated Temporary Immersion System (ATIS) and a Multi-stage Farmer Participatory Multiplication Program. DURAS, 139,500 Euros for 3 years, in collaboration with NRCRI, Nigeria

Securing the Harvest: Molecular-Assisted Intogression of Genes for Delayed Post-Harvest Physiological Deterioraton (PPD), high protein and beta-carotene content into African Cassava Gene Pools. DURAS, 150,000 Euros for 3 years, in collaboration with NRCRI, Nigeria.

Gene Flow Analysis for Environmental safety in the Tropics. CIAT – University of Costa Rica – Hannover University and BBA, Germany. Donor: BMZ. EURO 1,219,513. Submitted May 31, 2004. In Evaluation.

Precision transformation technology using heterologous recombination systems in crops. CIP-CIAT-IRRI-EMBRAPA- Berkeley University. Donor: Generation Challenge Program. USD 740,000. Submitted May 15, 2004.

3.6.3 Projects funded and their Donors (Oct 2002 – Sept 2003)

Canada

International Development Research Centre. (IDRC)

Seed aid and Germplasm restorat
Strategies for integrating small-scale end-users in cassava biotechnology research (Latin America)
Gines – Mera Fund Postgraduate Studies in Biodiversity

Colombia

Fundación para la Investigación y el Desarrollo Agrícola. (FIDAR)

Rice Functional Genomics Consortium

Ministry of Agriculture and Rural Development. (MADR)

Federación de Cafeteros

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
Bean Genomics in Central America

New Zeland

Government of New Zeland (NZ)

Rome

Food and Agriculture Organization. (FAO)

Strengthening seed security

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 IV – 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

Expanding the range of uses of cassava starch: A source of income generation

Nutritional Genomics

Maize – Vita-Biofortification

Food Safety Meeting

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.

IFAR

First capacitation building Fellowship

IFPRI

Biofortified Crops for Improved Human Nutrition

Characterization germplasm DNA Chip

WB-IFPRI

Biofortified Crops 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

List of Partners

BMZ-Germany - "Bean genomics for improved drought tolerance in Latin America", a. 750,000 USD (2003-2006).

Colciencias – "Obtención de nuevas variedades de fríjol común con atributos de rendimiento y potencial para nuevos mercados, utilizando selección convencional y asistida por marcadores moleculares" submitted by Universidad Nacional with CIAT - 22,000,000 Col (2004-2007).

Generation Challenge Program - "Genotyping, molecular marker development and QTL analysis of common bean" 266,000 USD (2004-2005)

Harvest Plus Challenge Program – "Biofortified crops for human nutrition" various donors a. 300,000 USD/yr (2003-2008).

USAID – "Breeding staple crops for improved micronutrient value", a. 400,000 USD (2002-2004)

Louisiana State University, Cornell University, Fedearroz, Flar, Inta-Nicaragua, Yale University, Universidad del Tolima, IVIC, etc.

Jan Custer, Wageningen University. The Netherlands.

Peng Zhang. ETH. Zurich. Switzerland.

Changhu Wang. South China Botanical Garden, Academia Sinica (SCIB), Guangzhou, China

Hans-Jorg Jacobsen, University of Hannover, Germany.

Joachim Sciemman. Federal Biological Research Centre for Agriculture and Forestry (BBA), Germany

Nilgun Tumer. Biotechnology Center. Rutgers University. USA

María Angélica Santana. USB. Caracas, Venezuela

Ana Mercedes Espinoza, University of Costa Rica

Embrapa/CNPAF – C. Brondani and C. Guimaraes

WARDA – H. Gridley and M.-N. Ndjiondjop

IRD – A.Ghesquière and C. Tranchant

Cirad – M. Châtel, N. Ahmadi and B. Courtois

Cornell University – S.R. McCouch

IRRI – K. McNally

Arizona Genomics Institute – R. Wing

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	Research Assistant
Galindo, Leonardo F.	Research Assistant
Villamizar Johnna Patricia	Research Assistant
González, Eliana	Research Assistant
Ladino, Janeth Julieta	Research Assistant
Manrique, Norma	Research Assistant
Muñoz, Liliana	Research Assistant
Tabares, Eddie	Research Assistant
López, Danilo	Research Assistant
Echeverry, Morgan	Research Assistant
Juan Jairo Ruiz	Research Assistant
Fory, Luisa	Rice Biotechnology Research Coordinator
Rios, Auradela	Technician
Bolaños, Eugenio	Technician
Dorado, Carlos	Technician
Herrera, Pablo	Technician
Ríos, Alexander	Technician
Tigreros, Humberto	Technician
Marco Brito	Technician
Criollo Arturo	Technician

Genome Diversity

Gallego,Gerardo.	Asociate – Laboratory Coordinator
Gaitán, Eliana.	Asociate
Barrera, Edgar.	Research Assistant
Gutiérrez, Janeth P.	Research Assistant
Bohorquez, Adriana.	Research Assistant
Vargas, Jaime	Research Assistant
Quintero, Constanza.	Research Assistant
Giraldo,Olga X.	Research Assistant
Galindo, Lonardo M.	Research Assistant
Londoño, Claudia	Technician
Romero Catalina	Research Assistant
Posso Duina	Research Assistant

