Annual Report Project SB-2
2005

Conservation and Use of Tropical Genetic Resources

CIAT
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CONTENT

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 Three new phaseolin types found in populations of *Phaseolus vulgaris* L. collected in its primary center of diversity

Debouck, D.
SB-2 Project

Introduction

Phaseolin (Phs), the major seed stotage protein of common bean, has proved to be an excellent, cheap and polymorphic marker in evolutionary studies (Gepts, 1988). Although this globulin has a narrow range of molecular weights (45-52 kD) and isoelectric points (5.6-5.8) (Brown *et al.* 1981), up to now several types and subvariants of phaseolin have been found in wild, landraces and improved bean genotypes (Gepts & Bliss, 1986; Gepts *et al.*, 1986; Koenig *et al.*, 1990; Debouck *et al.*, 1993; Tohme *et al.*, 1995; Beebe *et al.*, 1997). The present report indicates several Phs types not reported previously, and establishes standards for Phs morphotypes which are available internationally as genetic stocks (Ocampo & Toro, 2005).

Materials and Methods

The accessions reported here were obtained from the worldwide collection held in CIAT (Table 3). The samples were analyzed in 1D-SDS-PAGE (Brown *et al.* 1981) and confirmed later in 2D-IEF-SDS-PAGE (O'Farrel, 1975).

Results and Discussion

New phaseolin diversity found in beans from Mesoamerica. Using 1D/2D-IEF-SDS-PAGE, two new phaseolins were identified in wild and cultivated accessions from Mesoamerica (Figure 37). The first phaseolin was found only in G11027, a wild population from Mexico, in the state of Durango. We suggest that this pattern be designated as "Durango" pattern (abbreviated "Dur") making reference to the name of the Mexican state where it was found. The second new phaseolin type was first observed among wild accessions from Honduras (G50722B) and Costa Rica (G51062A). However, it was later found in several cultivated accessions (for example G18970) from the Costa Rican locality of Telire (Table 3). This is the first time that the Mesoamerican cultivars are reported to have a phaseolin type other than the "S", "CH" and "B"

types (Gepts and Bliss, 1986; Koenig et al., 1990). Accordingly we suggest that this phaseolin be designated as "Telire" ("Tel").

A novel simple phaseolin found in beans from Colombia. Using 1D/2D-IEF-SDS-PAGE, a simple phaseolin type was observed for the first time and exclusively in a Colombian cultivar (G24674) (Figure 3). Along with the previous result, we suggest that this phaseolin be designated as "Quincho" ("Qui") making reference to the name of the cultivar where it was found. This study confirms again that germplasm from Middle America and Colombia has the greatest amount of phaseolin types (Gepts & Bliss, 1986; Gepts *et al.*, 1986; Koenig *et al.*, 1990; Beebe *et al.*, 1997).

Table 3. The molecular weight (MW) of bands in 1D-SDS-PAGE and isoelectric focusing (IEF) of peptides within each band are described for the new phaseolin types.

Phaseolin Types	Biological population			1D-SDS-PAGE (bands)		2D-IEF-SDS-PAGE (peptides)	
	Identification	Status	Gen. St[A]	Number	MW (kD)	Number	Total
Durango (Dur)	G11027	Wild	Mexdu-01	2	58.94	5	10
					56.00	5	
Quincho (Qui)	G24674	Cult.	Fi-4421	2	56.00	6	11
					53.20	5	
Telire (Tel)	G18970 G51062A G50722B	Cult. Wild Wild	Fi-5791 Fi-5765 Fi-3925	2	54.12	5	9
					50.55	4	

A: The accessions which are reported here as the source of each Phs morphotype will be maintained in CIAT as genetic stocks.

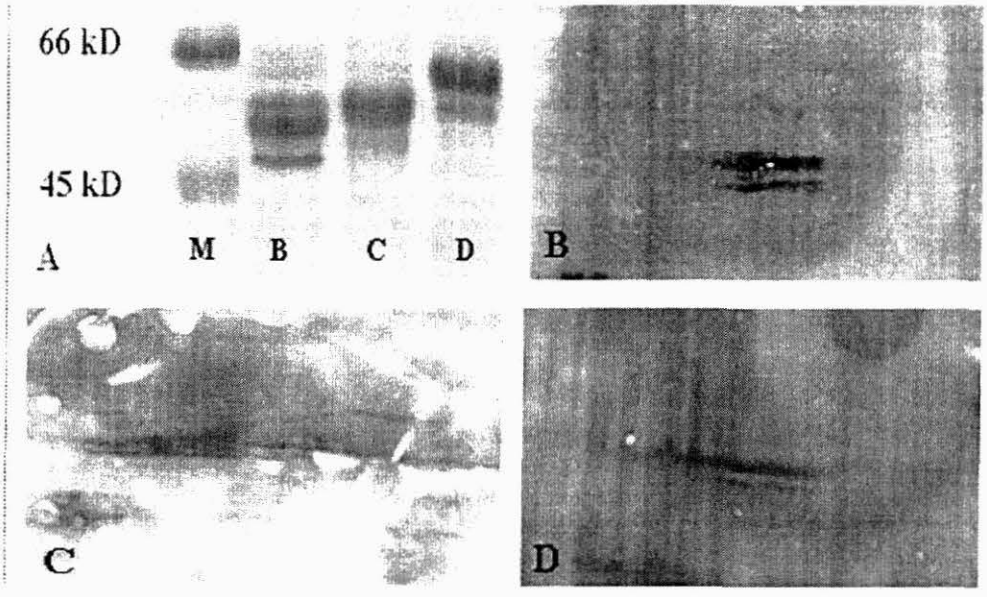

Figure 3. 1D-SDS-PAGE (A) and 2D-IEF-SDS-PAGE (B-D) electrophoresis of newly found phaseolins. A: molecular weight marker (M) with its value of kilo-Daltons (kD), B: "Tel" phaseolin type, C: "Qui" phaseolin type, D: "Dur" phaseolin type.

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Contributors: C. H. Ocampo and O. Toro

1.1.2 Two novel globulin variants found in the wild teparies beans (*Phaseolus acutifolius* A. Gray)

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CIAT

Introduction

In teparies, the major seed storage protein is called globulin and has similar chemical characteristics (molecular weight, isoelectric points, serological cross-reactivity, etc.) as the phaseolin of common bean (*Phaseolus vulgaris* L.) (Sathe *et al.*, 1994). However the sequence homology between its coding genes has not yet been verified. Schinkel and Gepts (1988) report fourteen different patterns among wild forms, whereas only one pattern was identified in the cultivars. Another study contributed by Toro and Debouck (1989) increases the number of reported patterns to 25; 23 patterns were identified among wild teparies and two patterns in the domesticated form. Later Florez (1996) confirms these results and increases the number of patterns found to 27 (25 patterns among wild teparies and two patterns among the domesticated form). We are here reporting two novel globulin types found in wild populations of the tepary bean (Ocampo & Toro, 2005).

Materials and Methods

We made a further screening of the seed storage protein (globulin) variability, focusing on the material that was not worked out by the previous investigators: we analyzed 68 accessions (50 wild and 18 cultivated) of *P. acutifolius* from the worldwide collection held in CIAT. This variation was first analyzed using one-dimensional SDS/PAGE electrophoresis (Brown *et al.* 1981) and later confirmed in two-dimensional IEF-SDS-PAGE electrophoresis (O'Farrel, 1975).

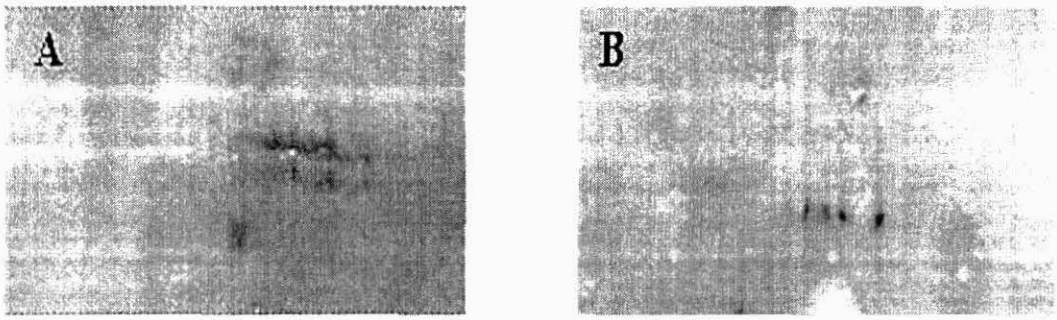
Results and Discussion

Finding new globulin variants. Two new patterns were observed among wild populations of *var. acutifolius* collected in Mexico. The first pattern (XXVIII) was found in G40103, a population collected in Sinaloa by D. G. Debouck, which showed previously the pattern XVII (Schinkel & Gepts, 1988), and the second pattern (XXIX) was found in the population G40298 (collected in Guerrero by Robert Reid). These patterns can be identified by one-dimensional electrophoresis parameters (Table 4): the pattern XXVIII by the presence of a pair of equally strained bands and with an average molecular weight (42.52 and 46.22 kD). The second pattern (XXIX) reveals a stronger band with a molecular of 44.70 kD. These patterns also can be observed in two-dimensional electrophoresis (Figure 4), using the isoelectric focus parameter. A single band in 1D-SDS-PAGE is often composed of peptides with slight differences in molecular weight but with some overlaping, creating a smear of peptides, which can be observed in 2D-IEF-SDS/PAGE. The IEF peptides within each band are presented for both patterns in Table 34. These results confirm the presence of two new globulin types for teparies beans. In addition the fact that these new globulin patterns have been found only in wild material confirms the previously described trend of a strong reduction in genetic diversity in the cultivated form of *P. acutifolius* Gray.

Table 4. Description of new patterns: the molecular weight (MW) of bands in 1D-SDS-PAGE and isoelectric focusing (IEF) of peptides within each band are presented for the globulins XXVIII and XXIX.

Pattern	Populations	1D-SDS-PAGE (bands)		2D-IEF-SDS-PAGE (peptides)	
		Number	MW (kD)	Number	Total
XXVIII	G40103	2	42.52	4	8
			46.22	4	
XXIX	G40298	1	44.70	4	4

Figure 4. Two-dimensional IEF-SDS-PAGE electrophoresis of newly found globulins. A, pattern XXVIII (G40103) and B, pattern XXIX (G40298).



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1.1.3 Biochemical characterization of the *Phaseolus* germplasm bank for improved and refined collections.

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CIAT

Introduction

Phaseolus vulgaris L., the common bean, contains a large amount of native genetic variability in its populations. Identification of the kinds and amounts of genetic variability is necessary to insure that all variability is conserved and available to users. Phaseolin, the major seed storage protein of common bean (Osborn, 1988), has proved to be an excellent - cheap and polymorphic - marker in evolutionary studies (Gepts, 1988). The description of phaseolin type is also becoming a routine descriptor and powerfull for the bean germplasm characterization. Given the usefulness and practicability of such marker, is necessary the caracterization of variation genetic at the population level of accessions both wild and cultivated *of Phaseolus vulgaris* L. colected in its primary center of diversity and maintained in CIAT ' s gene bank.

Materials and Methods

Plant material. The germplasm analyzed in this study consisted of wild and cultivated genotypes of *Phaseolus vulgaris* L. collected in its primary center of diversity and maintained in CIAT ' s gene bank. These genotypes (seeds) were analyzed as isotypes of phaseolin type found for each analyzed seed (nondestructive test of seed for proteins extraction). Later these seeds were planted in greenhouse, which made possible to conserve its genotype and to guarantee repeatability of the results obtained with this study. This samples were analyzed in one-SDS-PAGE electrophoresis for seed protein Phaseolin (Brown et al. 1981)

Results and Discussion

In 2005, 3,200 genotypes of the *Phaseolus vulgaris* L. germplasm collection held at CIAT were analyzed for seed storage proteins (phaseolin). This diversity in the phaseolins together with morphoagronomic characterization is a requisite for improving the representativeness of the designate collection.

Reference

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1.1.4 Use of the different phaseolin types for broadening the genetic base of crops: the case of the interspecific hybridization between common and tepary beans (service operation for the Biotechnology Unit).

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SB-2 Project

Introduction

The tepary bean (*P. acutifolius*) is known to possess high levels of resistance to several abiotic and biotic stresses. Through this collaborative activity with the Biotechnology Unit we are trying to transfer multiple desirable traits from selected genotypes of tepary bean into lines elite of common bean.

Materials and Methods

To assess introgression of tepary alleles in the common bean, the parents and its offspring were analyzed for seed storage protein of common bean (phaseolin). We use one biochemical technique (1D-SDS-PAGE) for the detection of the phaseolin protein polymorphism.

Results and Discussion

This year, 130 individuals of offsprings have been analyzed. The observed variation shows several phaseolin types in these genotypes, which display interesting characteristics to assess introgression of tepary alleles in the common bean. Additional studies on introgression of tepary DNA fragments were carried out using molecular markers.

1.1.5 Microsatellite marker diversity in common bean (*Phaseolus vulgaris* L.)

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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 were selected for genotyping in the Generation Challenge Program.

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: The microsatellite alleles were 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

Results of allele number and polymoprhism information content were presented last year. This year we did further analysis with the multiple correspondance analysis and analysis of intra-population diversity and geneflow among the two principal clusters of genotypes corresponding to the Mesoamerican and Andean gene pools and the subclusters corresponding to races which were evaluated (Tabel 1). Intra population diversity (Hsi) was higher within the Andean genepool than within the Mesoamerican genepool and this pattern was observed for both gene-based and genomic microsatellites. Furthermore, intra-population diversity within the Andean races (0.356 on average) was higher than within the Mesoamerican races (0.302). Within the Andean gene pool, race Peru had higher diversity compared to race Nueva Granada, while within the Mesoamerican gene pool, the races Durango, Guatemala and Jalisco had comparable levels of diversity which were below that of race Mesoamerica. The divergence of the larger number of races in the Mesoamerican gene pool (Durango, Guatemala, Jalisco and Mesoamerica) was low compared to the races in the Andean gene pool (Nueva Granada and Peru).

Conclusions and Future work

Microsatellite markers were found to be useful for distinguishing races within the two major gene pools, and therefore supplement information collected from RAPD and AFLP studies that have defined the basic structure of diversity within the species. The information has been used to design fluorescent microsatellite marker panels for ABI377, ABI3100 and ABI3730 platforms.

Table 1. Observed intra (H_s) and inter population (H_{si}) diversity for genotypes belonging to wild and cultivated common beans, to Andean and Mesoamerican gene pools and to races within each gene pool.

Category	N	Observed Heterogeneity			Value
		cDNA based (57)	Genomic (72)	Total	
Total	44	0.444	0.593	0.527	H_t
Species/Status[1]	44	0.429	0.575	0.511	H_s
Cultivated *P. vulgaris*	40	0.432	0.583	0.516	H_{si}
Wild *P. vulgaris*	3	0.388	0.477	0.437	H_{si}
Tepary Bean *P. acutifolius*	1	0.000	0.000	0.000	H_{si}
Gene pools	40	0.343	0.486	0.422	H_s
Mesoamerican	30	0.319	0.481	0.410	H_{si}
Andean	10	0.412	0.500	0.461	H_{si}
Races	40	0.253	0.363	0.314	H_s
Nueva Granada	4	0.215	0.352	0.292	H_{si}
Peru	5	0.397	0.436	0.419	H_{si}
Introgressed	1	0.000	0.000	0.000	H_{si}
Durango	4	0.154	0.325	0.249	H_{si}
Guatemala	2	0.246	0.292	0.271	H_{si}
Jalisco	3	0.257	0.367	0.319	H_{si}
Mesoamerica	21	0.289	0.430	0.368	H_{si}

1/ Status distinguishes wild versus cultivated *Phaseolus vulgaris*.

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

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Introduction

We continued analyzing a large number of Caribbean genotypes that are held in the FAO-designated collection of common bean at CIAT for microsatellite diversity. We focused especially a on genotypes from Cuba (black and light red kidney) and Jamaica (pink striped). We sampled from most Andean and Mesoamerican seed classes found in the Caribbean including the small seeded (red and black), large 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 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 a set of 30 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 30 microsatellite (20 genomic and 10 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 10 bp size standard ladders that were used two times per gel. The total number of alleles evaluated across all 30 markers was 143 and on average each marker revealed 4.76 alleles each. The average diversity value was 0.586. The number of alleles was significantly higher for genomic microsatellites (5.7) than for gene-based microsatellites (2.9) based on unpaired t-test (at $P<0.05$). Interestingly the discrimination power (D) values were similar for both genomic and gene-based microsatellites (0.59 vs 0.57). The most polymorphic markers in terms of number of alleles or high D values were BM152 (13 alleles, 0.821 D value), BM160 (13 alleles, 0.763 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, BMy6 and GATs54 of which four 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 0.19 Dice similarity

value. There were a greater number of accessions and a higher overall diversity within the Mesoamerican group (97 accessions, 0.50 to 1.0 Dice similarity values) than within the Andean group (85 genotypes, 0.57 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 two 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. A few 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 Future Work

This work will be extended by an analysis of Cuban genotypes from INCA that are part of the PHD dissertation of S. Lorigados. We plan to compare landraces, released varieties and farmer selected genotypes from participatory plant breeding program.

Table 1. *Diversity values and number of alleles identified by 30 microsatellite markers* used in the analysis of Caribbean bean germplasm.

	Microsatellite marker	Map Position	Range in sizes	Allele No. of Alleles	Diversity Value (d)
Genomic	BMd12	B06	158-174	5	0.207523
	BMd33	B11	98-110	3	0.509842
	BMd36	B03	164-188	6	0.603792
	BM139	B02	86-120	6	0.56376
	BM140	B04	160-200	6	0.515759
	BM142	B02	154-156	2	0.499457
	BM143	B03	116-162	10	0.753079
	BM152	B02	94-144	13	0.820795
	BM155	B05	116-118	2	0.501117
	BM160	B07	184-266	13	0.762982
	BM164	B02	150-184	4	0.591776
	BM170	B06	158-188	5	0.641378
	BM172	B03	82-118	6	0.642042
	BM175	B05	160-192	7	0.612698
	BM181	B03	180-188	5	0.685274
	BM183	B07	124-142	3	0.572817
	BM184	B09	152-166	5	0.592652
	BM189	B08	108-120	5	0.614237
	BM197	B03	198-202	3	0.530854
	BM205	B07	124-142	5	0.648986
Genic	BMc5	B10	134-142	2	0.580184
	BMd10	B01	140-144	3	0.552409
	BMd15	B04	166-204	3	0.644246
	BMd20	B05	120-130	4	0.649318
	BMd26	B04	136-142	2	0.49994
	BMy1	B04	152-172	5	0.705048
	BMy2	B011	148-160	3	0.521495
	BMy4	B01	162-166	3	0.480377
	BMy6	B04	136-142	2	0.504861
	GATs54	B03	116-118	2	0.570704

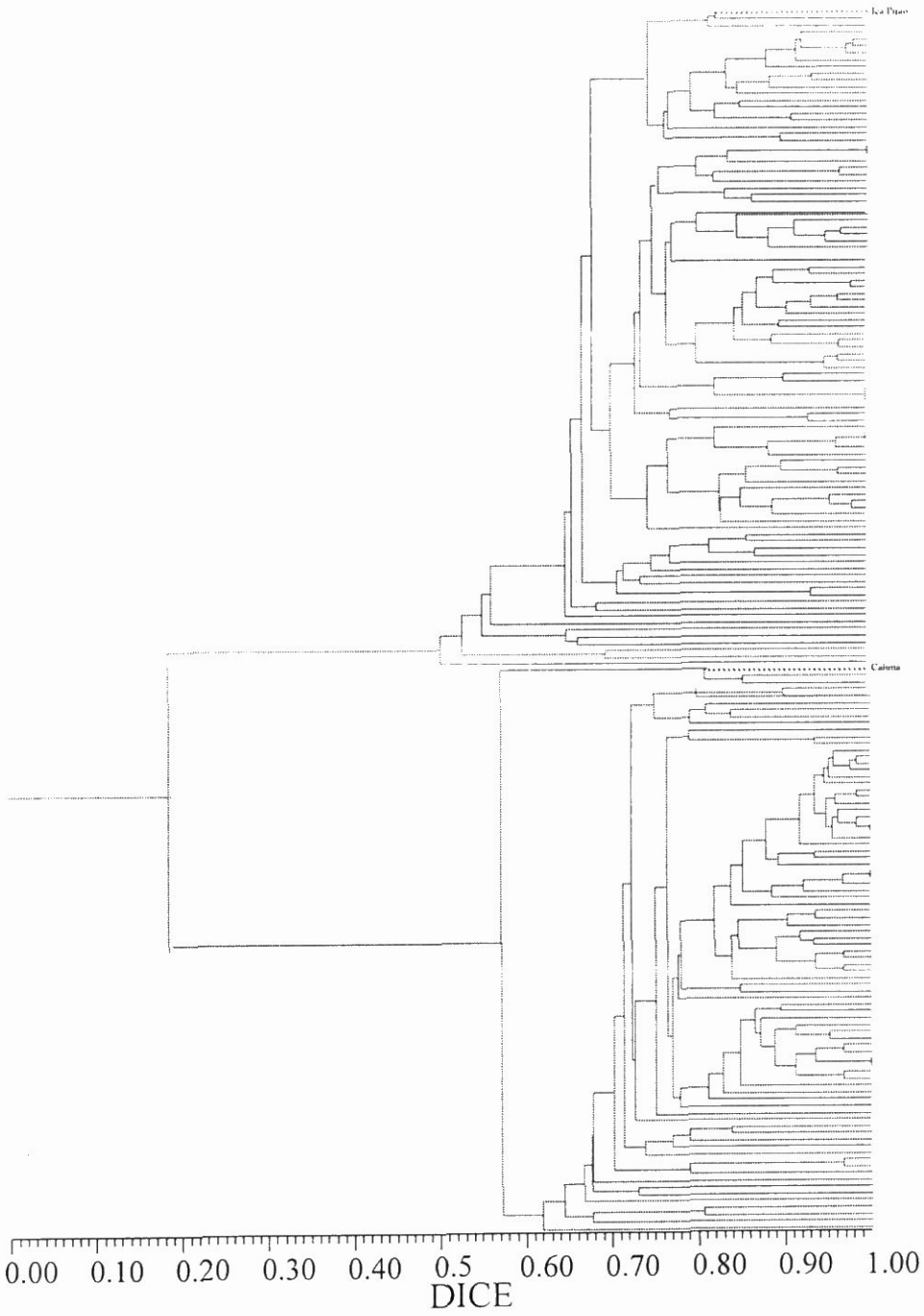

Figure 1. Dendrogram of relationships among Caribbean accessions of common bean uncovered by microsatellite marker analysis.

1.1.7 Generation of TILLING mutants in common bean

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Introduction

This project has initiated the development of TILLING (*Targeting Induced Local Lession in Genomes*) mutant resources for common bean given that fewer mutant stocks have been created for grain legumes compared to those for *Arabidopsis* (McCallum et al., 2000; Colbert et al., 2001; Till *et al.*; 2003; Greene et al., 2004), forage legumes (*Lotus* principally) (Perry et al., 2003) and the cereals (Till et al. 2004; Henikoff et al., 2004; Slade *et al.*; 2005). TILLING mutant collections are both a forward and reverse genetics tool that can be used to either find new phenotypic mutatnt or to screen for mutations in candidate genes through a PCR approach based on pools of total DNA from mutated individuals. Mutants for TILLING projects are generally generated by EMS treatment which causes a high frequency of point mutations.

Materials and Methods

Seed multiplication: We multiplied seed for the target TILLING genotype BAT93 over the course of two seasons in Colombia to increase the amount of seed to a total of over 20 kg. BAT 93 was selected as it is one parent of the mapping population reported on by Freyre et al. (1998) and has a high multiplcation ratio given that it is small ceded and broadly adapted. The multiplication of BAT93 was based on type specimens which were purified and fingerprinted with 150 microsatellite (SSR) markers to ensure homogeneity of the mutagenized genotypes and quality control during TILLING. The large-scale seed multiplication was done in order to ensure a seed supply for the mutagenesis work (next point).

Chemical mutagenesis: 9 ethyl methane-sulfonate (EMS) was used to create a M1 population of 300 fertile individuals in Univ. of Geneva which was shipped to CIAT for multiplication. In addition Univ. of Geneva has initiated further mutagenesis with an additional 4 kg. of seed that we prepared at CIAT as described above and are using an optimum EMS concentration that was determined by Pankhurst et al. (2003).

Generation advance and phenotypic screening: was performed in an enclosed screenhouse for the M1 to the M2 generation while the following generation was advanced in the field at CIAT headquarters. The M1:2 plants were screened for phenotypic differences compared to the non-mutated control genotype, BAT93. Phenotypic mutants (dwarfing, leaf fasciation, leaf variegation, spindly growth, etc.) were documented and photographed.

DNA extraction: a miniprep DNA extraction technique has been developed to extract DNA from 400 of the mutant M1:2 plants. The DNA quantity and quality has been checked using flourometry and gel-comparisons to stock (λ) DNA (Figure 1) in preparation for plans for pooling of individuals from different mutant families or pooling of the individual plants analyzed in the phenotyping described above.

Results and Discusión

We have produced an initial number of mutants for troubleshooting all the steps in the DNA extraction and pooling process but will need to increase the number of EMS mutants to start TILLING (targeted induced local lesions in genomes) protocols. So far 350 M1 families have been increase to the M2 generation (harvesting 6 to 8 plants per family and evaluating each for phenotypic mutations in the greenhouse as well as extracting total genomic DNA). From the first set of 100 families (each with 8 plants per family), the M2 generation (a total of 800 single-plant selections) was planted in the field to harvest M3 seed both as single seed descent and as bulks. From the second set of 250 families (each with 6 plants per family), the M2 generation was harvested (a total of 1000 single plant selections) and is awaiting field planting. Many leaf and non-germinating mutatnts were identified in the greenhouse; while a number of stable maturity date and dwarf or semi-dwarf mutants were confirmed when evaluated in the field. A database of photographs is being created with images taken in both field and greenhouse plantings similar to the database created for tomato TILLING mutants by Menda et al.(2004) (Figure 2, 3 and 4). DNA extractions has resulted in concentrations averaging 200 ng/µl and a total of 800 of the mutant lines have DNA isolations completed.

Conclusions and Future Work

A well-developed mutant stock, particularly in genotypes of common bean (*Phaseolus vulgaris*), a simple diploid species with a small genome (650 Mb), will serve the broad community involved in tropical legume improvement aiding gene-discovery both in common bean, the most widely consumed grain legume for human consumption and a major protein and mineral source in East Africa and Latin America, as well as in two tropical legume relatives: cowpea (*Vigna unguiculata*), a food crop important in West African agriculture and soybean (*Glycine max*) a major industrial and feedstock crop around the world. To reach this goal we will increase the number of mutants in the TILLING population. These mutant stocks will allow researchers in common bean to conduct both forward and reverse genetics experiments aimed at understanding the genes involved in abiotic and biotic stress tolerance as well as those genes involved in biological nitrogen fixation in the tropical legumes. Mutations will be sought in common bean genes that have been isolated at CIAT and shown to be associated with drought tolerance. The upcoming year's work will involve extraction and /or testing of CEL1 endonuclease on the heteroduplex mixtures of PCR products from wild type and mutant pools for genes of interest. This screening is expected to identify different mutants for each candidate gene. The phenotypic effect of these mutations will be analyzed as a proof of concept for the value of the mutant stocks generated by this project. Systematic phenotypic screening will be used to evaluate if the TILLING stocks show different morphology in the field (especially dwarfing or maturity mutants) and in abiotic stress tests in the greenhouse (Aluminum toxicity screen).

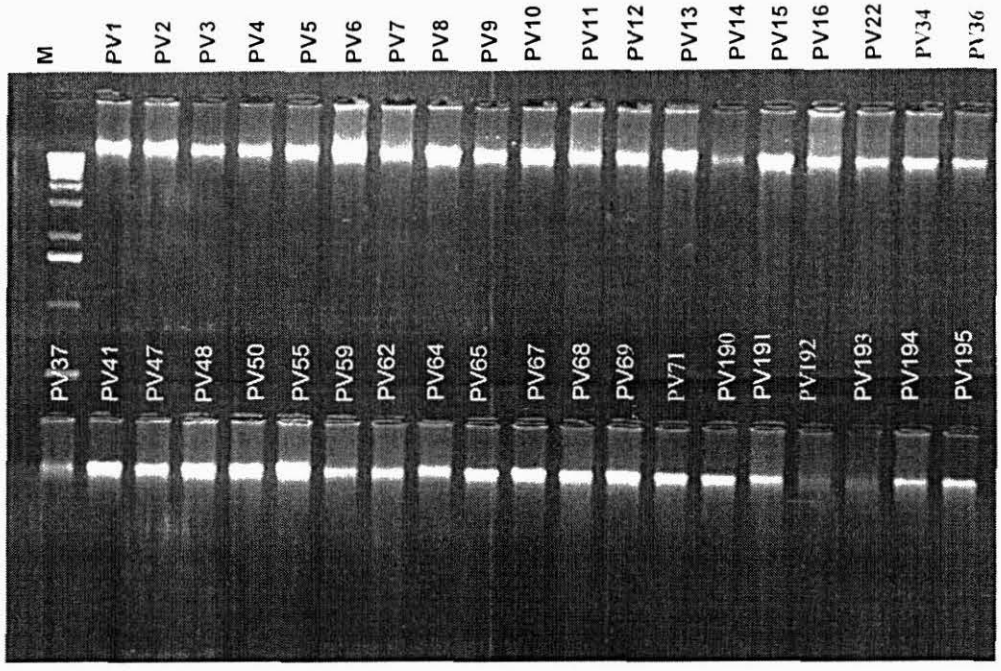

Figure 1. DNA quality evaluation for TILLING mutants.

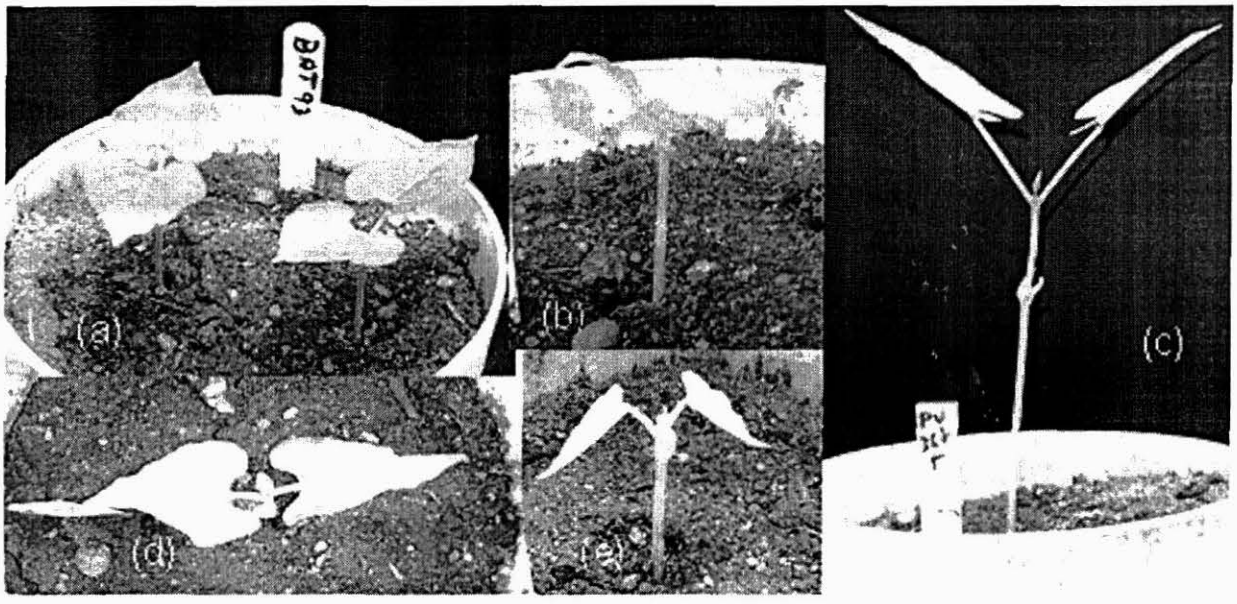

Figure 2. Mutant seedling phenotypes observed in the screenhouse evaluation of TILLING mutant M1 families. Comparison of wild type BAT93 (a) with mutant genotypes showing light green leaves (b), stem elongation (c) interveinal chlorosis (d) albinism (e).

Figure 3. Mutant leaf phenotypes observed in the screenhouse evaluation of TILLING mutant M1 families. Comparison of wild type BAT93 (a) with mutant genotypes showing leaflet fusion (b)(c)(d)(e), and chlorosis (f)(g)(h).

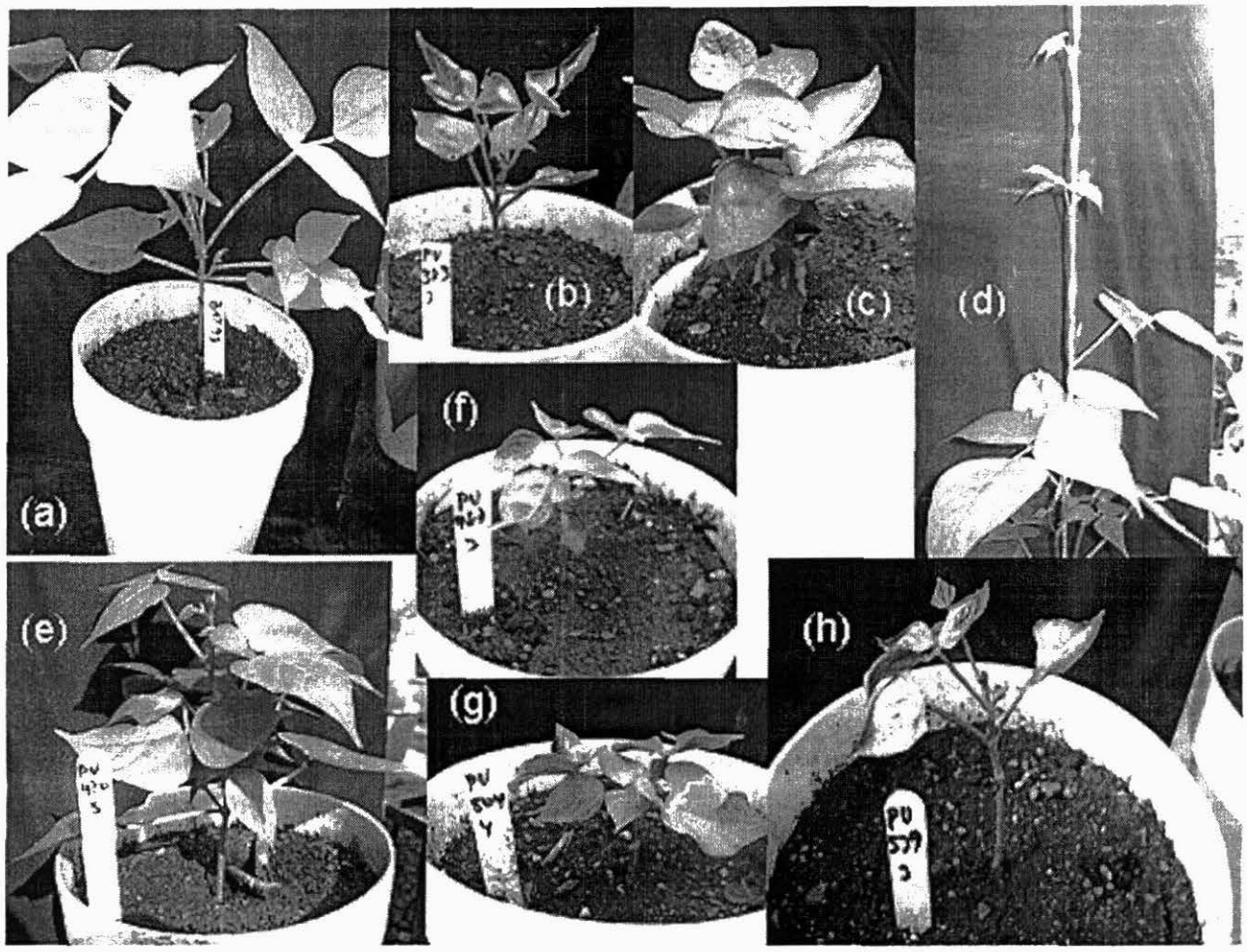

Figure 4. Mutant plant phenotypes observed in the screenhouse evaluation of TILLING mutant M1 families. Comparison of wild type BAT93 (a) with mutant genotypes showing short internodes or dwarfing (b)(c)(f)(e)(g)(f) and longer internodes or spindly phenotypes (d).

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1.1.8 Seed mineral analysis for bean landraces from Cauca

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Introduction

Iron deficiency anemia and zinc deficiency affect large number of people worldwide and in Colombia. Legumes are a good source of iron and other essential micronutrients that are found only in low amounts in the cereals or root crops. Unlike many cereals that are polished before eating, resulting in significant loss of nutrients, beans and other grain legumes are usually consumed whole, thus conserving their nutritional content. An ongoing project, has shown that bean seeds are variable in the amount of minerals (iron, zinc and other elements), vitamins and sulfur amino acids that they contain and that these traits are likely to be inherited quantitatively. In this study we tested a total of 31 improved varieties and local landraces grown in the department of Cauca for iron and zinc content. These genotypes are being evaluated as part of a collaborative project with FIDAR to promote high mineral beans found in local collections in various regions of Colombia and follows a similar analysis of local landraces from Nariño which we carried out last year.

Materials and Methods

The genotypes in the study included white, red, red mottled seed mostly but also liborino (yellow-mottled), andino (pink mottled) and mortiño (purple mottled) types. Seed sizes ranged from small (one navy and one black bean) to medium / large (the remaining Andean genotypes). Seed mineral content was evaluated at CIAT by 1) grinding 4 g of grain from each plot into a fine powder using a modified Retsch mill with 25 ml volume teflon chambers and zirconium grinding balls and 2) analyzing 2.5 g of the resulting powder in double replicates using a wet digestion method and Atomic Absorption spectrophotometry in the Analytical Services laboratory of CIAT for both iron and zinc concentration measured in parts per million (ppm).

Results and Discussion

Iron and zinc content for the 31 genotypes are shown in Table 1. The range in iron content was from 44 to 69 ppm while the range for zinc content was 25 to 44 ppm. The means for iron and zinc content were 56.1 and 31.6 ppm, respectively. The genotypes with highest concentration of iron were Blanquillo (68.55 ppm), MAC13 (67.95 ppm), Limoneño (67.80 ppm), El Piojo (66.09 ppm) and ICA Cafetero (65.20 ppm) while low iron genotypes included Caraota (46.91 ppm) Cargamento cafetero (46.76 ppm) Regional la maria (46.65 ppm) and Sangretoro calentano (45.50 ppm). Blanquillo (43.58 ppm) Sangretoro (38.30 ppm) and Palicero (37.34 ppm) were

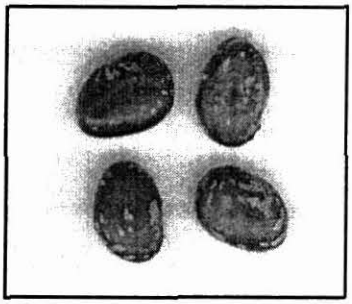
high in zinc, while the correlation between the two minerals across all the genotypes was r=0.4783 ($P<0.0001$).

Table 1. Iron and Zinc content in common bean landraces from the Cauca department grown in Pescador in semester 2005A.

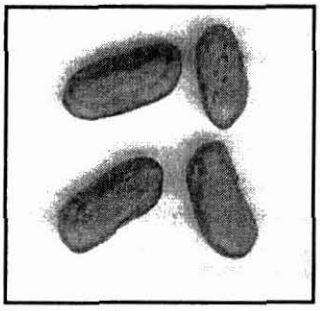
Genotype	Seed color	Seed size	Fe	Zn
Blanquillo	White	Small	68.6	43.6
MAC 13	Red mottled	Large	67.9	30.5
Limoneño	Pink mottled	Large	67.8	36.2
El piojo	Yellow mottled	Small	66.1	34.0
ICA cafetero	Red mottled	Medium	65.2	30.3
Liberal	Red	Medium	63.6	28.7
Sangre toro	Red	large	63.3	38.3
Sangre toro	Red	large	63.0	34.4
Morado	Purple mottled	Large	60.9	36.8
Frijol cacha amarillo	Yellow	Large	60.2	23.5
Africa 612	Red mottled	Medium	59.1	35.6
Mediavara	Red mottled	Small	58.1	29.4
Pinche	Vaquita	Large	56.6	26.0
Liberal	Red	Small	56.5	36.9
Calima	Red Mottled	Large	56.4	32.4
Cargamanto	Red Mottled	Large	56.1	31.9
Honduras	Red	Small	54.6	32.7
Perrito cansamuela	Red	Small	54.5	30.0
Palicero	Red	Small	54.3	37.3
Cacha	Red	Large	53.6	32.9
ICA cativo	Red mottled	Large	51.3	29.2
Cargamanto moraleche	Red mottled	Large	49.6	30.3
Pinto morado	Purple mottled	Medium	49.5	34.6
Guarzo	Red mottled	Large	49.5	27.4
Tio canela	Red	Small	49.2	31.8
Chocho rojo	Red	Large	48.6	27.9
Huerteño	Cream mottled	Large	48.2	28.0
Caraota	Black	Medium	46.9	25.4
Cargamanto cafetero	Red mottled	Large	46.8	27.7
Regional la maria	Red	Large	46.6	27.7
Sangretoro calentano	Red	Large	45.5	27.2
Average			56.1	31.6
SD			7.21	4.60

Figure 1. High mineral genotypes from the Cauca department grown in Pescador in semester 2005A

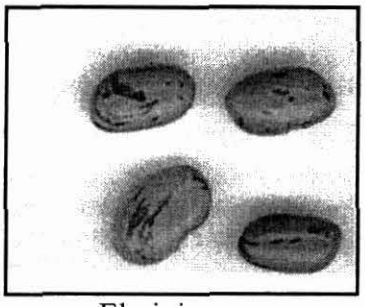
Blanquillo
Iron 68.5 ppm
Zinc 43.5 ppm



MAC 13
Iron 67.95
Zinc 30.5 ppm



Limoneño
Iron 67.8 ppm
Zinc 36.23 ppm



El piojo
Iron 66.0 ppm
Zinc 34.0 ppm

1.1.9 Evaluation of Geminivirus and drought resistance sources for legume conserved orthologous sequence (COS) markers.

LM Rodriguez, MW Blair
SB2-CIAT

Introduction

The objective of this project was to evaluate a set of Conserved Ortholog Set (COS) markers that were previously reported, in order to find highly conserved, single copy gene based markers to use in comparative mapping between legumes and for filling out genetic maps of various bean populations we are testing. These new markers were tested on a set of genotypes we have used for breeding heat tolerance, drought tolerance and BGYMV resistance. 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 where it is worsened by both drought and heat stress in which the vector is more active and problematic.

Materials and Methods

Plant material and DNA extraction: A panel of genotypes previously constructed with all the known sources of resistance to BGYMV and some sources of heat tolerance and resistance to drought was evaluated (Table 1). A total of 18 genotypes were included and represented the parents of nine populations. In addition, we used two bulks for BGYMV reaction from the DOR476 x SEL1309 population. DNA was extracted with a miniprep technique used in our laboratory.

Marker genotyping: the first set of COS markers included 19 markers which were selected from linkage group b03 based on *Medicago truncatula* core genetic map (linkage group b08) (Choi et. al. 2004). The second set of markers included 20 additional COS markers derived from *M. truncatula* ESTs. All markers were run on 2% agarose gels.

Results and Discussion

Only 59% of the COS markers amplified, but those that did were single copy and valuable for SNP or CAPS analysis. These methods will be necessary since all the markers were monomorphic as tested here, except for the COS marker named ASNEP which was useful as a dominant SCAR marker since it distinguishes all Andean genotypes tested.

Future work

The 41% that did not amplify using standard conditions will be re-tested. Based on parental amplification we will select COS markers for enzyme digestions (CAPS tests). Meanwhile, based on population polymorphism and bulked segregant analysis, we will try mapping COS markers on the populations represented by this survey. In addition, we will test another COS marker set from ICARDA on this same parental survey.

Figure 1. Parental survey showing monomorphic (AAT) and dominant polymorphic (ASNEP) markers on the entire geminivirus/drought survey.

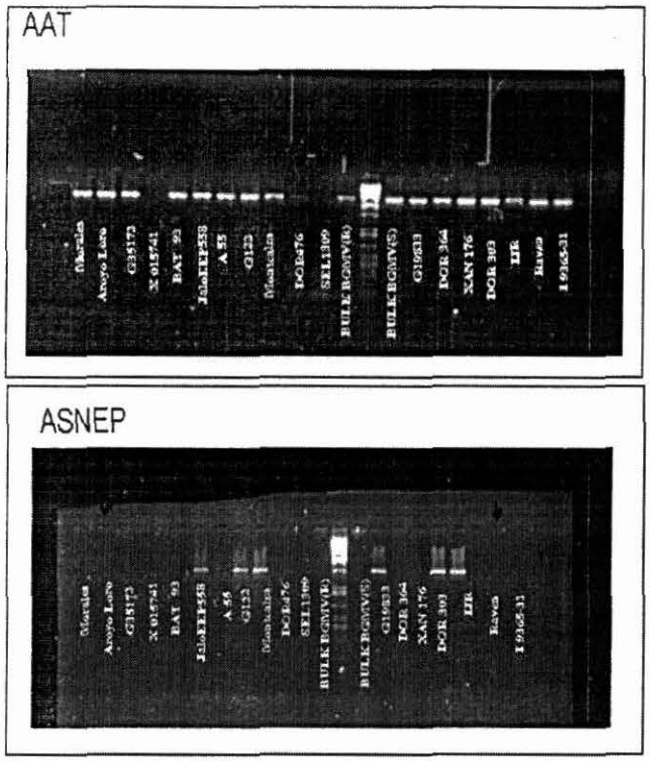
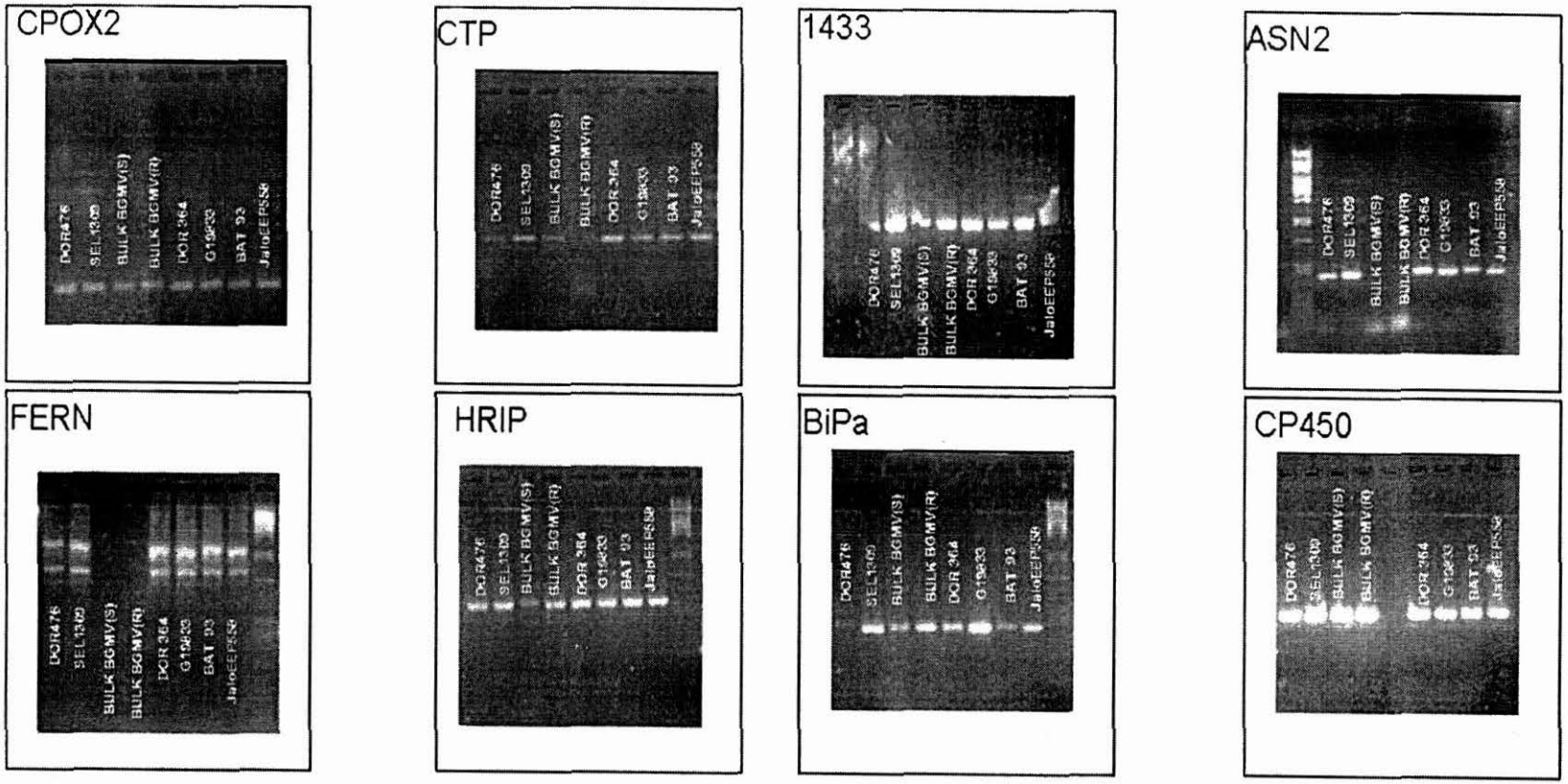


Table 1. Evaluation of COS markers on the geminivirus/ drought parental survey.