Plant-Stress interactions

Chaves Alba L.	Research Associate
Soto Mauricio.	Research Assistant
Leonardo Miguel Galindo	Research Assistant
Andrés Felipe Salcedo	Research Assistant
Maria Eugenia Recio	Technician
Maria Eugenia Buitrago	Visiting Research
Mauricio Quimbaya	Visiting Research
Maria Fernanda Montenegro	Visiting Research
Felipe Sarmiento	Thesis Student
Jacobo Arango	Thesis Student
Andres Bolaños	Technician
Lina Quesada	Thesis Student

Administrative

Cruz, Olga L.	Bilingual Secretary
Zuñiga, Claudia S.	Bilingual Secretary
Duque, Myriam C.	Statistical Consultant
Rojas Fernando	System Analist

Institute A. von Humboldt

Palacio, Juan D.	Coordinator
Carolina Villafañe	Research Asistant
Juan Felipe Calderon	Undergraduate Student
Javier Restrepo	MSc Student
Diego Amorocho	Doctoral Student
Viviana Montilla	MSc Student
Ivan Restrepo	Visiting Research
Lindamar Camacho	Visiting Research

CORPOICA

Sanchez, I., PhD.	Visiting Researcher
Angela Zárate	Visiting Researcher

3.7.2 Current Graduate Students

Edgar Barrera; MSc. Molecular markers for ACMD resistance- MSc Plant Breeding, Universidad Nacional de Colombia, Palmira, Colombia.

Fabio Escobar; Molecular markers to certify seeds of rice - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

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.

Yamileth Cortés. MSc Plant Breeding. Analyzing the genetic diversity of the Colombian plantain collection (*Musacea collection*) using microsatellites. Universidad Nacional de Colombia, sede Palmira

Rosana Paola Pineda. MSc. Medición de flujo de genes con microsatelites en arroz. Universidad Nacional de Colombia, Sede Medellín.

Jaime Marin, Universidad de Tolima, Ibague. (QTL mapping of early bulking)

Paola Fory, MSc. Universidad Nacional. Recursos Fitogenéticos.

Juliana Chacón. MSc. Patrones filogenéticos en el género *Manihot* Mill. (Euphorbiaceae): Biogeografía y ecología comparada de las especies de la amazonía y la región andina. Universidad de los Andes.

Yamilet Cortés, MSc. Fitomejoramiento. Universidad Nacional, Sede Palmira

Constanza Quintero, MSc.Universidad Nacional de Palmira

Ivan Ochoa – Pennsylvania State University, USA – (Jan 2004) genetic mapping of low phosphorus tolerance in common bean.

Juan Manuel Diaz – Universidad Nacional – Palmira, Colombia (since Jan 2004) - evaluation of genetic diversity in Andean accessions of the common bean core collection.

Leon Dario Velez – Universidad Nacional – Bogotá, Colombia (July – Dec 2004) – studying the inheritance of intercropping ability between common bean and maize.

Lucy Diaz – Universidad Nacional – Palmira, Colombia (since Jan 2004) – evaluation of genetic diversity in Mesoamerican accessions of the common bean core collection.

Luz Nayibe Garzón - – Universidad Nacional – Bogotá, Colombia (Sept – Dec 2004) – development of molecular markers for anthracnose resistance in common bean.

Oscar Checa – Universidad Nacional – Palmira, Colombia (to Aug 2004) – studying the inheritance of climbing ability in common bean and the importance of genotype x environment interaction in this trait (returned to teaching position in Univ. de Nariño).

Wilfredo Pantoja - – Universidad Nacional – Palmira, Colombia (since Jan 2004) – evaluation of genetic diversity in Tepary bean accessions.

Andrea Frei – ETH, Switzerland – (February 2004) preparation of a publication on quantitative trait loci involved in resistance to the leaf-feeding insect, *Thrips palmi* in common bean

Carmenza Muñoz – University of Lyon (October – November 2004) preparation of a publication on genetic diversity of tepary bean.

3.7.3 Undergraduate students (current)

Andrés Bolaños, Universidad Nacional de Colombia, Sede Palmira

Jacobo Arango, Universidad Javeriana-Bogotá (cassava gene cloning)

César Augusto Posada

Felipe Sarmiento, Universidad de los Andes (cassava gene cloning)

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

Hector Fabio Buendía, Universidad de Tolima

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

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

Fausto Villafañe Rodríguez. Biología Molecular

Carolina Rosero, Universidad Javeriana, Bogotá

Hugo Arley Jaimes, Universidad del Valle

Jesús Alonso Beltrán, Universidad del Tolima

Ana Correa, Universidad del Valle.

Gina Viviana Caldas –Universidade del Valle (July 2003 – Sept 2004) – Tannin QTL mapping

Henry Lozano – Universidad Nacional – Palmira, Colombia (July 2003 – Dec 2004) Advanced backcross - micronutrient content.

Lina Maria Rodríguez – Universidad de Los Andes – (since July 2004) Geminivirus resistance markers.

Anna Maria Correa (Colombia) Universidad de Valle, Cali, Colombia (expected finish date October 2004)

Milena Sepúlveda (Colombia) National University of Colombia, Palmira (expected finish date September 2005)

Angie Ayala (Colombia) National University of Colombia, Palmira (expected finish date September 2005)

Kiliany Arcia, . Thesis. Universidad del Tolima

Juan Carlos Florez, . Thesis. Universidad del Tolima

Angela Mina. Ángela Mina. B.Sc. El Valle University. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Yamid Sanabria. B.Sc. Universidad del Tolima. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives

Julie Orjuela. B.Sc. Universidad del Tolima. Training on microsatellites analysis of rice and their use for gene flow analysis into wild/ weedy relatives