No	Genotype	Source [1]	EIF 5A	SUSY	CPOX2	CP450	CTP	HRIP	PGDH	1433P	ASN2	BiPA	FENR	PTSB	EST763	PGKI	AAT	ASNEP	CALTL	C
1	Morales	MA	N.E.	N.E.	N.E.	-	N.E.	N.E.	+	-	-	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+
2	G35172	-[2]	N.E.	N.E.	N.E.	-	N.E.	N.E.	+	-	-	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+
3	Arroyo loro	MA	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	+	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+
4	X 015741	MA	N.E.	N.E.	N.E.	-	N.E.	N.E.	-	-	-	N.E.	N.E.	N.E.	N.E.	N.E.	-	-	-	-
5	BAT	MA	+	+	+	-	+	+	+	(+/-)	+	+	+	+		+	+	-	+	+
6	JALO	A	+	+	+	-	+	+	+	(+/-)	+	+	+	+		+	+	+	+	+
7	A55	MA	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	+	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+
8	G122	A	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	+	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	+	+	+
9	Montcalm	A	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	+	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	+	+	+
10	DOR476	MA	+	+	+	-	+	+	+	-	-	+	+	+		+	(+/-)	-	+	+
11	SEL 1309	MA	+	+	+	+	+	+	+	+	+	+	+	+		+	-	-	+	(
12	BBGMVR	--	+	+	+	+	-	+	+	-	+	+	-	+		-	+	-	+	+
13	BBGMVS	--	+	+	+	+	+	+	+	-	-	+	-	+		-	+	-	+	+
14	G19833	A	+	+	+	+	+	+	+	-	+	+	+	+		+	+	+	+	+
15	DOR364	MA	+	+	+	-	+	+	+	-	+	+	+	+		+	+	-	+	+
16	XAN 176 (3)	MA	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	-	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+
17	DOR 303	A	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	(+/-)	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	+	+	+
18	IJR	A	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	(+/-)	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	+	+	+
19	Raven	MA	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	(+/-)	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+
20	I 9365-31	MA	N.E.	N.E.	N.E.	+	N.E.	N.E.	+	(+/-)	+	N.E.	N.E.	N.E.	N.E.	N.E.	+	-	+	+

Abbrv: 1/ MA: Mesoamerican, A: Andean, 2/ *P.coccineus*, N.E/ Not evaluated. 5 more COS makers amplified (data not show) but are subject of PCR condition improvement.

Figure 2. Parental survey of COS markers



1.1.10 Evaluation of parental polmorphism for drought and heat tolerance populations using microsatellite markers

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1. SB2-CIAT 2. UPR

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. BGYMV tends to occur in regions where drought and heat stress are problematic and where whitefly pressure is more intense. Therefore geminivirus resistance must be combined in a background of drought tolerance and adaptation to hot growing conditions. Our objectives for this study were to screen sources of geminivirus resistance and drought or heat tolerance for polymorphic microsatellites selected from across the entire genome in preparation for construction of genetic maps for recombinant inbred line populations derived from these sources.

Materials and Methods

A panel of 17 genotypes was constructed with sources of resistance to BGYMV and drought or heat resistance and included DOR303, DOR364, DOR476, G35172, Morales, X015741 as sources of geminivirus resistance, G122 and IJR as sources of heat tolerance and A55, Arroyo loro, G19833, Montcalm, SEL1309 and XAN 176 as susceptible parents. These genotypes represent the parents of nine populations that have been used to determine the genetic control of resistance and tolerance (two at CIAT and four elsewhere). The parents of a BCMV mapping population, Raven x I 9365-31 were also included. Miniprep and medium scale DNA extraction techniques were used based on a CTAB- phenol/chloroform extraction from Dellaporta et al. (1983). All CIAT microsatellites with known map locations from the DOR364 x G19833 population were screened on 4% PAGE gels with silver staining.

Results and Discussion

In the microsatellite survey, the parental combinations varied in their level of polymorphism (Table 1). The cross A55 x G122 being inter-gene pool was much more polymorphic than the intra-gene pool cross G122 x Montcalm which had the lowest polymorphism rate among the combinations analyzed. These two populations were of major interest for the heat tolerance study since G122 is a source of heat tolerance and both populations produced interesting phenotypic results in a drought/heat stress trial in Palmira in 2003 B (non stress) and 2004A (under both heat stress and drought stress in separate trials) (Table 2). Other populations were intermediate in polymorphism. It was notable that the DOR303 x IJR population was almost as polymorphic as DOR364 x G19833 population in this study.

Future work

Based on parental polymorphism and bulked segregant analysis, we will select microsatellite markers for mapping on the populations represented by this survey, mainly for drougth/heat resistance populations

Once a genetic map is prepared, QTL analysis will combine phenotypic data described above with the genotypic data for the A55 x G122 and G122 x Montcalm populations to identify loci important in heat and drought tolerance.

Table 1. Number of polymorphic microsatellite markers in every population and overall percentage population polymorphism. No. of markers tested =153.

Population	Number of polymorphic markers	% polymorphism on each population
Arroyo loro x X -015741	74	48
DOR 476 x SEL 1309	59	39
DOR 364 x G19833	107	57
DOR x IJR	56	56
A 55 x G122	113	74
G122 x Montcalm	36	24
Raven x I9365	50	33
DOR364 x XAN176	48	31

Table 2. Yield evaluation (kg/ha) for the A55 x G122 and G122 x Montcalm populations under drought, heat and non-stress environments in Palmira in 2003B and 2004A.

	A55 x G122		G122 x Montcalm		
	Drought	Non-stress	Drought	Heat	Non stress
Total no. individual	60	60	96	96	95
Mean	724.28	1523.40	424.45	1696.4	1585.8
SD	227.57	292.67	141.32	294.52	184.4
Minimum	350.21	792.41	146.7	1125.2	1236.2
Máximum	1397.9	2296.4	770.3	2320.9	2237.7

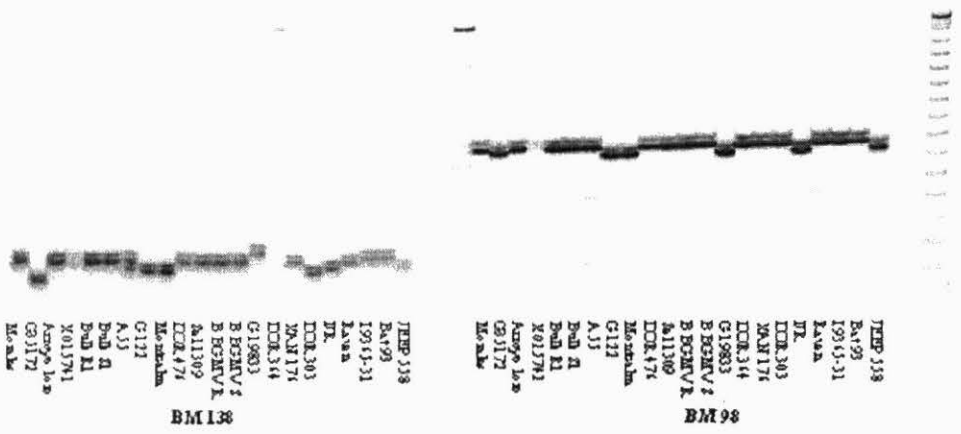

Figure 1. Parental survey of Geminivirus, heat and drought resistance sources.

1.1.11 Development of climbing beans with higher seed iron and zinc content from a single backcross using the G14519 source of mineral accumulation.

[1]MW Blair, [1]C. Astudillo, [1]A. Hoyos, [1]S. E. Beebe, [2]O. Mosquera
1. IP-1, CIAT 2. Analytical Services, CIAT

Introduction

Iron deficiency anemia and other micronutrients deficiencies affect over 3.5 billion people and due to a generalized decrease in the quality of poor people's diet has actually increased over recent decades, even in developed countries and even in areas where food is not limiting. Legumes are good source of iron and others essentials micronutrients that are found only in low amounts in the cereals or root crops. An ongoing Project, has show that bean seeds are variable in contain and that these traits are likely to be quantitatively inherited. These results suggest that is will be possible to breed for better micronutrients content in common bean. The objective of this work was to use backcrossing to increase the mineral content of two varieties of climbing, red seeded beans of the Mesoamerican genepool (G685, G2333) that will be useful in Africa, the Caribbean and South America. The source genotype for the high iron trait was G14519 a medium sized brown climbing bean that has been used successfully in recurrent backcrossing program for bush, red mottled beans (CIAT, 2004). The new line code for the genotypes derived from this work will be nutritional climbers (NUV series). The high iron source has been used for molecular tagging of QTLs for iron accumulation so these lines will be useful for QTL confirmation.

Materials y Methods

Crosses were made between the high mineral source, G14519, and both target genotypes, G685 and G2333, followed by a single backcross to the target genotypes. Bulk selections were made

in the BC1:F1 through BC1:F4 generation and individual BC1:F4:5 lines were derived in Palmira. The individual selections were then grown in two locations, Darien (1450 masl) and Palmira (1000 masl) for general and heat adaptation, respectively. The first group will heretofore be called the non-heat tolerant group while the second group will be called the heat tolerant group. Mineral content was determined for each line and the top 100 lines for iron content from Darien were grown for confirmation in Popayán; while the entire set of lines tested in Palmira were grown in Darien to obtain a more reliable estimate of mineral content since estimates in Palmira are not as reliable as those in Darien or Popayan due to the higher pH found in Palmira. All mineral analysis was done as described previously with ground seed processed in a Retsch Mill and evaluated for iron and zinc by Atomic Absorption Spectrophotometry in the Analytic al Services Laboratory of CIAT.

Result and Discussion

A total of 255 lines were selected for the non-heat tolerant group and grown in Darien of which 100 lines with higher iron were grown for confirmation in Popayan along with nine control genotypes (G14519, G2333, G685 each planted in three replicates). A total of 374 lines were selected for the non-heat tolerant group and grown in Palmira of which 366 were repeated in the Darien planting. For the non-heat tolerant group in both Darien and Popayan the range observed for seed iron content across all lines was from 34 ppm to 94 ppm, with averages of 60.63 ppm for Darien and 56.13 for Popayan (Table 1). For the heat tolerant group harvested in both Palmira and Darien the range in seed iron content was similar from 36 ppm to 104 ppm for iron content with averages of 62.6 ppm for Palmira and 55.90 for Darien.

Analysis of variance (data not shown) for both non and heat-tolerant groups showed significant genotype x environment interaction and correlations between sites were moderate ranging from r = 0.163 to r = 0.359 (Table 2). Correlations between iron and zinc content ranged from r = 0.230 to r = 0.395 but were higher for the heat tolerant group of lines grown in Palmira 2002B and Darien 2004B than for the non-heat tolerant group of lines grown in Darien in 2002B and Popayan 2004B.

Both the crosses with G 2333 as well as those with G 685 produced BC1:F4:5 lines that were higher in seed iron and zinc content than their respective recurrent parents and close but not quite as high as the donor parent, G14519 which had up to 92.1 ppm iron in Palmira, 87.9 ppm in Darien and 74.4 ppm in Popayan. The recurrent parents had lower iron content than the average of the lines with G685 having 52.8 ppm in Pamira, 53.1 ppm in Darien and 42.4 in Popayan; and G2333 having 65.0 ppm in Palmira, 53.6 in Darien and 52.8 in Popayan. From these results it appears that G685 has lower baseline seed iron content than G2333.

Conclusions and future work

The fact that higher mineral lines were obtained from the backcrossing program confirmed the utility of the higher iron source parent G14519 we had used previously. However the background of the climbing bean recurrent parents which had lower starting content of minerals than the Andean beans we had worked with previously made it difficult to obtain the very high mineral content we were looking for. The results suggest once again that the accumulation of seed iron and zinc appears to be a quantitative trait. More effort will be needed to pyramid sources of high mineral accumulation to obtain the expected increases for climbing beans. It will also be interesting to analyze whether mineral content is related to yield potential in climbing

beans and whether there is a dilution effect of higher yield on mineral accumulation in this growth habit.

Table 1. Descriptive statistics for iron and zinc content of backcross lines (G 685 x (G 685 x G 14519) and G 2333 x (G 2333 x G 14519) in Darien, Popayan and Palmira in the semesters 2002B and 2004B.

	IRON CONTENT				ZINC CONTENT			
	FEDAR02B	FEPOP04B	FEPAL02B	FEDAR04B	ZNDAR02B	ZNPOP04B	ZNPAL02B	ZNDAR(
N	255	109	374	366	255	109	373	366
Sum	15460	6117.7	23416	20460	6484.3	3181.6	10126	8536.8
Lo 95% CI	59.52	54.59	61.47	55.13	24.87	28.41	26.75	23.03
Mean	60.63	56.13	62.61	55.90	25.43	29.19	27.15	23.33
Up 95% CI	61.74	57.66	63.75	56.68	25.99	29.97	27.55	23.62
SD	9.02	8.08	11.24	7.54	4.56	4.11	3.93	2.89
Variance	81.31	65.26	126.34	56.78	20.81	16.92	15.48	8.34
SE Mean	0.56	0.77	0.58	0.39	0.29	0.39	0.20	0.15
C.V.	14.87	14.39	17.95	13.48	17.94	14.09	14.49	12.38
Minimum	39.11	34.31	36.09	34.50	14.47	19.77	18.08	14.57
1st Quarti	54.56	50.96	55.01	50.85	21.75	26.24	24.20	21.40
Median	59.92	56.43	62.12	55.96	25.46	29.01	26.81	23.27
3rd Quarti	66.23	61.77	69.94	60.80	28.22	31.48	29.62	25.23
Maximum	88.01	81.47	104.10	82.75	38.01	41.32	40.01	35.37
MAD	5.88	5.35	7.58	4.96	3.24	2.64	2.64	1.90
Biased Var	80.99	64.66	126.00	56.63	20.73	16.76	15.44	8.32
Skew	0.30	-0.09	0.26	0.24	0.34	0.39	0.49	0.21
Kurtosis	-0.02	0.43	0.16	0.12	-0.39	0.41	0.10	0.65

Table 2. Correlation between iron and zinc content in Darien, Popayan and Palmira for the heat and non-heat tolerant backcross lines from the crosses (G 685 x (G 685 x G 14519) and G 2333 x (G 2333 x G 14519).

		IRON			ZINC			IRON vs ZINC
GROUP	LOCATION	1	2	3	1	2	3	
NON - HEAT	Darien							0.282*
	Popayan	0.359*			0.180*			0.230*
HEAT TOLERANT	Palmira	0.163*	0.170*		-0.054ns	-0.175ns		0.395*
	Darien	0.331*	0.178*	0.266*	0.338*	0.000 ns	0.124 ns	0.377*

Significance at P=<0.05(*), 0.01 (**), 0.001(***) or not significant (ns)

1.1.12 Genotype x Environment interaction for condensed tannin content of common bean grain grown in a multi-locational trial in Nariño, Colombia.

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Introduction

Tannins are products of secondary metabolism in plants, and are anti-nutrients in that they reduce mineral availability for the human diet. Seed tannins are found predominantly in the seed coat rather than in the cotyledon or embryo and can make up as much as 30% of this part of the seed. Previously we found that seed tannin content (both soluble and insoluble condensed tannins) was inherited quantitatively and varied with seed color in recombinant inbred line populations of common bean. In this study we analyze seed coat tannin in a set of high iron lines from the NUA series and compare their seed coat tannin levels to a series of Andean control genotypes all grown in a replicated field experiment in three locations in Nariño. This work is based on a new methodology to reliably determine condensed tannin contents in common bean seed coats that we developed and improved over the past two years. The overall objective is to determine whether seed tannin contents in the high iron red mottled lines we have been developing is similar to tannin content in standard varieties of red colored Andean beans, in preparation for a bioavailability studies that will be carried out with Univ. del Valle and Cornell University.

Materials and Methods

A total of 11 genotypes were planted in randomized complete block design experiments in the municipalities of Consacá, Sandoná, and Yacuanquer, Department of Nariño, Colombia. Two samples of 10 seeds each were analyzed for each genotype. Only the peeled seed coats of these samples were ground for analysis, while the cotyledons and embryos of the peeled seed were discarded.Condensed tannins were extracted using 70% acetone and analyzed spectrophotometrically using the butanol–HCl method. Two replicated measurements were used to assess sample variability and repeatability of the method. Where coefficients of variation were above 10%, two addition determinations were conducted. Tannin concentrations were determined by comparing absorbance values to a standard curve created for purified tannins from large red seeded beans. The data obtained were analyzed, using Statistix v. 8.0.

Results and Discussion

The analysis of variance for soluble and insoluble condensed tannins is shown in Table 1. Source of variance were significant for soluble and insoluble condensed tannins for genotype but not for location x genotype interactions. The effect of location was significant for total condensed tannins and somewhat significant for soluble condensed tannins (P=0.0515) but not for insoluble condensed tannins alone (P=0.1422). The genotype effect was also significant for total condensed tannins as it was for the components. The analyses of variance between the determinations or laboratory replications, did not show significant differences and coefficients of variation were low (soluble condensed tannins = 12.66%; insoluble = 18.90%; and totals = 11.45) demonstrating

the reliability of the butanol–HCl method for quantifying condensed tannins in bean seed coats. It was also notable that this method was also reliable for laboratory replications performed on different days allowing the extracted samples to be stored between determinations.

Genotypic differences in average total condensed seed coat tannins seemed not to be due to the genotype's seed color (red mottled versus large red) since both Radical Cerinza and Sangretoro, three red beans, had no higher tannin content than AFR612, Calima and CAL143, three red mottled check beans (Table 2). Among the NUA lines, NUA 45 was variable and presented high total condensed tannin content in Yacuanquer and Sandona but lower content in Consaca. The other NUA lines, except for NUA50 were more stable for total condensed tannin content. NUA35 was notable for being lower in total condensed tannin with 12 to 13.2% only compared to standard red mottled genotypes, such as AFR612 and Calima which had 18 to 19.6 %. This characteristic of NUA35 combined with its high iron content may make it a promising genotype to promote for nutritional quality and for the bioavailability trials. We also point out that differences due to the effect of location (Figure 2) resulted from genotypes with highly variable values for the three locations. Yacuanquer had higher values than Sandoná and Consacá, which presented similar means. However, as mentioned above most of the genotypes exhibited certain stability within locations, as confirmed by results of analyses of variance for the means, which suggested that no differences existed between locations, except for total tannins, where the sum of soluble and insoluble tannins increased these differences.

Table 1. Analyses of variance for contents of soluble, insoluble, and total condensed tannins in common bean varieties planted at three sites, Department of Nariño, Colombia.

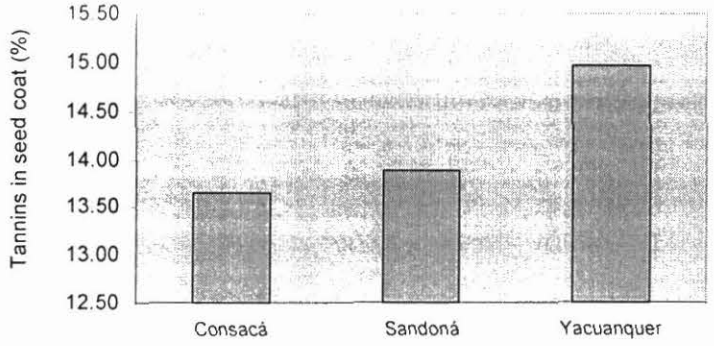
Source	df	SS	MS	*F*	*P*
Soluble condensed tannins					
Site	2	21.901	10.9507	3.41	0.0515
Genotype	10	222.992	22.2992	6.94	0.0001
Site × genotype	20	79.575	3.9788	1.24	0.3124
Genotype × replication	11	11.768	1.0698	0.33	0.9687
Error	22	70.739	3.2154		
Total	65	406.976			
Insoluble condensed tannins					
Site	2	0.8813	0.44063	2.13	0.1422
Genotype	10	31.3677	3.13677	15.20	0.0000
Site × genotype	20	3.3992	0.16996	0.82	0.6670
Genotype × replication	11	0.4319	0.03926	0.19	0.9966
Error	22	4.5415	0.20643		
Total	65	40.6215			
Total condensed tannins					
Site	2	31.294	15.6469	4.34	0.0257
Genotype	10	286.080	28.6080	7.94	0.0000
Site × genotype	20	85.273	4.2636	1.18	0.3488
Genotype × replication	11	14.720	1.3382	0.37	0.9540
Error	22	79.248	3.6022		
Total	65	496.614			

Table 2. Total condensed seed coat tannins (measured as % of seed coat) in 11 genotypes of common bean grown in three locations in the Department of Nariño, Colombia.

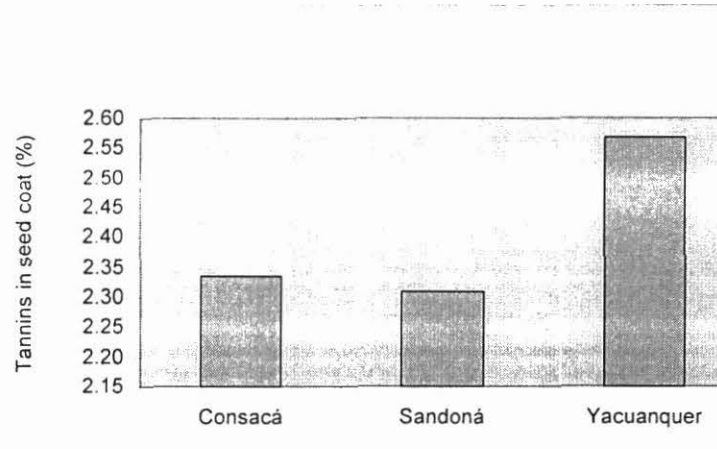
	Location		
Genotype	Consacá	Sandoná	Yacuanquer
AFR612	18.14	18.04	19.53
Choco	17.91	17.98	17.95
Diacol Calima	19.18	19.59	18.99
Sangretoro	14.95	14.81	15.12
Radical Cerinza	17.04	17.90	17.44
CAL143	15.13	15.72	14.00
NUA50	13.65	12.49	17.87
NUA35	12.05	13.05	13.20
NUA4	17.30	16.54	18.53
NUA56	14.54	13.79	16.59
NUA45	15.95	18.11	23.67

Figure 1. Means of tannin contents in common beans growing at three locations in the Department of Nariño, Colombia.

(a) Soluble condensed tannins



b) Insoluble condensed tannins



1.1.13 Seed multiplication of high mineral NUA lines for nutrition studies

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Introduction

High iron NUA lines were developed as described in previous years' annual reports. Four sister lines each were selected to represent high iron and zinc versus low iron and zinc. All of these were developed from backcrosses with the recurrent parent CAL9 and have good red mottled seed type. The four high iron lines and CAL96 have been in high demand for bioavailability studies in Colombia and East Africa as well as nutritional quality tests at Cornell University. Therefore over three seasons we have multiplied the amount of seed of the NUA lines. Our overall objective is to have over 750 kg of high and low iron beans for a nutrition study with Universidad del Valle and good seed stocks for rapid multiplication in case of future studies with animal models as well as for variety promotion and extension work. Several of the lines are also being compared to established Colombian varieties for the accumulation of minerals both with and without fertilization (see previous sections) in agronomic trials in Nariño, Cauca and Valle. CAL967 has proven to be a popular variety for farmers in these areas and is being promoted. The seed color of the NUA lines likewise is very acceptable.

Materials and Methods

Five genotypes were chosen for multiplication – these were the high iron lines NUA30, NUA35, NUA45 and NUA56 as well as the recurrent parent CAL96. The genotypes were grown in multiple row plots in Darien 2004B, 2005A (Table 1) and then in large plots in 2005B (data not shown). Rouging was used to remove any off-type plants with unexpected growth habit from the multiplication plots.

Results and Discussion

pending

Conclusions and Future Work

We are evaluating the mineral content by row of the genotypes that were multiplied. This is to maintain seed purity and nutritional quality. CaCoII bioavailability tests will be conducted on the NUA lines to see if their *in vitro* bioavailability differs from CAL96. The NUA lines themselves will be promoted with farmers in several regions of Colombia. Comparative work in Kenya, Malawi and other countries of Eastern and Southern Africa is underway.

Table 1. Multiplication plots of four high iron NUA lines and CAL96 in Darien in 2004B and 2005A.

Line code	Source	2004B rows	2004B yield (kg)	2005A yield (kg)	Purpose
Low Iron Parent					
CAL 96	DAR 2004 A/B	NA	10.0	100.0	Mass increase for Univalle nutrition FIDAR multiplication
High Iron Lines					
NUA 30	DAR 2003 A	4471-80	2.0	23.0	FIDAR multiplication
NUA 30	NAR 2004 A	4583-46	4.0	41.0	Basic seed - Cornell tannin analysis
NUA 35	DAR 2003 A	4481-86	1.0	4.0	Reserve
NUA 35	DAR 2004 A	4487-45	2.0	27.0	Basic seed - Cornell tannin analysis
NUA 35	NAR 2004 A	4601-19	3.7	29.0	FIDAR multiplication
NUA 45	DAR 2004 A	4501-15	2.0	22.0	Basic seed - Cornell tannin analysis
NUA 45	PAL M3 2004 A	4551-60	2.0	19.0	Mass increase for Univalle nutrition
NUA 45	NAR 2004 A	4620-36	3.7	49.0	FIDAR multiplication
NUA 56	DAR 2004 A	4516-34	4.0	36.0	Basic seed - Cornell tannin analysis
NUA 56	PAL M3 2004 A	4561-69	1.0	13.0	Cornell Bioavailability
NUA 56	NAR 2004 A	4637-52	3.6	32.0	Cornell Bioavailability
NUA 59	DAR 2004 A	4535-50	2.0	30.0	Reserve
NUA 59	PAL M3 2004 A	4570-82	2.0	25.0	Basic seed - Cornell tannin analysis

1.1.14 Purification of tannins in color varieties of common bean

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Introduction

Among the compounds that affect the nutritional quality of common bean seeds are condensed tannins, which are able to chelate minerals and to interact with proteins reducing mineral bioavailability and protein digestibility. Tannins are phenolic compounds and they are synthesized in the flavonoid pathway. Therefore, tannins contribute to the coloring found in the seed coats of common beans. In the previous year we began tannin quantification through the Butanol-HCl method, which requires a calibration curve with purified tannins. Since calibration curves realized with purified tannins could have a different behavior among color classes of beans, it was important to us to determine specific calibration curves for each color class that we could use to quantify tannins more accurately. For that reason, we selected bean genotypes from the CIAT core collection that differed in seed coat color and we realized calibration curves with each one in order to determine differences among them and improve the accuracy in spectrophotometric quantification of condensed tannins through the Butanol-HCl method.

Materials and Methods

Plant Material and Seed Coat Preparation: 10 accessions from CIAT core collection were analyzed in this study (Table 1). The accessions represented variety of color classes. Seed coats were peeled from seed of the varieties after soaking in n-heptane for 24 hours and ground into a fine powder by hand to use in the analysis. Approximately 10g of ground seed coat were used for each seed color sample.

Tannin Purification: The tannins were extracted from the ground seed coats first with 70% acetone and then with pure diethyl ether. Tannins were then purified with a Sephadex LH-20 column. The tannins were then lyophilized and treated with Butanol-HCl in the same way as done previously with the samples for quantification. A Shimadzu UV-1601 spectrophotometer was used for detection. The calibration curve was established by plotting average absorbances against tannin concentrations using a dilution series made from a stock solution of purified tannins and distilled water.

Results and Discussion

The purified tannins showed similar color to the seed coat color of the source genotype; meaning that the darker the seed coat of the genotype the darker the purified tannins. The same purification process was realized in white beans, but with higher amount of seed coat due to the expected low levels of tannins found in this commercial class of beans. Indeed, no tannins were detected in the white beans even with the larger sample for extraction, which confirmed the absence or minimal presence of these compounds in white beans. Because of this, it wasn't possible make a calibration curve for white beans.

Concentration of tannins were measured based on the calibration curve relating concentration to absorption. Since the absorption is measured based on the Beer-Lambert law, estimates of concentration are only valid for solutions in which interactions between the absorbent compounds are minimal. So far, these interactions appear only at the upper levels of concentrations above 0.01M concentration where a non-linear relation between absorbance and concentration can be expected. For this reason, taking into account the linearity, we chose to evaluate concentrations only to a maximum value of 0.60mg tannin/mL water with a maximum absorbance of 1.30 which is below the critical threshold molar concentration given above. At high concentrations, interaction between the tannin species or subcomponents would deviate the light path in a non-linear manner and create inaccurate results in the spectrophotometric analysis. This can be taken care of in a few extreme cases of higher than threshold tannin concentration by using a dilution factor.

When comparing the concentration curves, all commercial classes had similar slope except for the curve for black beans, which had a higher slope (Figure 1). The difference among the slopes for each curve is probably based on the proportion and type of tannins in the samples from the different genotypes, since the absorbance depend in large proportion on the structural shapes of the molecules in the analyzed sample. In general, the curves had good linearity and good detection and quantification limits (Table 2, table 3). The regression, slope deviations and Sc value allowed us to calculate the error for any datapoint based on the respective curve. A 95% confidence interval was estimated using three repetitions per datapoint and an average of ten points per curve.

Table 1. Purified genotypes for calibration curves of condensed tannins.

Genotype	Color
G18372	White
G23073	White/Brown
G7150	White/Red
G11419	Cream
G1844	Cream
G21725	Cream
G12610	Cream
G5706	Black
G11350	Red
G15137	Red

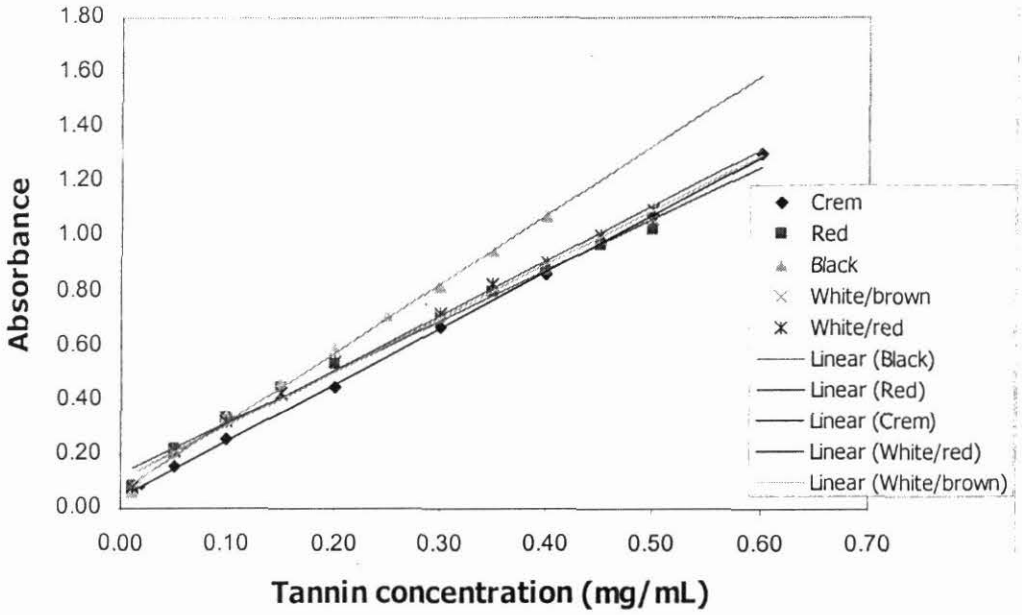
Table 2. Statistics values (b= Slope, a= Intercept, Sr= standard deviation of y estimate, Sb = standard deviation of the intercept) for calibration curves of different common bean color classes.

Color	b	a	Sr	Sb	Confidence Interval
Crem	2.0665	0.0458	0.0119	0.0192	0.0176
White/brown	1.9726	0.1099	0.0278	0.0536	0.0084
Red	1.8645	0.1304	0.0327	0.0629	0.0164
Black	2.5347	0.0677	0.0150	0.0357	0.0090
White/red	2.0104	0.1079	0.0249	0.0480	0.0166

Table 3. Detection and quantification limit for calibration curves of different common bean color classes (X= Tannin Concentration, Y= Absorbance, DL= Detection Limit, QL= Quantification Limit).

Color	LDy	LDx	LCy	LCx
Crem	0.0816	0.0173	0.1652	0.0578
White/brown	0.1934	0.0423	0.3882	0.1411
Red	0.2284	0.0526	0.4571	0.1752
Black	0.1125	0.0177	0.2172	0.0590
White/red	0.1827	0.0372	0.3572	0.1240

Figure 1. Calibration curves of different common bean color classes.



1.1.15 Tannin content of commercial classes of common bean.

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Collaborators: P. Fajardo, C. Lascano (CIAT-Forage project)

Introduction

Common bean seed (*Phaseolus vulgaris* L.) is a significant source of protein, calories, vitamins and minerals to diets of Latin America and Africa. However, seeds of some varieties of beans may contain high amounts of phytate, protease inhibitor and tannins, which adversely affect the nutritional quality of beans (House et al., 2002). 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 / proanthocyanidins (derived from polymerized flavonoids), which are measurable by different techniques. In 2002, bean genotypes that differed in their apparent ability to accumulate minerals and antinutritional factors were selected from the CIAT core collection (House et al., 2002). In that study, tannin content was determined using the vanillin method (Desphande et al., 1987), methanol extraction and a commercial standard for the calibration curve. While this previous method was useful for general analysis, the butanol-HCl method has advantages over the vanillin method in that acetone extraction is known to be a better solvent for tannin extraction and purified tannin from beans can be used as a more appropriate standard than any of the commercially available ones. In addition, photometric detection is more accurate with this second method compared to the first. Because of the differences between methodologies we decided to evaluate the same 27 genotypes for tannin content using the Butanol-HCl method as those that had been measured with the Vanillin method. In addition we created calibration curves from purified tannins for more accurate quantification of bean tannins and chose a further set of genotypes to represent additional commercial classes and seed colors (see Table 1).

Materials and Methods

Plant Material: A total of 38 accessions all from the CIAT core collection were selected to represent a variety of commercial classes and seed colors. Of these accessions, 22 had been analyzed previously by House et al. (2002) and 16 were chosen to further study of the white and dual white and non-white patterned seed classes.

Seed Coat Preparation: Seed coats were peeled from the grain and ground into a fine powder by hand to use in all subsequent analysis. To facilitate seed peeling, the seeds had been soaked in n-heptane for 24 hours. A total of 10 mg of ground seed coat and three replicates were used per seed coat sample.

Tannin Extraction: Total condensed tannin extraction and analysis of soluble and insoluble condensed tannins were as reported last year. Photometric tannin analysis was realized with the Butanol-HCl method and the blank was a butanol-water (5%) mix. The calibration curve used was chosen from a variety of curves described in a previous section of this annual report made for determining differences among purified tannins from bean seeds of different color. The absorbance of the samples was determined at 550nm in a spectrophotometer Shimadzu UV-1601.

Results and Discussion

The amount of soluble, insoluble and total condensed tannins in the beans that were analyzed ranged from a minimum of 0.00 % (for almost all the white bean accessions) to maximums of 16.8%, 4.3% and 18.95%, respectively for non-white beans (Tables 1 and 2). The genotype with the highest soluble condensed tannins was a red bean (G11530) while the genotype with the highest insoluble condensed tannins was a yellow bean (G13220); however when these two tannin types are added together the genotype with the highest total condensed tannins was a brown bean (G3971). Black, purple and black mottled beans although they are the darkest in the color range have intermediate to intermediate/high amounts of total tannins. Cream beans especially lighter seed coated cream beans had low soluble and total tannins. Correlation between

soluble and insoluble tannins was high (r=0.861, P>.001) across all genotypes. Mean separation with least significant differences is shown in Table 2.

Among the 15 white accessions analyzed only one, G18372, contained tannin in its seed coats and this was due to insoluble tannins. In this case we used the calibration curve for cream colored beans given the difficulty in generating a calibration curve specific for white beans due to the lack of tannin in that seeds for sufficient purification. This may have under-estimated the tannin content for white beans however the results coincide with previous reports about absence of tannins in white common bean seeds. On the other hand, white beans with colored patterns, heretofore called white mottled beans, had varying amounts of tannin depending on the proportion of the seed coat that contained color and the proportion lacking color as well as the type of color present with white and red combinations having more tannins than white and black, white and brown or white and cream beans. As far as white seeds were concerned, their lack of tannins was probably related with their lack of pigment, so it is probably impossible to transfer the null or very low tannin trait from white beans into other colors without also transferring white seededness which is not always preferred by consumers. Nevertheless, the apparent variability within color classes establishes the possibility of alter the tannin content in the future achieving the lower tannin levels in other colors of beans as well. Because the pigmentation plays an important role in breeding, the study of the relationships between seed coat color and tannin content is an important step in the elucidation of genes from the biosynthetic pathway and in the effort to improve the bioavailability of mineral content, which is affected by tannins. It will be interesting to determine if tannin content and seed coat color are correlated in common beans as has been observed with some other legumes, notable *Vicia faba* (Cabrera and Martin, 1989).

Overall, tannin measurements were consistent between repetitions showing that the accuracy range of the Butanol-HCl method was good. Compared to the Vanillin HCl method which had been carried out previously on the first set of 22 non-white seed coat beans as described in House et al. (2002), the Butanol method was more accurate as the ranges coincide with values previously reported for common bean of 0% to 18% tannin per seed coat weight(Ma y Bliss, 1978). The differences between the ranges shown for the two methods is shown in Table 3. In this respect, the Butanol-HCl method had the higher ranges than the Vanillin methods; perhaps because of the detection limits and the specific calibration curves used. With both methods there was more variation within color classes than between classes.

Conclusions and Future work

In conclusion, the results indicated that much variability exists for this trait but that tannin concentration is related to seed color. Despite this trend there is more research to be done: 1) to evaluate a greater number of genotypes from the yellow, brown, red and pink seed classes; 2) determine if red and pink genotypes do indeed always have higher amounts of soluble condensed tannins and whether this is related to color intensity within the reds; 3) evaluate more black beans to see if they are uniformly lower in soluble condensed tannins; 4) compare red and black beans for their concentration of insoluble tannins; 5) study the yellow bean genotypes further for their amount of tannin to better understand if insoluble tannins accumulate in this seed class in greater amounts than in other seed classes; 6) evaluate the variability in cream beans for both soluble and insoluble tannin fractions; 7) determine the inheritance of total tannin content and its component fractions in seed classes we have not studied previously; and 8) determine the tannin subcomponents and flavonoid precursors involved in creating tannins as some have been

suggested to have a positive effect on health through anti-oxidant activity and it will be important to have a greater understanding of their biochemistry.

Table 1. Condensed tannin content in 38 bean accessions from CIAT core collection through Butanol-HCl analysis.

Genotype	Color*	Soluble	Insoluble	Total	Mean
G11229	White[1]	0.00	0.00	0.00	0.03
G18811	White	0.00	0.00	0.00	
G2883	White	0.00	0.00	0.00	
G17427	White	0.00	0.00	0.00	
G13092	White	0.00	0.00	0.00	
G13043	White	0.00	0.00	0.00	
G19326	White	0.00	0.00	0.00	
G22286	White	0.00	0.00	0.00	
G23804	White	0.00	0.00	0.00	
G16829	White	0.00	0.00	0.00	
G16794	White	0.00	0.00	0.00	
BRB197	White	0.00	0.00	0.00	
CAB19	White	0.00	0.00	0.00	
Kaboon	White	0.00	0.00	0.00	
G18372	White	0.00	0.52	0.52	
G11708	White/Purple[1/7]	0.00	0.40	0.40	6.37
G23073	White/Brown[1/4]	0.15	1.42	1.56	
G12061	White/Cream[1/2]	4.25	2.08	6.33	
G21055	White/Black[1/8]	6.26	1.71	7.97	
G7437	White/Red[1/6]	7.57	2.12	9.69	
G7150	White/Red	9.96	2.28	12.24	
G12610	Cream[2]	5.66	2.63	8.29	14.56
G1844	Cream	8.10	2.92	11.01	
G21725	Cream	12.71	4.15	16.86	
G11419	Cream	15.29	2.61	17.91	
G734	Cream	15.41	3.29	18.70	
G5034	Cream/Yellow[2/3]	11.39	3.21	14.60	14.64
G19022	Cream/Black[2/8]	10.50	2.93	13.43	
G2774	Cream/Brown[2/4]	13.69	2.20	15.90	
G13220	Yellow[3]	11.91	4.33	16.24	16.24
G3971	Brown[4]	15.44	3.51	18.95	18.95
G16267	Pink[5]	14.90	2.23	17.13	17.23
G15137	Red[6]	14.01	1.92	15.93	17.28
G11350	Red	16.83	1.80	18.63	
G23063	Purple[7]	11.67	2.22	13.90	13.37
G1678	Purple Mottled	10.15	2.69	12.84	
G5706	Black[8]	11.22	2.97	14.18	12.38
G3096	Black Mottled	8.55	2.03	10.58	

***CIAT Color Code**

Correlation Soluble and Insoluble Tannin Content= 0.86

Table 2. Least significant difference (LSD) Comparisons of a) soluble and b) insoluble tannin content in seed coats of 22 common bean genotypes with different seed colors.

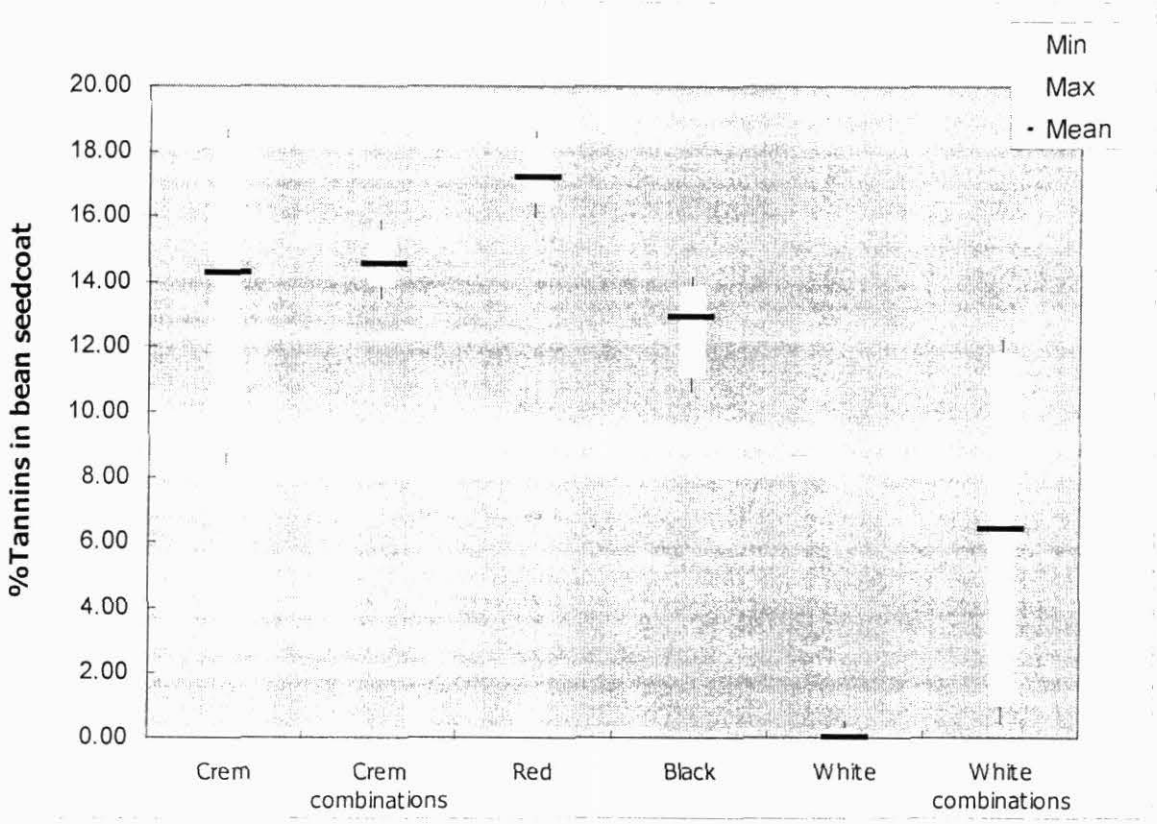
Seed color	Genotype	Mean	Homogeneus group
cream/purple	G21242	22.825	A
cream/white	G21078	17.275	B
red	G11350	16.825	B
cream/brown	G4825	15.55	C
brown	G3971	15.44	C
cream	G734	15.403	C
cream	G11419	15.293	C
pink	G16267	14.897	C
red	G15137	14.007	D
cream-ray	G2774	13.694	D
cream	G21725	12.715	E
yellow	G13220	11.913	EF
purple	G23063	11.675	FG
cream-yellow	G5034	11.385	FGH
black	G5706	11.215	FGH
brown	G14519	10.88	GHI
cream-black	G19022	10.505	HI
purple mottled	G1678	10.15	I
black mottled	G3096	8.545	J
cream	G1844	8.0933	J
cream	G12610	5.6567	K

Seed color	Genotype	Mean	Homogeneus group
yellow	G13220	4.3252	A
cream/purple	G21242	4.1517	AB
cream	G21725	3.9466	AB
cream/brown	G4825	3.7867	B
cream	G734	3.2948	C
cream-yellow	G5034	3.2084	C
black	G5706	2.9651	CD
cream-black	G19022	2.9295	CDE
cream	G1844	2.9151	CDE
brown	G3971	2.7457	DEF
purple mottled	G1678	2.6872	DEF
cream	G12610	2.6309	DEF
cream	G11419	2.6147	DEFG
cream/white	G21078	2.5435	EFGH
brown	G14519	2.3308	FGHI
pink	G16267	2.2286	GHIJ
purple	G23063	2.221	GHIJ
cream-ray	G2774	2.2037	HIJ
black mottled	G3096	2.0271	IJK
red	G15137	1.9208	JK
red	G11350	1.8013	K

Table 3. Comparison of tannin concentration based on vanillin and butanol assay results for 20 genotypes from five commercial color classes (cream, yellow, pink, red and black) studied at Cornell and CIAT, respectively; along with 18 white or white mottled genotypes analyzed for comparison to these color beans studied at CIAT only.

Color	Vanillin assay g tannin/g seed coat		Butanol assay g tannin/g seed coat	
	Mean	Range	Mean	Range
Crem	0.116	0.036-0.168	0.143	0.083-0.187
Yellow	0.116	0.060-0.184		
Pink	0.12	0.072-0.164		
Red	0.128	0.048-0.196	0.172	0.159-0.186
Black	0.124	0.048-0.196	0.129	0.105-0.141
White			0	0-0.005
White comb.			0.064	0.004-0.122

Figure 1. Comparison of average and range of tannin concentrations among 38 genotypes from six commercial seed classes of beans (cream, cream mottled, red, black, white and white mottled).



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1.1.16 Phytate analysis in parents of five common bean mapping populations.

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Introduction

Phytic acid and derivatives, jointly known as phytates, are important nutritional components of seeds both as nutrients and anti-nutrients. Their anti-nutritional qualities are due to their strong interaction with essential minerals such as iron with which they form insoluble complexes which cannot be taken up during digestion, thus removing micronutrients from the diet. Phytates however have been suggested to have a role in human health and are also essential for plant nutrition, since they are the main storage form of phosphorus (P) in seeds and are used for growth during seedling development. Phytates can reach concentrations of 2% of total seed weight but vary greatly between genotypes, growing seasons and species and therefore sensitive quantification techniques are needed to determine the amount of phytates present in seeds. With this in mind, we have been developing a technique to evaluate the concentration of phytates in common bean seeds with the aim of conducting QTL analysis for the traits with five mapping populations at CIAT. This section outlines the results with parental genotypes.

Materials and Methods

The parental genotypes evaluated included: G2333 and G19839 grown under medium and high P conditions; DOR364 grown in low and high P conditions and G21242 and G21078 grown under high P conditions. Seeds of each genotype were prepared by lyophilizing and grinding in a Retsch mill as described previously for mineral analysis. A total of 0.1 g of ground seed powder was mixed with 3 ml of 0.5M HCl and mixed mechanically for 2 hours. This sample was then diluted with 15 ml of distilled water and centrifuged at 25000 rpm for 2 hours. Supernatants from the samples were then loaded onto SAX solid phase columns. Phytates were eluted from the columns with 3 ml of 2M HCl and then diluted with 18 ml of distilled water. Following extraction, 3 ml of the diluted phytates were mixed with 1 ml of Wade reagent (0.030% $FeCl_3$ in 0.3% sulfosalicylic acid) and analyzed on a Shimadzu UV-1601 spectrophotometer with absorbance at 500 nm. Previously, a spectrum of absorbances in the range of UV-visible light were evaluated using the Wade reagent at the given concentration. A standard curve was set up using known concentrations of phytic acid ranging from 5 to 100 µg/ml. When mixed with Wade reagent, these phytic acid dilutions caused a reduction in absorbance resulting in a negative slope for the plot of absorbance versus concentration in the calibration curve. Once established, this relationship was used to estimate phytates in the seed samples described above.

Results and Discussion

A phytate quantification method was tested in the first part of the study and we found that maximum absorbance for the Wade reagent was at 500 nm, that the calibration curve showed linearity in the range of phytate concentrations used and that quantification of small amounts of

phytates was possible using the dilution factors tested (Figure 1). In the second part of the study where we analyzed the bean samples, phytate concentration estimated had a low error (below 1%) for replicate determinations. Phytate concentrations were expressed both as net content and percentage of the seed (Table 1). The genotypes with the highest phytate content were the P inefficient genotypes DOR364 (average 0.499% seed phytate), G21242 (0.567%) and G21078 (0.439%), while the lowest phytate content were the P efficient genotypes G2333 (average 0.295%) and G19839 (average 0.235%). This has been observed previously in studies of low P tolerance where under low P conditions efficient genotypes produce a larger amount of grain for a given amount of soil P. In this study the same genotypic differentiation was observed under both medium, high and even low soil P levels, especially for the genotype DOR364, although interestingly G19839 had higher phytate content in the medium P than in high P unlike G2333 that had higher phytate content in high P compared to the medium P treatment. Given this instability and the similarity between these two parents the G2333 x G19839 population will be de-emphasized for phytate analysis. Similarly, the contrast between the high and low iron genotypes, G21242 and G21078, was low and may make it difficult to use the G21242 x G21078 population for phytate analysis. It remains to be seen if G19833, another P efficient genotype which is crossed with DOR364 in the DOR364 x G19833 population presents low phytate levels as might be expected. If this is so then this population would be very useful for the analysis of phytate levels.

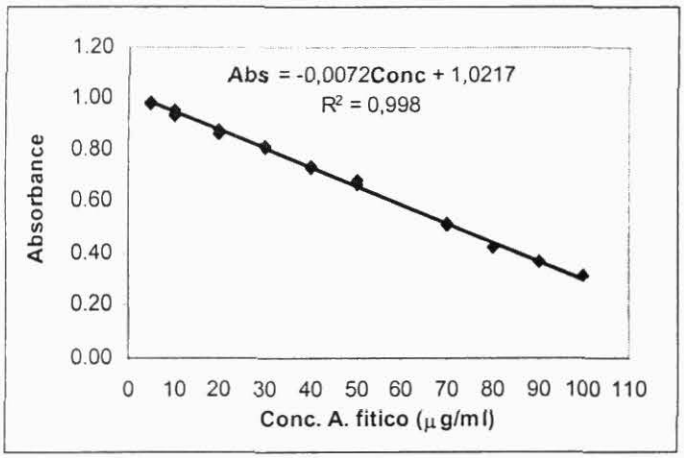
Conclusions and future work

There is still a need to optimize the extraction process and analyze further samples of the parental genotypes, especially G19833. Future work will concentrate on making the technique reliable and rapid so that the full recombinant inbred line populations can be analyzed especially for the DOR364 x G19833 population and perhaps for the G2333 x G19839 population grown under different P levels. After establishing the Wade reagent technique we also plan to analyze the specific phytate species and quantify them with HPLC analysis.

Table 1: Average phytate content in common bean parental genotypes evaluated in the study.

Genotype	Soil phosphorus level	Phytate content (µg)	% seed phytate
G19839	high	209,125	0,210
G19839	medium	257,042	0,260
G2333	high	358,000	0,360
G2333	medium	233,500	0,230
G21242	high	567,026	0,567
G21078	high	439,269	0,439
DOR 364	high	539,240	0,539
DOR 364	high	451,324	0,451
DOR 364	low	507,609	0,508

Figure 1. Calibration curve for phytic acid versus absorbance at 500nm using the Wade reagent test and 95% confidence interval.



1.1.17 Simple sequence marker (SSR) evaluation of global germplasm resources in cassava

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Funding: Generation Challenge Program (GCP)

Important Outputs

Genotyping of 3000 cassava accesions from 3 germplam banks (CIAT, IITA, EMBRAPA) using 36 SSR markers

Structural characterization of diversity in global cassava genetic resources using SSR markers

Introduction

One of the main goals of sub-programme 1 of the GCP is to define the genetic structure of germplasm collections as a first step to looking for new genes and alleles that contribute to solving the challenges of modern agriculture. Eleven first tier crops were initially selected to characterize structural and functional diversity as the entry point of the SP1 activities. Cassava is one of the 11 mandate crops and it was decided, at a meeting to select plant material and marker systems for selected crops held at the Plant and Animal (PAG) genome 2004, that 3000 accessions should be characterized using 36 markers. Accessions would be 1500 accessions from CIAT's world germplasm collection, 1000 accessions from Africa (IITA) and 500 accessions

from EMBRAPA. Responsibilities for molecular characterization were divided as follows: CIAT and EMBRAPA will characterize all 3000 accessions with 22 SSR markers, IITA will do same with14 SSR markers. Data analysis was based on SSR marker data from 36 loci and it includes assessment of genetic structure using principal coordinate analysis (PCoA) and multidimensional scaling (MDS) based on individuals, cluster analysis based on country samples, and an estimation of genetic diversity and allelic richness.

Methodology

Assembling Cassava germplasm

Plant material for the analysis of genetic diversity in global germplasm resources of cassava is subset of 3000 accessions from over 10.000 varieties held in the three collections at CIAT, IITA and EMBRAPA (Fig 1). The selection of the subset was based on criteria that emphasize location, to capture the broadest 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 was described earlier (CIAT, 2004).

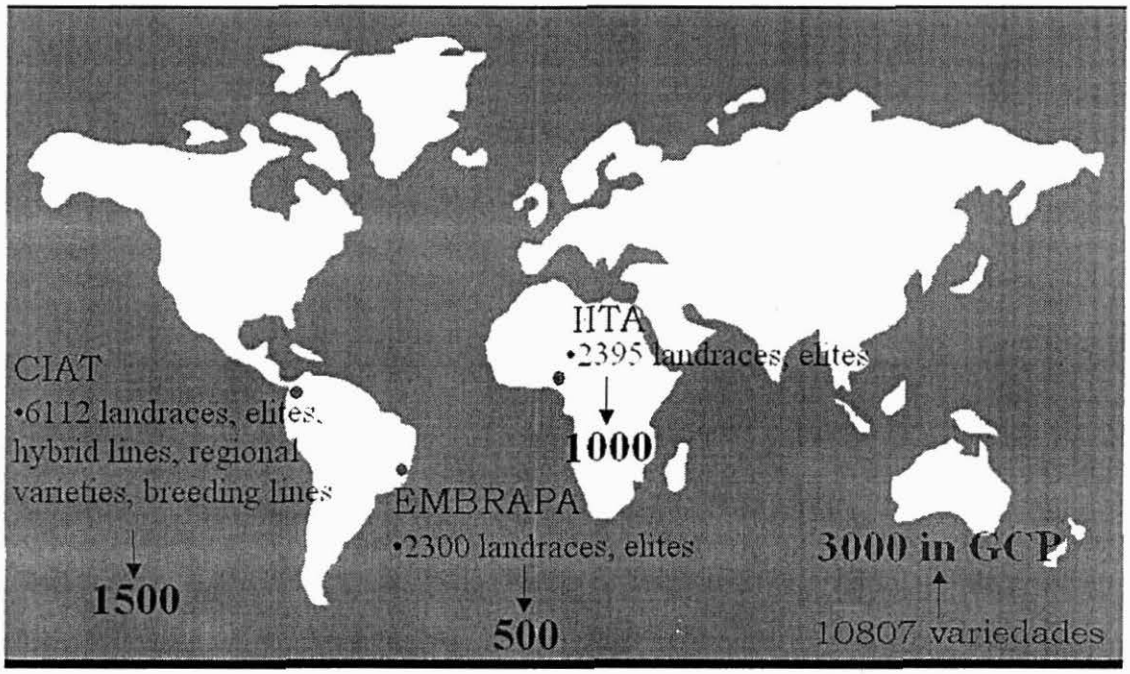

Figure 1. Source of the "composite set" of 3000 accessions for assessment of genetic diversity of global cassava germplasm resources

The subset of accessions include 1000 accessions from the International Institute of Tropical Agriculture (IITA) in Nigeria, principally local varieties from West and East Africa and elite IITA varieties; 1500 from CIAT germplasm collection, mainly landraces from the Neotropics, elite and breeding lines; 500 CNPMF Brasilian accessions held at CIAT world cassava collection. The subset represents cassava germplasm from 50 countries from all over the world, 18 countries in America, 23 in Africa and 6 in Asia.

DNA from each accession was isolated at the respective institution, with the exception of accessions from EMBRAPA that were extracted at CIAT from duplicate copies in the CIAT

germplasm bank and shipped to CIAT (lead institution) for re-distribution to the participating centers in order to do the genotyping work at IITA (Nairobi) and CIAT.

Selection of SSR markers and genotyping

Over 850 SSR markers exist for cassava and 67 were used to assess diversity in previous studies (Fregene et al, 2003). 36 SSR markers of the 67 were selected to assess the diversity of global cassava genetic resources. The selected markers have clear and reproducible allele patterns as well as high PIC and are spread over the 18 linkage groups of cassava. SSR marker analysis has been carried out using the set of 36 markers. CIAT genotyped the 3000 accessions with 22 SSR markers, corresponding to 16 previously assigned to it and 6 markers allocated to EMBRAPA-CNPMF. Due to difficulties in securing the release of DNA from selected accessions from EMBRAPA-CNPMF, an agreement was reached with CNPMF to do the analysis of the Brazilian selection at CIAT with the participation of a CNPMF scientist (Ms Claudia Fortes Ferrerira) who visited CIAT for two months (October- December, 2004) and participated in the analysis of the Brazilian accessions held in CIAT's cassava world collection. SSR marker analysis at CIAT was done by silver stained polyacrylamide gel electrophoresis. IITA analyzed 3000 accessions using 14 SSR markers by automated fluorescent capillary electrophoresis (ABI sequencer).

Data analysis

CIAT as lead institute collated and analyzed the molecular data generated from 30 markers, 22 analyzed at CIAT and 8 analyzed at IITA. Three different molecular marker data files were generated for statistical analysis: allele molecular weight information by locus and genotype, allele number by locus and genotype, binary data by genotype. The appropriate files were then analyzed using the relevant software (NTSYS, SAS and Popgene) to assess genetic relatedness of all individuals using principal coordinate analysis (PCoA) and multidimensional scaling (MDS), based Jaccard's similarity matrix, and relatedness of country sample based on Nei's (1972) genetic identity and genetic distance. Data analysis was done with genotypes having more than 80% of complete data per SSR locus.

Passport data and phenotypic information

CIAT as lead institute has compiled passport data, including the local names, source (Country/State/Province/Region/Village), geographical position (Longitude, Latitude, Altitude) and the main agronomic traits, of the 3000 accessions into a data base. A preliminary passport data template for IITA, EMBRAPA and CIAT cassava accessions was sent to Guy Davenport (Bioinformatics SP5) for processing and storage, as well as to make it accessible to the entire cassava research community through the GCPweb site.

Results

Preliminary results and statistical analysis were conducted on a first data set that corresponds to 2494 genotypes evaluated at CIAT using 22 SSR markers (genotypes with more than 80% of complete data per set of SSR markers). A second data set consisting of 2575 genotypes evaluated at IITA using 8 SSR markers (it has between 50-100% of missing data per genotype) was also analyzed. The remaining data from 6 SSR markers is still being processed at IITA, so the complete analysis of all 36 SSR markers will be carried out by the end of the year. A cluster analysis based on country of origin was done using each data set to establish if the data sets could

be combined (22 SSR analyzed at CIAT and 8 analyzed at IITA). Independent analysis of the 2 data sets showed similar results (Figure 2) indicating that all alleles could be put together in one matrix to increase the resolution of genetic diversity structure.

These preliminary results from cluster analysis (Fig3 , Fig 4, and Table1), as well as, a multi-dimensional scaling based on individuals from data set 1 reveal a clear separation between accessions from Africa and the rest of the world confirming findings from previous studies that shows that global cassava germplasm diversity is structured by region. Sources of this genetic differentiation could be selection for adaptation to agro-ecologies, particularly diseases, found in Africa. Accessions from Asian countries also show high differentiation that could be explained by the new introduction of the crop to this continent as well as the smaller sample sizes.

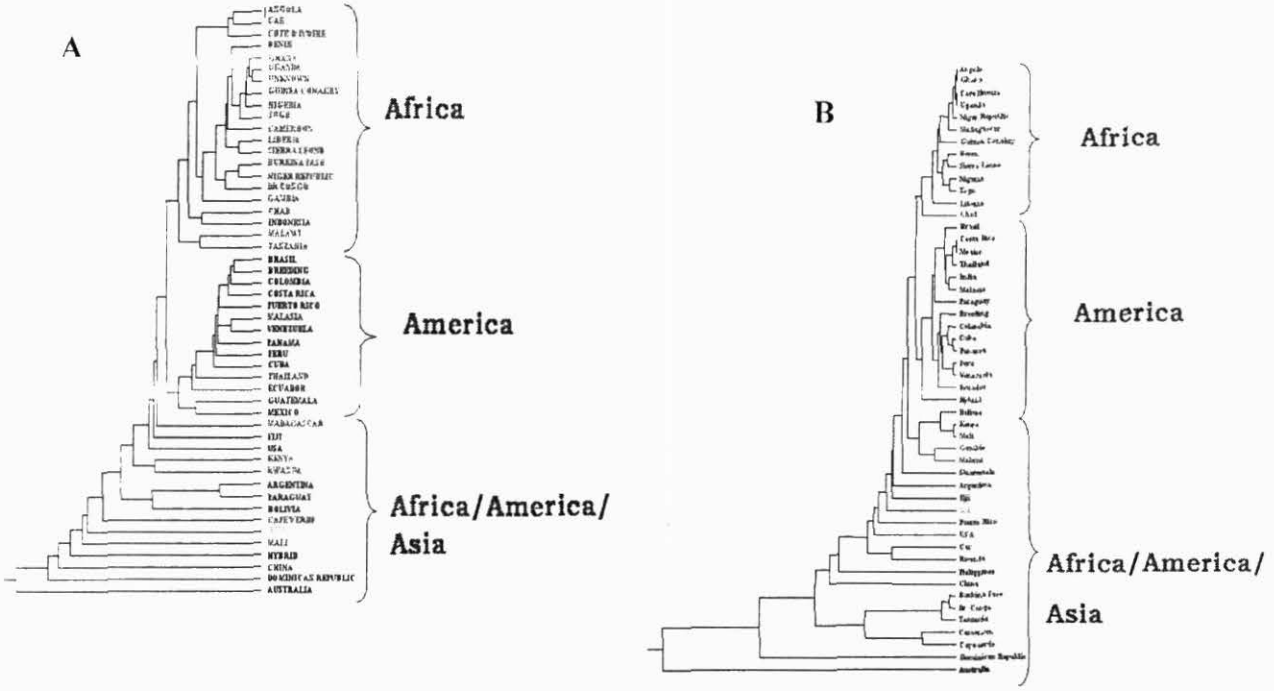

Fig. 2 A based on 50 countries using Nei's (1972) genetic distance. A. data set 1 (2494 genotypes evaluated at CIAT using 22 SSR markers). B. data eet 2 (2575 genotypes evaluated at IITA using 8 SSR markers).

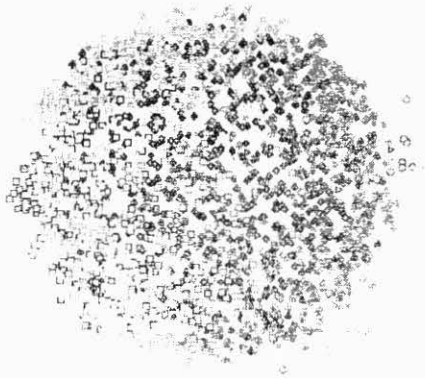

Figure 3. Multidimensional scaling plot from 2494 genotypes analyzed with 22 SSR markers (set 1). Diversity structure explaining 70% of the genetic variance. Additional MDS analysis shows how the data set 1 is divided in 8 groups explaining 70% of the genetic variance).

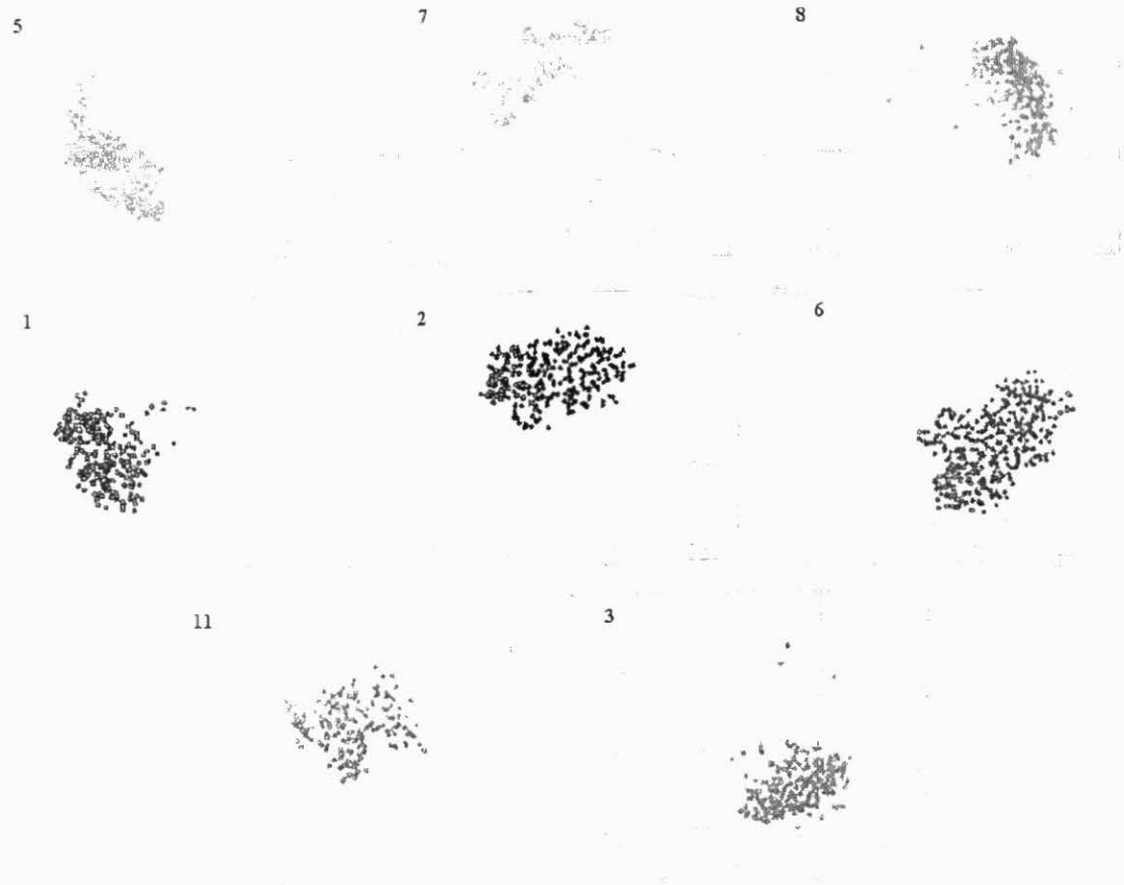

Figure 4. 8 groups generated by multidimensional scaling using data set 1. Each color represents a group and the different shapes correspond to the germplams collecion (CIAT, IITA, EMBRAPA represented by club, square and flag respectively) and the wild accessions (represented by circle).

Even with 40% missing data, African accessions are showing a clear structure by MDS analysis with a separation of some accessions from Ghana and Nigeria accessions from the rest of the continent as indicated in previous diversity studies. Cassava was only recently introduced to Africa in the 1600s, it is possible that the separation between African accessions could be explained by selection intensity for resistance to biotic and abiotic stresses. The American accessions showed a clustering between Brazilian and Central American genotypes. The source of the observed structure could be introgression from wild relatives, as well as, independent domestication events. Wild accessions are distributed in all the American groups, especially in group 3 and 8 where the separation is clear between South and Central American accessions that could be represent introgression of some wild genes.

Table 1. Most frequent country per group defined by MDS

Group	Country	Frequency (%)
1	Nigeria/Ghana	20/23
2	Brasil	54
3	Wild/Mexico/Gutemala	2/4/6
5	Nigeria/Ghana	31/27
6	Brasil	63
7	Ghana/Brasil	21/23
8	Wild/Peru/Brasil	4/7/22
11	Colombia/Ghana	15/20

Conclusion and perspectives

Preliminary results reveal global cassava germplasm diversity is structured by origin of accessions with those from Africa showing the highest differentiation from those from the Neotropics. Sources of the observed genetic differentiation could be selection for adaptation to agroecologies, particularly diseases, found in Africa, as well as domestication events and introgression from wild relatives in accessions from Guatemala and others from Central America. Other principal findings include

The results agree with previous diversity studies in Cassava where the structure is along the lines of origin of accessions.
The broad diversity in Nigerian accessions could be explained by migration and selection for tolerance to drought and diseases.
The cluster composed by African/Asian/ American accessions could be explained by small sample size or recent introduction.
The accessions must be analyzed using 36 SSR markers covering the genome to obtain a better structure of diversity.

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Landraces: Genetic Structure in a Predominatly Asexually Propagated Crop Theor Appl Genetics 107:1083-1093.

1.1.18 Phylogeographic study of the origin of Local Guatemalan cassava varieties

Cesar Azurdia, Luis Montes and Martin Fregene
CIAT

Important outputs

Collection of accessions of wild *Manihot* species in Guatemala to assess genetic introgression into Cassava from wild relatives in Central America.

Definition of the actual distribution of *Manihot* wild species in Guatemala

Introduction

Although several studies have demonstrated a likely South American origin for cassava, the diversity of cassava and its wild relatives in Meso-America is great enough to suggest a second center in Meso-America. Several studies have revealed unique allele in cassava landrace from Guatemala and the potential of Meso-American diversity in cassava improvement has not been properly assessed. Previous SSR characterization of genetic diversity of local variety from Guatemala revealed a high genetic differentiation of some Guatemalan accessions compared to those from other parts of the world (CIAT 2003).

The objective of this study was to assess the relatedness of these highly differentiated accessions with those of wild *Manihot* wild species from Guatemala, phylogeographic analysis will be conducted on the collections using a region of the single-copy nuclear gene glyceraldehydes 3-phosphate dehydrogenase (*G3pdh*) to determinate if there is a introgression of wild species into local cassava varieties from Guatemala.

Methodology

Plant collections

Information on wild *Manihot* species and their localization were collected from different herbariums and documents describing this genus. With this information we generated maps of the distribution of these species by using diverse software for example DIVA-GIS, ArcView and Aptitud (CATIE version 1). The maps were used to plan the collections by first of all interviewing persons from the localities where the maps showed high probability to finding these species and searching in non-disturbed areas.

Molecular analyses

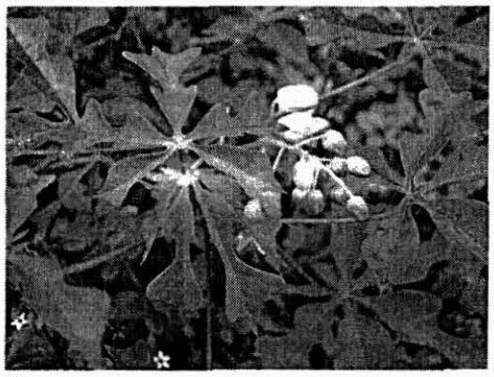
Dried leaf tissue collected from wild *Manihot* species was brought to CIAT for further analysis. DNA was extracted from dried leaves by using a modified Dellaporta (1983) protocol and representative samples from Africa and South America included for comparison. PCR amplification of the *G3pdh* region in the samples was using primers designed by Strand et al (1997). These primers were designed from conserved regions identified in published *G3pdh*

sequences of *Arabidopsis thaliana* and *Ranunculus acris* (Olsen K and Schaal 1,999). DNA sequencing will be done at the Iowa State University DNA sequencing facility and analysis carried out at CIAT.

Results

Plant Collections

There are two *Manihot* wild species in Guatemala *Manihot aesculifolia* and *Manihot rhomboide* (Fig 1). *M. rhomboide* shows two sub-species *microcarpa* and *romboidea*.

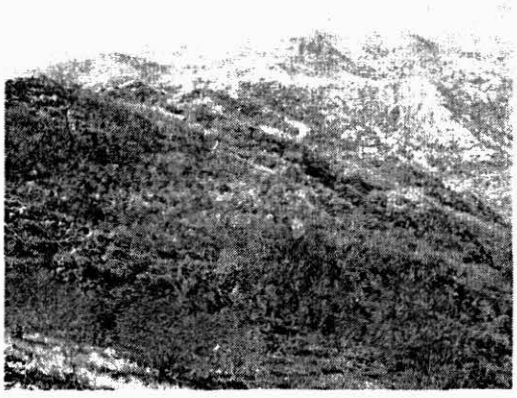


A

B

Figure 1. A) *Manihot aesculifoli.* B) *Manihot rhomboide*

These species are found in the arid regions of Guatemala (Fig. 2). *M. aesculifolia* usually grows along the foothills and lower slopes of the mountains between 300 and 900 m.



A

B

Figure 2. A) Arid region in dry season B) Arid region in rainy season

The location of *Manihot aesculifolia* accessions collected in Guatemala is shown in the Fig 3. This specie is widely distributed in the arid region in Guatemala.

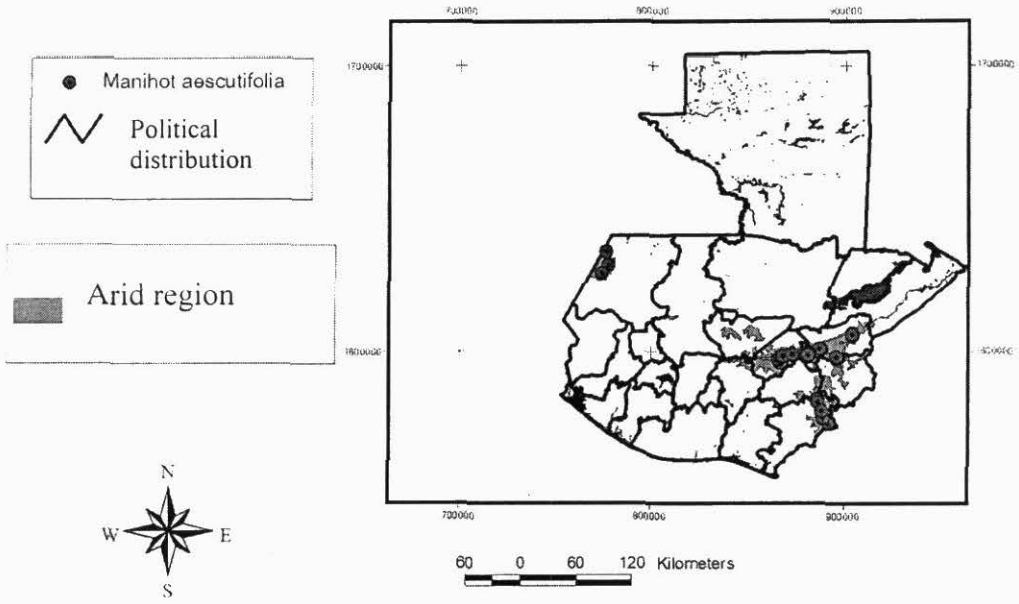


Figure 3. Distribution of *Manihot aesculifolia* collected in Guatemala.

Some populations of *M. aesculifolia*, were composed by vines of between 5-6 meters in length and 17 cm in diameter (Fig. 4). These populations were found in areas where Gila monster (*Heloderma suspectum)* has been protected in the departments of Zacapa and El Progreso (Fig 5)

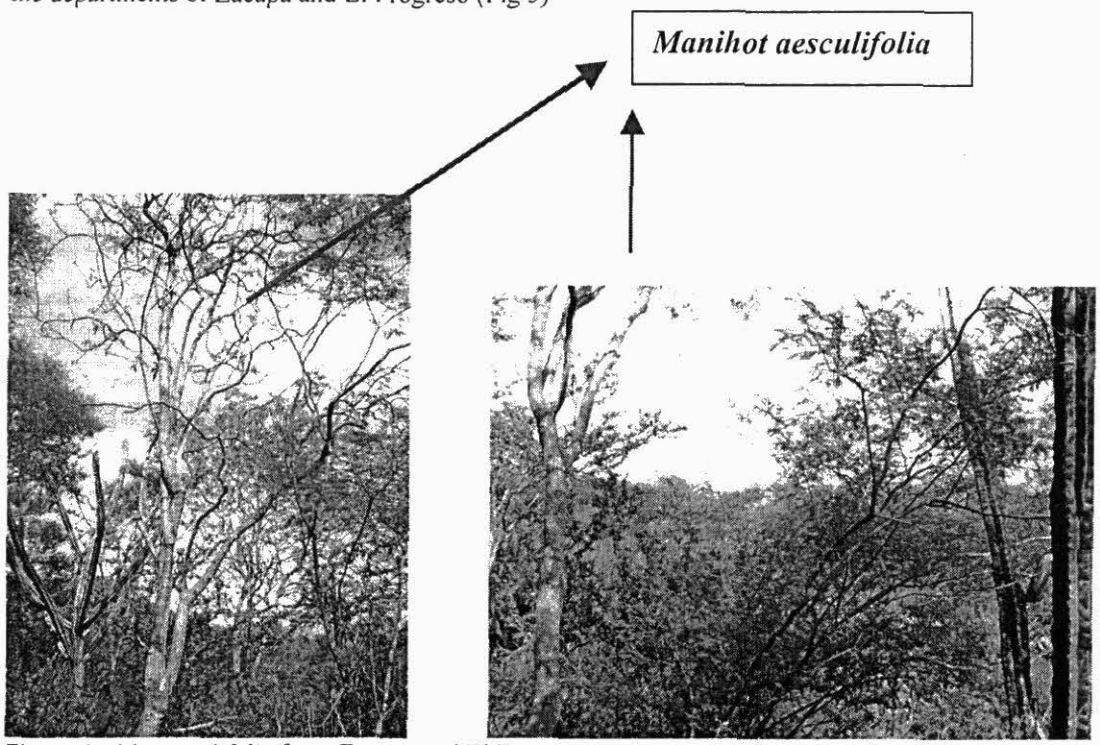


Figure 4. *M. aesculifolia* from Zacapa and El Progreso.

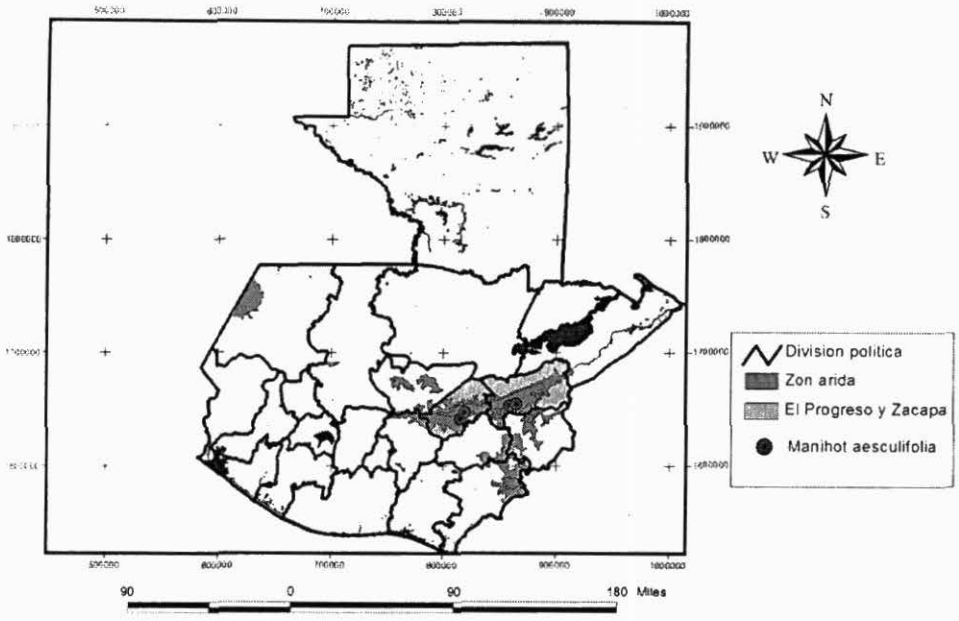


Figure 5. Localization of highly protected population of *M. aesculifolia* in the Zacapa and El Progreso

Other *M. aesculifolia* populations were found in small groups of plants, some times used as fences (Fig 6.)

Figure 6. *M. aesculifolia* used as fence

Manihot rhomboide grows in rocky arid region like *M. aesculifolia*. This specie has been reported as solitary plant in the collection of Steyermark 50614, 50866 and Pittier 132 (C. Azurdia personal communication) and it is not as frequent as *M. aesculifolia*. One problem with finding this species is that almost every place where *M. rhomboidea* was reported between 1904 and 1942, has been changed from forest to agricultural land
(Fig. 7).

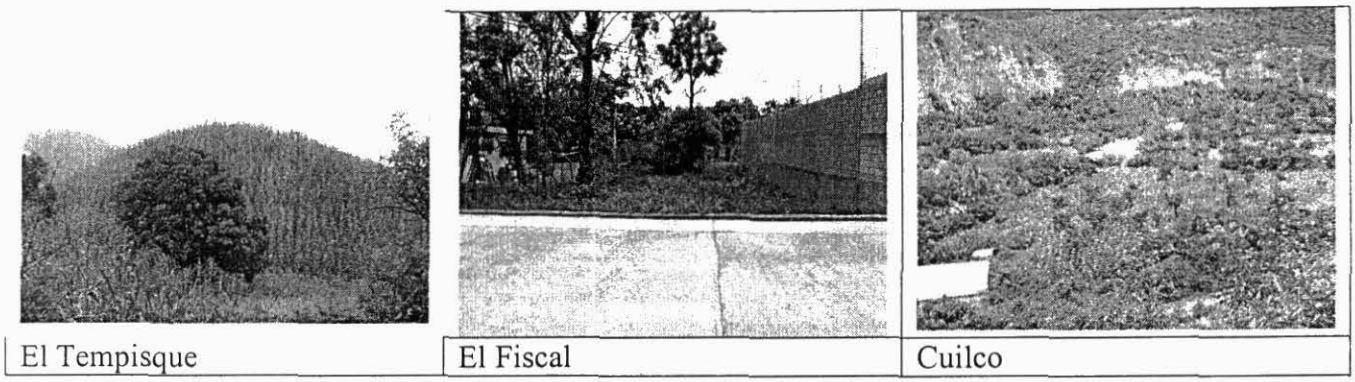


Figure 7. Current status of areas where *M. rhomboidea* was reported in 1942.

Only in one area was is possible to find *M. rhomboidea*, this place is in the Cerro Cachil in the department of Baja Verapaz and the plant was reported in 1905 by Pittier 132 (NY, US) (Fig. 8).

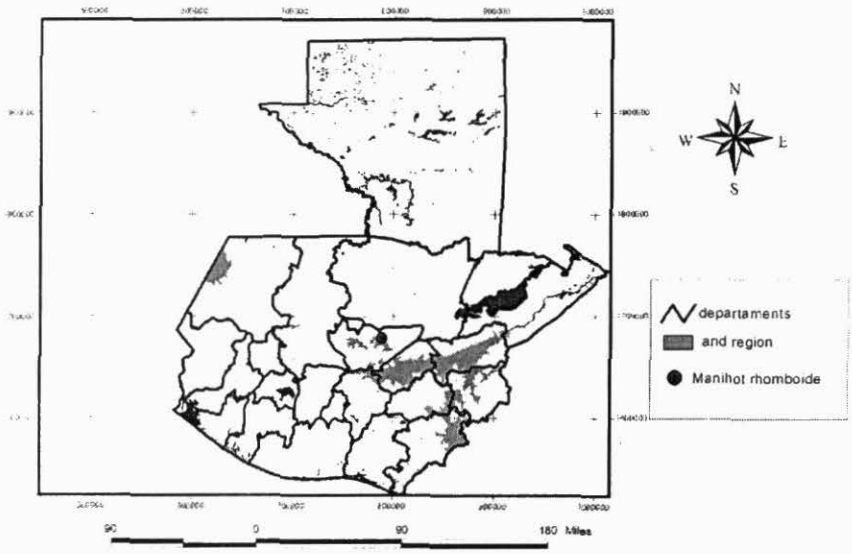


Figure 8. Localization of *M. rhomboidea* in Guatemala.

Some cassava plants (*M. esculenta*) were found with leaf shape like *M. aesculifolia* cultivated by farmers (Fig 9). These plants were also collected for this study.

Figure 9. Cassava (*M. esculenta*) with leaf shape like *M. aesculifolia*

Molecular analysis

DNA has already been extracted from the dry leaf tissues of all collected samples and controls. PCR amplification of the samples using the *G3pdh* gene, sequencing, and analyses has already been initiated.

Conclusion and perspectives

Two *Manihot* wild species were found in arid regions of Guatemala, *M. aesculifolia* and *M. rhomboidea* sub *microcarpa*. *M. aesculifolia* has a wide range of distribution in Guatemala, there are many populations, some of them highly conserved by the protection of another species, Gila monster (*Heloderma suspectum)*. On the other hand *M. rhomboidea* is not as frequent as *M. aesculifolia* and it has been losing its habitat because of agricultural activity. The plants collected were brought to CIAT for the molecular analyses. These analyses will be done by using a region of single-copy nuclear gene (*G3pdh*), to determinate the introgression of wild species in cassava race in Guatemala.

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1.1.19 Measuring impact of improved CIAT cassava varieties in Colombia by simple sequence repeat (SSR) marker characterization of genetic diversity of cassava of small holder in the Atlantic coast of Colombia

Adriana Alzate, Juan Carlos Perez, Hernan Ceballos, Martin Fregene
Funding: CBN (Gines Mera Fellowship)

Important Outputs

Collection of cassava varieties in 39 towns (35%) in 4 departments of the Colombian North coast and establishment at CIAT

DNA isolation and SSR characterization of the collections to determine the extent of adoption of CIAT improved varieties

Introduction

One of the challenges of breeding for small holders is combining adaptation to the diverse agro-ecologies and culinary preferences in a few improved genotypes. To increase adoption of CIAT varieties in the North Coast of Colombia, a farmer participatory research program was initiated that led to the adoption of several improved varieties (Hershey 2004). However, no quantitative estimate of what proportion of cassava acreage in the Colombian North Coast is grown to improved CIAT varieties. With funding from the Gine-Mera fellowship, a study was initiated to assess how frequent are CIAT improved varieties in the 4 departments of the North coast of Colombia.

Methodology

Collection of cassava varieties

Collection of the cassava varieties grown by small holders in the Colombian Atlantic Coast was carried out in the departments of Córdoba, Sucre, Atlantic and Magdalena. The department of

Bolivar was not visited due to problems of insecurity during the time of collection. A stratified sampling method, where each department was the stratum, was used. A statistically representative size of sample of the number of towns and farms to be visit in each department was calculated. Once the number of farms per town per department was decided upon, farms were visited and one or two woody stems of all varieties grown by farmers were collected and planted the in screenhouse at CIAT. Young leaves were harvested from the genotypes and subsequently dried in a oven to 40 °C for three days. For SSR characterization, DNA was extracted using a modified Dellaporta (1983) method. Nine SSR markers with high polymorphism information content (PIC), was selected from subset of 36 markers used routinely for genetic diversity assessment based upon the analysis of 30 accessions of the 1048 samples collected in the North coast. SSR analysis was as described by Fregene et al. (2003).

Results

A statistically representative sample, with a confidence level of 90%, for farms in the four departments under study, is a minimum 392 farms. Ten farms were therefore visited in each of 39 towns distributed all over the 4 departments (Table 1 and 2).

Table 1. Number of towns visited in each of the 4 department of Northern Colombia sampled

DEPARTMENT	TOTAL MUNICIPIOS BY DEPARTMENT	No. OF VISITED MUNICIPIOS	No. OF SURVEYS Y/OR VISITED FARMS
ATLANTIC	23	10	100
MAGDALENA	21	9	90
CÓRDOBA	26	11	110
SUCRE	24	10	100
TOTAL	94	39	400

P: 0.5; Q: 0.5; d: 0.06; Effect of design: 2

Table 2. Names of the towns visited in each department

ATLANTIC	MAGDALENA	CÓRDOBA	SUCRE
1. Repelón	1. Marta Santa	1. Buenavista	1. Toluviejo
2. Malambo	2. New Site	2. New People	2. San Antonio de Palmitos
3. Baranoa	3. Marsh	3. San Pelayo	3. San Pedro
4. Sabanagrande	4. Whirlwind	4. Planet Rich	4. The Hearts of Palm
5. Polonuevo	5. Aracataca	5. Cerete	5. Sincelejo
6. Santo Takings	6. Salamina	6. San Carlos	6. Betulia
7. Palmar of Varela	7. Pivijay	7. Gold Marsh	7. Corozal
8. Luruaco	8. Foundation	8. San Andres	8. Sincelejo
9. Sabanalarga		9. Momil	9. The Union
10. Ponedera		10. Sagun	10. Sampues
		11. Chinu	

Collection in the department of Magdalena, was carried out in rainy season and it was impossible to visit some towns due to the poor condition of the roads, therefore only 8 towns were visited in that department and 11 farms were visited in each town.
In each one of the farms surveyed questions were put to the farmer on the varieties planted and a stem of each variety considered different by the farmer was requested. The number of varieties ranged from 1 to 9 varieties per farm.

A total of 1048 accessions were collected as follows:
Córdoba 243 accessions
Sucre 192 accessions
Atlantic 330 accessions
Magdalena 283 Accessions
The stakes from all accessions were transferred to CIAT and planted in the greenhouse.

Molecular analysis

For the preliminary SSR marker analysis. DNA was extracted from 30 randmly selected accessions and quantified by a fluorometer and the quality determined by electrophoresis in a 1% agar gel. Dilutions were made to a final concentration of 10 ng/µl of DNA. PCR Amplifications PCR was carried out using 36 SSR markers and the program: 30 cycles of: 94 °C 30, 55 °C 30, 72 °C 1 min, and a final extension of 5 min to 72 °C; in a PTC-100 thermocycler. The product of the amplification was run on denatured polyacrylamide gels (6% acrylamide) and was stained with silver (Fig 1). The gels were scored for allele sizes and polymorphism information content calculated for each SSR marker using the computer software CERVUS. Based upon the results the following markers were selected: SSRY12, SSRY51, SSRY82, SSRY100, SSRY151, SSRY155, SSRY69, SSRY63, SSRY179.

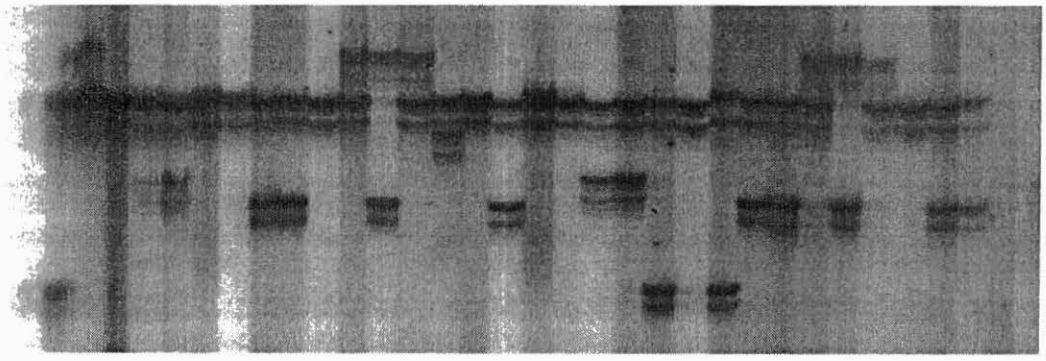

Figure 1. Silver stained PAGE of PCR amplification of 30 randomly selected samples using the SSR marker SSRY69

Conclusion and Perspectives

A total of 1048 cassava accessions were collected from 39 municipalities in 4 departments of the Colombian North Coast. Preliminary SSR characterization of 30 samples using 36 SSR markers led to the selection of 9 SSR markers for evaluation of the entire set of collections. DNA extractions, dilutions and the PCR amplifications of the total collected samples have been initiated. Data analyses include Principal Coordinates Analysis and Cluster analysis using Nei's

genetic distances, as well as genotype ID analysis, using a dissimilarity matrix, to estimate the level of adoption in the North Coast of Colombia of varieties released by CIAT.

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1.1.20 Assessment of Genetic Diversity of Local Cassava Varieties in the Cauca Department of Colombia based on Simple Sequence Repeat (SSR) Markers

Escobar R., P.Hurtado, Ch. Buitrago, J.Marin, C. Ospina, M. Fregene CIAT

Funding: CIAT

Important Outputs

Genotyping and analysis of 94 cassava accessions from Cauca using 36 SSR markers
Identification of duplicates in collections of local cassava varieties from Cauca cultivated cassava

Introduction

Genetic diversity of a collection of cassava accessions from the Cauca department of Colombia held in the CIAT germplsm bank was assessed using 36 SSR markers as a first step to identifying duplicates in the collection. Unique accessions of the collection will be employed in a tissue-culture multiplication scheme to replace diseased and pest infested germplasm being grown by farmers in the Cauca region.

Methodology

Plant material
In-vitro plants from 57 Colombian varieties and leaf tissue from 37 tissue culture plantlets were provided to Cassava Genetics program by Genetic Resources and Biotechnology Unit respectively. DNA from leaves tissue was extracted from the 94 accessions following a modified Dellaporta protocol (Dellaporta, 1983) and the quality was checked on a 0.8% agarose gel.

Genotyping using SSR markers

To assess genetic diversity, the 94 accessions were genotyped using 36 SSR markers that are routinely used for characterization of genetic diversity of cassava. DNA template for PCR amplification was 30 ng of each sample in a 15ul reaction mix containing 0.1 mM dNTP, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 uM of each primer and 1 U *Taq* DNA polymerase. PCR amplification was performed following a thermal profile: 2min. at 95°C, 30 cycles of 30s at 94°C, 55/45°C at 1

min. and 60 s at 72°C and a final step of 5 min. at 72°C. The PCR product was denaturated and electrophoresed on 4% polyacrilamide gels and visualized by silver staining. The raw SSR data were scored as allele sizes in a Microsoft Excel file for statistical analysis.

Data analysis

Three different molecular marker data files were generated for statistical analysis namely: molecular weight by locus and genotype, allele number by locus and genotype, and binary data by genotype. Data analysis was based on genotypes with more than 80% of complete data per set of SSR markers. The analysis included assessment of genetic structure using principal coordinate analysis (PCoA) and cluster analysis based on euclidian distances between individuals using the computer software package NTSYS.

Results

Statistical analysis was based on data from 94 genotypes corresponding to 176 alleles at 36 SSR loci. Principal coordinate analysis (Fig 1) and cluster analysis (Fig 2) revealed several groups and the presence of some genetic duplicates at 0.01 genetic dissimilarity.

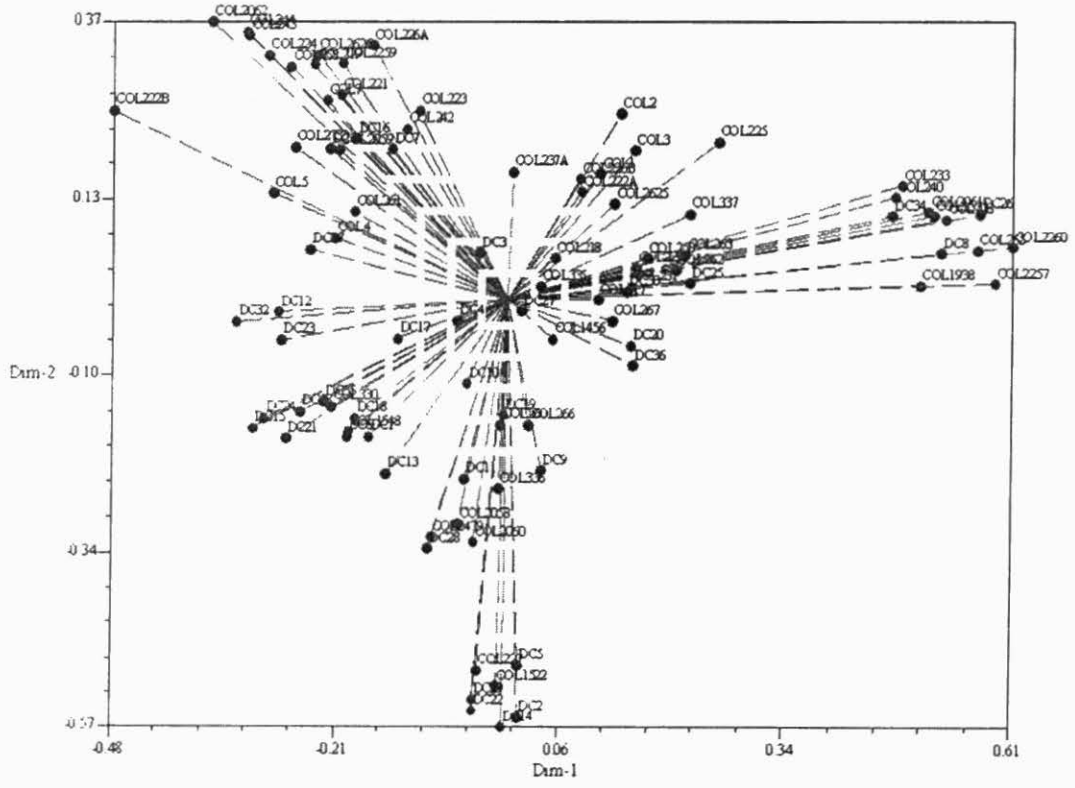

Figure 1. Principal Coordinate Analysis of 94 Colombian accessions evaluated with 36 SSR markers.

In figure 2 it is possible to distinguish 8 groups defined by cluster analysis with a threshold of 0.27 (dissimilarity coefficient). Five pairs of accessions were identified as genetic duplicates with a dissimilarity coefficient lower than 0.035.

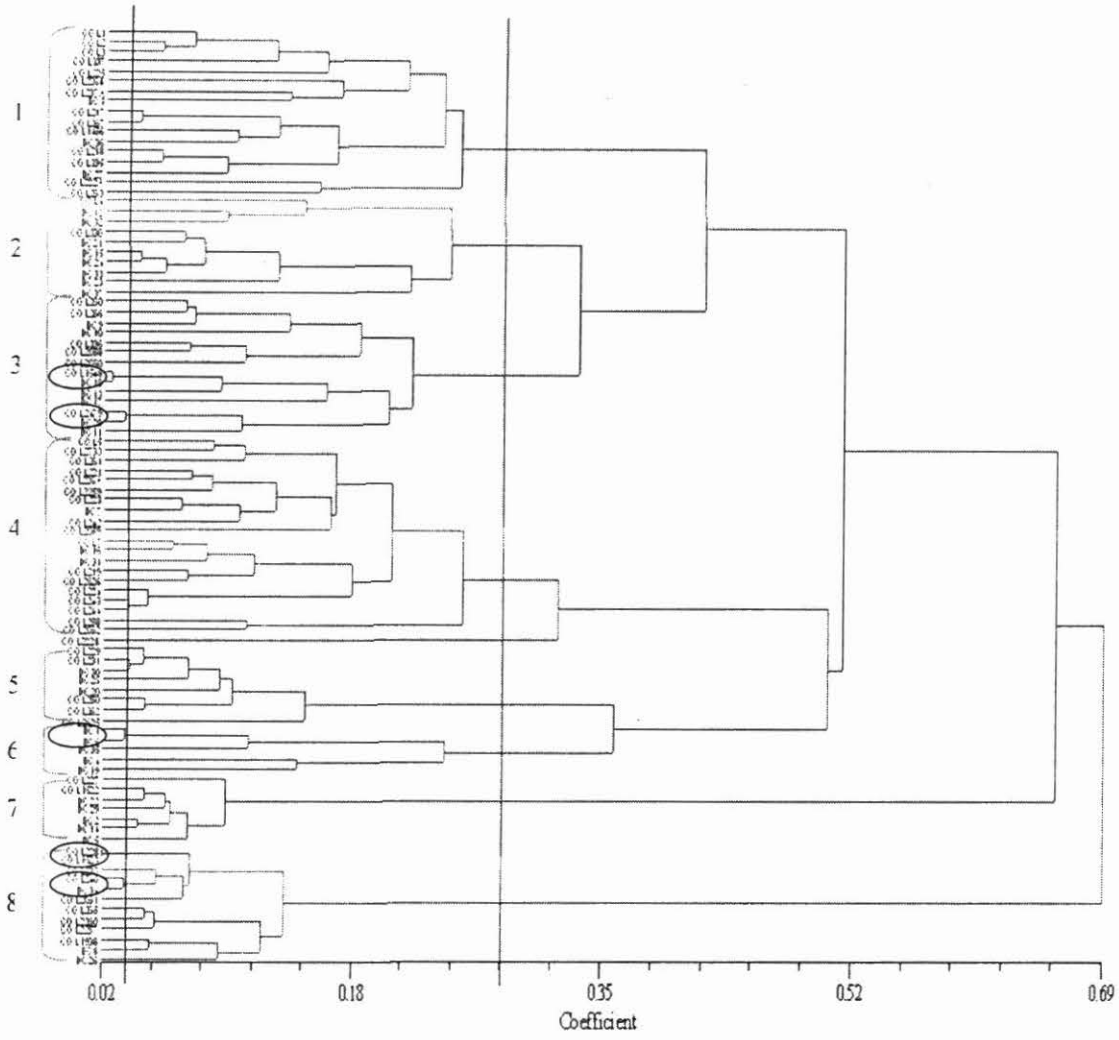

Figure 2. Cluster analysis showing 8 different groups generated by genotyping using SSR markers when the threshold is defined with a dissimilarity coefficient of 0.27 (red line). The blue line is defining the second threshold (dissimilarity coefficient 0.035) to identify genetic duplicates.

References

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1.1.21 Cassava Genetic Diversity in Tanzania based on Simple Sequence Repeat (SSR) Markers

[1]Esther Masumba, [2]Heneriko Kulembeka, Jaime Marin, Wilson Castelblanco, Charles Buitrago, Paula Hurtado, Isabel Moreno

1. SRI-Kibaha 2 ARI-Ukiriguru

Funding: The Rockefeller Foundation

Important Outputs

SSR genotyping, using 36 SSR markers, and analysis of a collection of Tanzanian local varieties from the 3 cassava producing regions and a collection of cassava brown streak disease (CBSD) resistant germplasm

Introduction

SSR characterization local varieties from the Eastern, Lake and Southern zones of Tanzania respectively was conducted to determine the structure of genetic diversity of local Tanzanian cassava varieties as well as select local varieties that maximize additive genetic variance for crosses to CIAT introductions. A second set of germplasm obtained from the East African Cassava Research Institute based at Amani, Tanzania that represent many decades of breeding for resistance to the cassava brown streak disease (CBSD) was also included. The cassava accessions were brought to CIAT by, Ms Esther Masumba and Mr Heneriko Kulembka, collaborators from Tanzania, and analyzed over 6 weeks.

Methodology

Plant material

Plant materials were 135 varieties of which 34, 39 and 22 were local varieties from eastern, lake and southern zones respectively and 35 improved varieties developed by plant breeders at the East African Cassava Research Institute based at Amani, Tanzania, they were brought to CIAT in April 2005. It also included 5 local varieties collected at Msabaha experimental station in Mombasa, Kenya). DNA from all the samples was precipitated with absolute Ethanol and Sodium Acetate 3M and the quality checked on 0.8% agarose gel.

Genotyping using SSR markers

To define assess genetic diversity, all samples were genotyped using 13 SSR markers (SSRY19, 34, 69, 82, 177, 12, 155, 103, 106, 151, 161, 179, 180) with high PIC value, clear and reproducible allele patterns located on different linkage groups of the Cassava genetic map. Thirty nanogram of DNA was used as template for SSR amplification in a 15ul reaction. PCR amplification was performed using the following thermal profile: 2min. at 95°C, 30 cycles of 30s at 94°C, 55/45°C at 1 min. and 60 s at 72°C and a final step of 5 min. at 72°C. The PCR product

was denaturated and electrophoresed on 4% polyacrilamide gels and visualized by silver staining. The raw SSR data were scored as allele sizes in a Microsoft Excel file for statistical analysis.

Data analysis

Three different molecular marker data files were generated for statistical analysis namely: molecular weight by locus and genotype, allele number by locus and genotype, and binary data by genotype. Data analysis was based on genotypes with more than 80% of complete data per set of SSR markers. The analysis included assessment of genetic structure using cluster analysis with the unweighted pair group mean (UPGMA) based upon Jaccard similarity and principal coordinate analysis (PCoA). Based upon clusters identified from the UPGMA analysis, estimates of genetic diversity were also calculated using the computer package Gene Survey.

Results

Results of UPGMA dendogram revealed three major clusters (Fig 1) as follows: Cluster 1, about 43 percent of varieties in this cluster were those collected from Lake Victoria zone; *Cluster 2,* this cluster had a dominance of Eastern and Southern zone varieties (69.2 percent); *Cluster 3*, in this cluster, about 74.2 percent were improved varieties developed at Amani. A few local varieties were found to be similar to some of the improved varieties. This included local variety Bwana mrefu and improved variety 5043/14. Variety Guzo found to be genetically close to Amani hybrid 4749. Varieties Kilusungu and Kalombe have found to be 100 percent identical, whereas Mdala, Kasumaili and Klorokwini were found to be different to the rest of the varieties in the cluster by being 56 percent dissimilar. Results from the genetic diversity of the varieties using SSR markers revealed the mean average gene diversity was 0.6937. The average gene diversity in the local germplasm is higher (0.7091) than that of improved germplasm (0.6782). The higher gene diversity was contributed by the local varieties from the lake zone (0.6049) than those collected from the eastern/southern zones (0.4944). The gene diversity of Amani varieties was also lower (0.4877). The findings implies that the genetic variation in Lake Zone varieties is higher than eastern/southern and Amani varieties confirms earlier observations that genetic diversity in farmers' field represents a valuable resource for cassava improvement. Local varieties from eastern/southern zones and Amani varieties seem to be related indicating that probably locals were used as the basis of breeding.

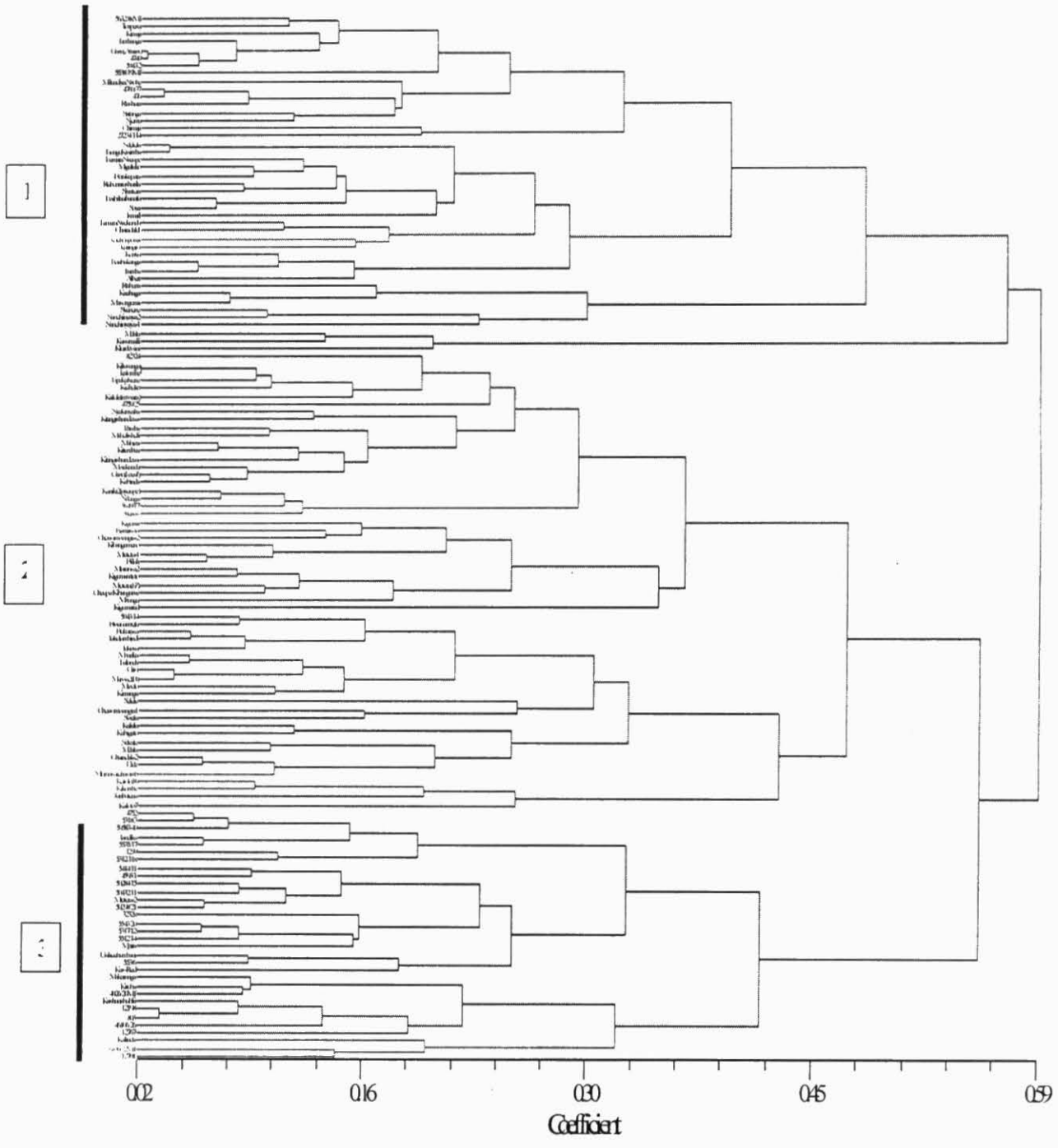

Figure 1. A unweighted pair-group method with arithmetic averaging (UPGMA) dendogram showing the three similarity clusters of the local varieties and improved varieties developed at Amani (the East African Cassava Research Institute), Tanzani

1.1.22 Relationships and potential origin of the weedy rice complex in Colombia

[1]E. Gonzalez, [1]L. Fory, [1]J.J.Vásquez, [1]P. Ruíz, [2]J. Silva, [1-2]MCDuque and [1-2]Z.Lentini
1.SB-2 Project; 2. IP-4 Funding from GTZ Germany

Introduction

Red rice (*Oryza sativa* L.) is a troublesome weed that infests most rice growing areas throughout the world (Pantone *et al.*, 1991). This weed shows a high similarity with cultivated rice varieties in the early growth stages. At maturity it has wide-ranging characteristics, including competitive ability, tillering capacity, flowering date, seed shattering and dormancy, pigmentation of several plant parts in particular of the pericarp (Diarra *et al.*, 1985). Different reports including our work Lentini and Espinoza (2005), show that some weedy rice can also have intermediate characteristics between wild *O. rufipogon* and cultivated *indica* or *japonica* varieties of *Oryza sativa* (Bres-Patry *et al.*, 2001) or could be divided into *O. sativa* ssp. japonica and *O. sativa* ssp. indica-like groups, with some intermediate accessions having characteristics from both groups (Cho *et al.*, 1995; Lentini and Espinoza, 2005). In addition, the high rate of fertility in crosses supports the traditional argument that red rice and cultivated rice are both *O. sativa*. However not everyone agrees with this traditional classification. It has been argued that in the tropics, weedy rice complex may also include other annual *Oryza* species such as *Oryza barthii*, *Oryza longistaminata*, *Oryza rufipogon*, *Oryza perennis*, or *O. punctata* (Kwon *et al.* 1991). These arguments are based on morphological characteristics, but the high degree of variation and lack of a clear classification system make it difficult to definitively categorize red rice. Another hypothesis is that weedy rice may have endo-ferally evolved through the dedomestication of cultivated rice to weedy types, where wild rice is not present (Vaughan *et al.*, 2003). The taxonomic composition of the weedy rice complex in Colombian has not yet been subject of study. In a ppreliminary work conducted by our research group (Gonzalez *et al.*, 2003. SB2 Annual Report 2003) we used 148 Colombian red rice accessions collected in farmer fields evaluated with 19 SSRs and the molecular data obtained were analyzed using a multiple correspondence analysis. This analysis generated five groups three of which grouped all red rice accessions. One of these groups clustered closely to *O. rufipogon*. The wild species *O. barthii* and *O. glaberrima* clustered together in the fourth group, and *O. glumaepatula* in the fifth group. The objective of this study was to evaluate the genetic variation among red rice types, hybrids between red and cultivated rice, some rice *O. sativa* cultivars japonica and indica types, Colombian landraces and wild species using microsatellite markers to have a better understanding of the relationships and the potential origin of the weedy rice complex in Colombia.

Materials and Methods

Plant Material. The materials used in this study consisted of: 154 accessions of weedy rice collected in Colombia; one generated in our laboratory; 40 commercial *indica* type rice varieties and 6 *japonica* type; 16 hand-made manual crosses between the RHBV-resistant transgenic Cica 8 line and non-transgenic variety purple or selected weedy rice accessions; 12 accessions of wild Oryza species, and 15 Colombian landraces.

Molecular analysis using SSRs Markers. The molecular characterization using microsatellites were the same as previously described in SB-02 Annual Report 2002 (González et al., 2002). The PCR products were resolved on silver-stained polyacrylamide gels and microsatellites alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

Statistical analysis. *A multiple correspondence analysis (MCA) was conducted. The MCA test established the level of significance for associations between categorical variables. MCA is a modeling technique to analyze associations in multi-entry data set. This analysis was conducted using SAS software (SAS, 1989). A dendogram was constructed using the Unweighted Pair-Group Mean Average (UPGMA) and the genetic distances were calculated using NTSYS-PC software version 2.02 (Rohlf, 1997).*

Results and Discussion

Multiple correspondence analysis (MAC) using SSRs markers generated five groups. All wild *Oryza* species were distributed in four different groups (Figure 1). Group 1: *O. glumaepatula* (accession from Costa Rica) was clearly distinct from the other species and formed one separated group. Group 2: *O. barthii (Chad) and* two accessions of *O. glaberrima (Africa)* clustered together in a second group. Group 3: A third group contained two wild species: *O. rufipogon* (accession from China) and *O. longistaminata.* Group 4: It is important to remark that *O. rufipogon* (India), *O. rufipogon* (Malaysia), *O. nivara* (China) and two crosses between both species from China were enclosed in the fourth group. This group included all weedy rice accessions (154), 85% of *O. sativa* indica type varieties (34), between the RHBV-resistant transgenic Cica 8 line and non-transgenic variety purple or selected weedy rice accessions (16), and 40% of the landraces (6). Group 5: Included all *O. sativa* japonica type varieties (6), the *O. sativa* indica type varieties from USA (5) and 60% of the landraces (9). These Colombian landraces come from the regions of Cordoba and Sucre. Varieties from USA (Starbonnet, Bluebelle, Bluebonnet 50, Cypress, Lemont and Rexoro) were in this group independently if they were japonica or indica. With the exception of the japonica variety from Brazil (Caiapo), no other South American variety was present in this group. The similarity between the Colombian landraces and USA varieties in this group may suggest that these landraces may be old USA varieties or derived from those materials.

In Colombia mainly indica type varieties are sown today. Therefore it is possible that the Colombian weedy rice had contact only with these varieties in the last years. In this study all the Colombian weedy rice accessions were related genetically to 85 % of the indica type varieties. Vaughan *et al.* (2001) also reported that all the weedy rice accessions were genetically related with indica type varieties, except the MS5 accession, which was enclosed with the japonica varieties. Three accessions of *O. rufipogon* were used in this study. Two of them (Malaysian and India) were included into the weedy rice group. The other accession (China) was included together with *O. longistaminata* in other group.

An additional multiple correspondence analysis was conducted excluding groups 1,2 and 3 to have a better understanding of the variation among the weedy rice, landraces, rice varieties and wild *Oryza* species (Figure 2). Eighty five percent of the weedy rice/ rice crosses are genetically distinct from 94% of the weedy rice (Figure 2; compare the red dotted oval with the green dotted oval). Two clearly genetically distinct groups (groups 1 and 5) of weedy rice are identified respect to the main) weedy rice group (75% accessions) which clustered with 75% of Latin

American indica rice varieties. (Group 2). Interestingly, Group 2 also included the accession of *O. rufipogon* from Malaysia and one of the *O.nivara/ O. rufipogon* crosses. And 60% of landraces are similar to USA varieties (Figure 2) suggesting the possible origin of these landraces derived from USA varieties.

Conclusions

The structure of the weedy rice complex in Colombia was studied and the MCA analysis showed five main groups. The weedy rice was found only in one group and was genetically related with six wild accessions belonging to *O. nivara* and *O. rufipogon* and crosses between them. Similar results were obtained by Vaughan *et al.* (2001).Colombian weedy rice was genetically similar to the indica varieties, and separated from japonica varieties. The two *O. glaberrima* and *O. barthii* accessions were enclosed together in a distant group. This result confirmed their African common origin, since *O. barthii* is the ancestor of *O. glaberrima*, a cultivated variety. Cultivated varieties from North America and the Colombian landraces varieties (60 %) were grouped with japonica varieties.

Future Activities

1) To study the relation between seed traits and the molecular analysis. 2) To analyze the genetic variation based on: alleles number, observed heterozygosis (Ho), genetic diversity (He) and population structure (Fst). 3) To conduct comparative analyses of genetic diversity of weedy rice and wild *Oryza* Colombian species.

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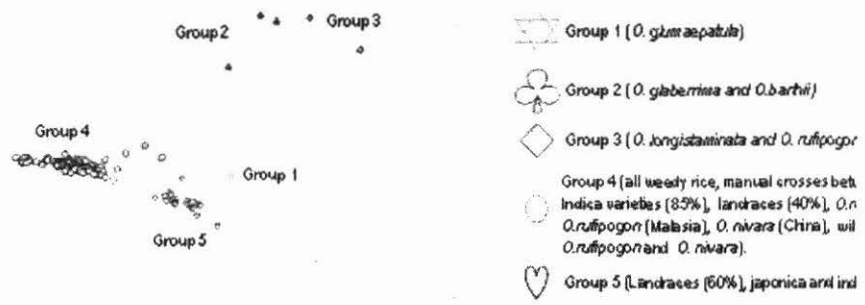

Figure 1.- Multiple correspondence analysis (MAC) using SSRs markers. Group 1: *O. glumaepatula*. Group 2: *O. barthii and O. glaberrima*. Group 3: *O. rufipogon* (accession from China) and *O. longistaminata*. Group 4: *O. rufipogon* (India), *O. rufipogon* (Malaysia), *O. nivara* (China) and two crosses *O. nivara* /*O. rufipogon* rice varieties, all weedy rice accessions, crosses between weedy rice and rice (transgenic and non-transgenic). Group 5: all *O. sativa* japonica type varieties, all USA rice varieties and 60% of Colombian landraces

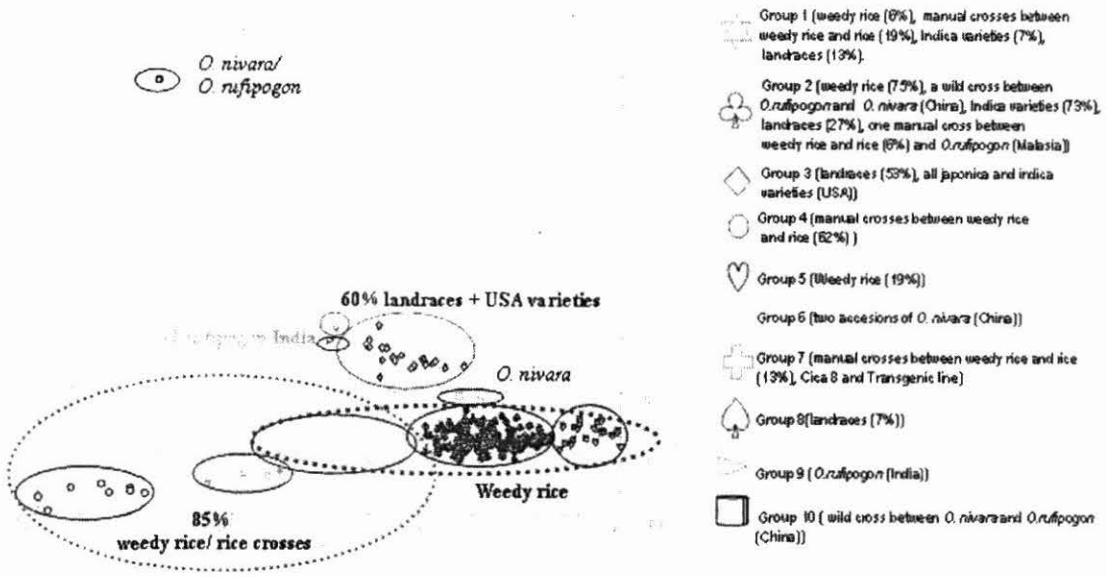

Figure 2. - Multiple correspondence analysis (MAC) using SSRs markers of samples excluding Groups 1, 2 and 3 of Figure 1.

1.1.23 Assessment of gene flow from transgenic and non-transgenic rice into weedy rice under experimental field conditions

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Introduction

It is important to study the potential environmental impact that the introgression of genes may cause in potential recipient wild populations and related parents. Weedy rice is among the main candidate for gene flow and introgression from cultivated rice since it is compatible and usually intermingled with the rice crop. Outcrossing rates < 1% from transgenic/non-transgenic rice to weedy rice/rice varieties had recently been reported by other authors under temperate conditions (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. In the tropics,

weedy rice is considered complex and broadly diverse, and apparently composed by numerous *Oryza* annual species. We have studied weedy rice populations from Colombia, its morphology and genetic trying to understand the genetic population structure. Genetic and morphological markers were identified distinguishing rice varieties from the weedy rice accessions from Colombia. These markers were used to study gene flow in the field. This work is part of a project "Gene Flow Analysis for Assessing the Safety of GMOs in the Neo-Tropics" directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species.

Last year, we reported 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 (sample about 6,000 plants) and non-transgenic rice (sample about 17,000 plants) into weedy rice under controlled experimental field conditions. This year we finished the analysis of total 54,713 plants by SSRs and 40,537 plants using morphological markers.

Materials and Methods

The experimental field designs as well as methods used to trace gene flow from transgenic and non-transgenic rice into weedy rice are according to Fory *et al.*, 2004 SB2 Annual Report 2004.

Results and Discussion

Hybridization rates from 0.0 to 0.4 % were obtained when SSR markers were used to trace gene flow in 54.713 plants derived from crosses between six weedy rice types and rice (either transgenic line Cica 8 or non-transgenic purple variety) (Table 1). The analysis by microsatellites indicates that not differences in hybridization rate are found between transgenic and non-transgenic rice (mean values of 0.10 and 0.12 respectively) when used as pollen donor. However, differences are found among weedy rice biotypes. In general, weedy rice biotypes classified as variety or intermediate types (accessions 1-21-3 and 5-38-5) (according to report in 2004) the highest hybridization rate (values between 0.12 and 0.39 %; whereas the wild type biotype accession 1-3-4 similar to *O. rufipogon* (according to report in 2004) showed the lowest hybridization rate (0.03- 0.0 %). These results suggest that perhaps there is different combinatory ability among weedy rice biotypes. This tendency probably occurs due to differences in flower structure between weedy types or incompatible barriers with the rice crop.

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 (Table 2). Furthermore, when plants were analyzed by bulk DNA without taking into account their anthocyanin profile, a higher outcrossing rate (0.12 %) was estimated respect to that based on the presence of the purple color (0.05%). These results suggest that the scoring of phenotypic trait alone could over- or sub-estimate the level of hybridization rate. These results show that the molecular markers SSRs are likely not linked to the genes that control the anthocyanin expression. 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), which can be located in different loci within the genome in such a way that it would be difficult to find the microsatellite markers and the gene within the loci location.

Future works should take into account the parental molecular characterization prior gene flow analysis as conducted in this study since the same allele can be presented by convergence evolution in different species/ genotype backgrounds. These events could under/over estimate hybridization rates or give wrong estimates of gene dispersion. It is also important to consider weedy rice diversity for the biotypes selection as parental lines since the hybridization rate can be influenced by genotype as shown herein (*rufipogon* type weedy rice had the lowest hybridization rates with rice respect to variety and intermediate types). Although the weedy rice is distributed at random in parches, it is important to take into account the homogeneity presence of pollen receptor individual at minimum distance from the pollen donor maximizing the probability of the hybridization events. We must consider the flowering periods to assure flowering synchronization between the receptor and donor. It is also important to evaluate environmental parameters which can influence the hybridization rate. Since the gene flow levels in rice can be as low as zero, the size of the progeny to be analyzed should be large enough to be able to detect these low rates. According to the statistical analysis in rice we should evaluate at least 20.000 plants by treatment to obtain 80 % confidence and 20 % permissible error. In our initial work we only analyzed 6.000 plants. Similar rates and patterns were obtained with that population to those seen after analyzing about 50,000 plants. Thus the number of individuals to be analyzed must be revaluated in future gene flow assays. These results suggest that alternatively when analyzing gene flow at large scale in the field, small plot samples can be taken distributed at random in the field to minimize the effect of environmental factors due to location of the plots, presence of physical barriers and soil conditions, wind, among others. Another important aspect to evaluate is the size of samples when doing DNA bulk analysis. Rung et al. (2004) were able to detect one hybrid per bulk of 10 DNA individuals when evaluating gene flow between varieties and rice hybrids using SSRs. In our study, we standardized the analysis for larger samples detecting one hybrid per bulk of 40 DNA individuals using SSRs (Gonzalez et al., SB2 Annual Report 2002) allowing to analyze a larger number of sample is less time. Higher numbers of hybrids were noted in treatments affected by a high wind speed during anthesis; however a clear cut effect of wind on the level of hybridization rates could not be established due to the strong compatibility effect on hybridization by genotype. Apparently, winds affected more hybridization of weedy biotypes with high or intermediate compatibility with rice whereas not influence was noted on those biotypes with the lowest compatibility which showed the lowest hybridization rates. Future gene flow studies should take into account the potential effect of genotype X environment interaction (especially wind in the case of rice) in the field.

Future activities

According International and Colombian biosafety regulations, it is necessary to carry out risk assessment evaluation case by case, when a transgenic organism is introduced into the environment. Our results shows not difference in gene flow rate between transgenic and non-transgenic rice into weedy rice. Thus it might be possible to get a comprehensive understanding of gene flow dynamic in farmers' field using non-transgenic crops at the onset of deployment the transgenic ones. In these studies it is also important to determine the level of gene introgression in subsequent generations. For this reason, hybrid fitness analysis between rice/ weedy rice hybrids should be evaluated at the landscape. Microsatellites would facilitate tracing the gene flow/introgression dynamics in crop/wild/weedy complexes and the potential impact on biodiversity.

Table 1. Hybridization rates between weedy rice and transgenic (A3-49-60-12-3/Cica 8 line) or non-transgenic rice (purple variety) rice transgenic under field conditions

Weedy rice	Type[1]	Mean gene flow T + P [2]	Transgenic line			Purple variety		
			Plants	H	% gene flow	Plants	H	% gene flow
4-12-2	V	0.15	5,123	3	0.06	4,263	2	0.04
5-38-5	V		5,014	6	0.12	4,051	16	0.39
1-21-3	I	0.16	4,869	13	0.27	3,989	9	0.22
5-36-4	I		3,381	1	0.03	5,311	6	0.11
1-3-4	R	0.04	5,526	1	0.03	5,935	0	0.00
5-48-2	R		4,284	3	0.07	4,967	3	0.06
Total/ Mean		0.12	26,197	27	0.10	28,516	36	0.12

[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. H = hybrid plants. [2]Mean gene flow rate per weedy type with transgenic or non-transgenic rice

Table 2. Hybridization rates between weedy rice and the non-transgenic rice purple variety under field conditions.

Weedy rice	Type[1]	plants	Plants first identified by purple stem color and then by SSRs		Plants analyzed without considering purple stem color
			% Hybrids by stem color	% Hybrids by SSRs	% Hybrids by SSRs
4-12-2	V	6,738	0.04	0.03	0.04
5-38-5	V	5,616	0.27	0.19	0.39
1-21-3	I	6,443	0.34	0.12	0.22
5-36-4	I	7,982	0.03	0.00	0.11
1-3-4	R	8,736	0.03	0.01	0.00
5-48-2	R	5,019	0.00	0.00	0.06
Total/ Mean		40,537	0.11	0.05	0.12

[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.

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1.1.24 Scaling up analysis gene flow analysis from rice into weedy rice at landscape under farmers' commercial conditions

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[1]SB2, [2]IP4, [3]FLAR. .Funding from GTZ, Germany.

Introduction

This work is part of the 2nd phase of a project entitled "Gene Flow Analysis for Environmental Safety In the Tropics", which main goal is to generate baseline genetic information for the development of guidelines on the safe introduction and use of novel agriculture traits (biotechnology derived or not native from the place of introduction), while reducing potential environmental impact on native biodiversity in the Neotropics, using two staple crops, bean and rice, as models. During the first phase, a systematic analysis of the composition and genetic diversity of weedy rice in Costa Rica and Colombia, including morphological, phenological and molecular characterization of 900 accessions from the main rice cropping areas of these two countries was accomplished, gene flow analyses under experimental conditions and optimization of methodologies based on molecular markers were optimized as described in sections by Gonzalez, e. et al., and Fory L., et al. of this report. The objective of this second phase is to assess the impact of specific non-transgenic traits on biodiversity (genetic structure of recipient population) due to gene flow over time at landscape in countries that harbor land races, weedy/wild species of these two crops. In the case of rice, herbicide is among the current methods preferred by farmers to control weedy rice, a major bottleneck for rice production in this region. Herbicide resistant rice varieties had been released in several productions sympatric to natural environments harboring native wild relatives of rice. Herbicide resistance in rice here derived from mutagenesis (imidazolinone resistance, Clearfield®) had been bred into elite local materials and released as improved varieties in Central America and Colombia. Because of its easy tracing, herbicide resistance provides an excellent model to evaluate the unintended transfer of traits deployed in the crop by cross-pollination to the sexually compatible weedy rice complex for which the herbicide is used as a form of chemical control (positive selection), and in the wild *Oryza* compatible relatives that are found in natural environments in the crop contact zones (neutral selection). In addition, the use of non-transgenic herbicide resistance source is an ideal case study for the comparison of the same trait in transgenic vs. non-transgenic allowing to elucidate the effects due to the trait itself independently from the gene source. This model will give information on impact of introgressed non-transgenic resistance genes that may affect fitness of derived hybrids, invasiveness, population dynamics and genetic structure of the corresponding wild/weedy. It will also serve for anticipating a potential impact from a transgenic situation. The information generated will be useful for *in situ* conservation of rice diversity. Protocols and methodologies using molecular markers will be established for assessing gene flow at landscape level (rate and direction) allowing comparison to those under controlled conditions. Finally, information will be used to develop guidelines for environmental safety and co-existence of different types of agriculture systems in the Neotropics.

Materials and Methods

Plant Material: The system Clearfield rice developed by BASF and Louisiana State University was deployed in USA as varieties Clearfield (CL): CL121, C161 and CF8 where the imidazolinone (IMI) gene was introduced by mutations. The variety Clearfield CF 205 (CF205) was developed by Fedearroz, Colombia, using USA Clearfield progenitors to introduce the IMI tolerance into Colombian commercial varieties. CF205 has been grown commercially in Colombia for the last 3 years in the Tolima region, and first sown in Jamundi, Valle del Cauca in 2005.

Characterization of CF205 by molecular markers: The molecular analysis was conducted with the 19 SSR markers identified in previous years to be highly polymorphic and allowing distinguishing rice from weedy rice.

Characterization of the weedy rice accessions from Jamundi-Alsacea. In order to identify the alleles of weedy rice, we collected weedy rice samples prior planting for the first time in the field the CF205 variety in the *Jamundi-Alsacea*. The plants were labeled and transplanted under greenhouse conditions. The molecular and morphological characterization of the collected seeds are in progress following similar methodologies previously used to characterized weedy rice accessions collected from the Tolima region in year 2001 prior the commercial introduction of CF205 in Tolima in 2003.

Plot selection, localization of plants and seed harvest. Four plots planted with CF 205 were identified in Tolima and Valle del Cauca departments. The parameters used for sampling the weedy rice population included: 1) plots with known agronomic history and with high weedy rice infestation up to 60 %; 2) synchronization of flowering between CF205 variety and weedy rice; 3) sampling area larger than 6 ha; 4) in the Tolima, the two plots selected (7 and 20 ha) had two consecutive cropping cycles of CF205. In Tolima (Saldaña and Espinal), weedy samples were collected at random through each plot from weedy rice spread in patches and intermingled and in contact with CF205. In Jamundi, samples were collected at random from an area between 200 and 250 m^2 from each of the two plots. Each plot was sub-divided into squares of 52 m^2 (Figure 1). The samples were collected and located by GPS, and the topographic map constructed in each case. In all cases (Tolima and Jamundi) weedy samples collected flowering panicles were in physical contact with flowering panicles from CF205. Each mother plant was labeled; progeny seeds harvested, leaf tissue samples collected for molecular analyses, and the original plant transplanted from the field to greenhouse conditions.

DNA extraction and analysis by SSRs. Genomic DNA of parental was extracted from rice leaves according to McCouch (1988). One polymorphic microsatellite markers clearly distinguishing weedy rice and Clearfield CF 205 were used to determine the profile of the materials collected. The characterization with additional six SSRs polymorphic previously shown to distinguish weedy rice from rice varieties is in progress.

Results and Discussion

A total of 1,660 plants were harvested in Tolima and Valle del Cauca. Preliminary results from a total of 114 weedy plants collected from Tolima using RM 212 indicated the presence of 9 weedy rice accessions of variety type showing the specific bands for rice varieties and weedy rice suggesting a potential hybrid nature, but more analyses need to be done to elucidate the potential

origin of the pollen donor. Most alleles of weedy rice ranged from 116 pb to 118 pb with this SSR marker, but some weedy types showed the same allele of 136 pb as for Fedearroz 50 and CF 205. The complete analysis with several polymorphic SSRs markers is underway.

Future Activities

The F1 generation of self-progeny seeds derived from the original weedy plants collected in Huila and Valle del Cauca farmer's fields will be planted in the field and will be tested for its susceptibility to the herbicide using lethal dosages according to the MASTERKEY® DG (BASF) and confirmed by our own tests. The surviving plants will be analyzed by SSRs and seeds harvested.

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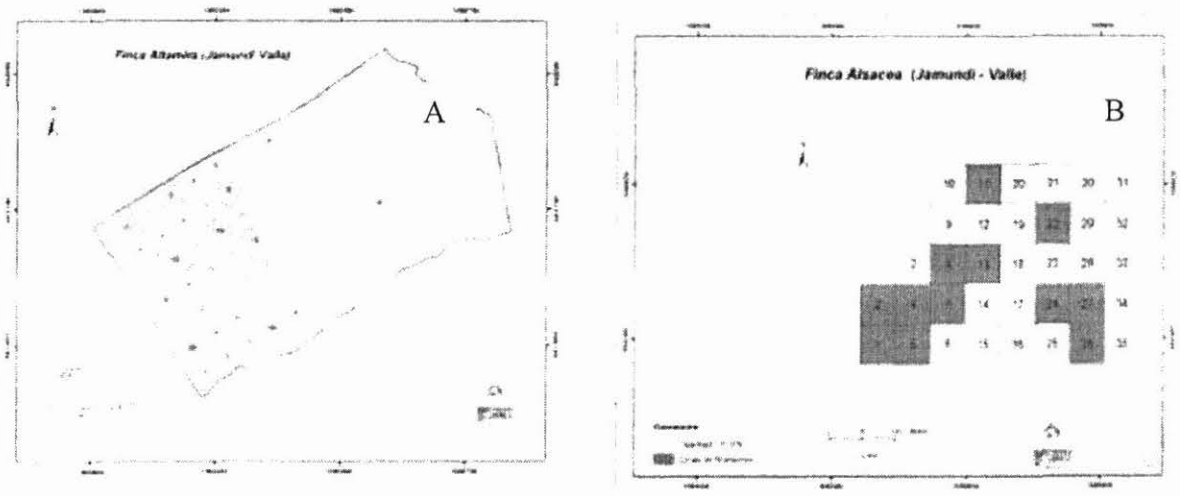

Figure 1. Maps of sampling plots in Jamundi, Valle del Cauca. A) Farm Altamira and B) Farm Alsacea. Blue squares represent the sampled areas within each plot.

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1.1.25 Standardization of methodologies for assessing gene introgression at large scale using molecular markers PCR based analyses

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[1]SB2, [2]IP4.Funding from GTZ, Germany.

Introduction

This work is part of a project "Gene Flow Analysis for Assessing the Safety of GMOs in the Neo-Tropics" directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species. During the first phase of the project, we established an efficient methodology based on DNA bulk analysis for the evaluation of gene flow from rice (transgenic and non-transgenic) into weedy rice using a population of 54,713 progeny plants derived from experiments under controlled conditions. The objective of this second phase is to assess the impact of specific non-transgenic traits on biodiversity (genetic structure of recipient population) due to gene flow over time at landscape level. Protocols and methodologies using molecular markers will be established for assessing gene flow at landscape level (rate and direction) allowing comparison to those under controlled conditions. Finally, information will be used to develop guidelines for environmental safety and co-existence of different types of agriculture systems in the Neotropics. PCR real time is an attractive alternative method which could facilitate the expedite evaluation of the large number of plants (Gachon, 2004). Although this technique shares a conventional PCR characteristic, it shows a high sensibility and quantitative precision. The applications include: viral detection, genes expression, and transgenic tissue identification by selecting transformation events with a low number of transgenes and the monitoring or the transmission of transgenes in subsequent generations and nucleotides polymorphisms detection (Schmidt and Parrott, 2001). The technique has been used to determine transgenes in various crops. The current report summarizes the progress attained using real time PCR and SSRs analysis towards setting up tools for assessing gene flow from transgenic and non-transgenic rice into wild/ weedy.

Materials and Methods

Plant materials included two rice commercial varieties (Clearfield CF205 and Purple), a RHBV resistant Cica 8-transgenic rice line and a weedy rice accession collected from Colombia. These materials were selected based on the high level of polymorphism indicated by three microsatellites. Genomic DNA was extracted from rice leaves according to McCouch (1988).

Mixtures of transgenic and non-transgenic tissues were made manually following ratios (transgenic: non-transgenic): 1:30, 1:100, 1:250, 1:500, and 1:900. Mixtures of tissues between weedy rice and rice varieties (Clearfield CF205 or Purple) of the same experimental ratios as above were used as negative control. Two replicates of each treatment were analyzed. The three SSRs (RM 211, RM 212 and RM 220) were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The PCR products were resolved on silver-stained polyacrylamide gels and microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega). For the real time analyses, the reaction was made using the kit SYBR® Green de Dynamo® following the recommendations of the manufacturer and the conditions standardized by (Beltrán, 2005). The *gus* transgene, a fragment of 240 pb was amplified using 1 µM uid 3 (5'atgaagatgcggacttacgtgg 3') and uid 4 (5'atggtgatgcagcgttgaactgc 3'), 120 ng de ADN, 10 µl Master mix. The reactions were carried out in the equipment DNA Engine Opticon 2 MJ Research following the program: 94°C 1min, 94°C 10s, 54-60°C 20s, 72°C 30s, 83°C 20s, during 44 cycles. In order to generate a PCR specific peak of the product the melting curves range from 65°C to 95°C. The final extension of the reaction was carried out during 10 min at 72°C. PCR was visualized in 1.2 % agarose gels, and transferred to nylon membranes (N Amersham). DNA probes were labeled with random primers and hybridizations were carried out overnight a 65°C.

Results and Discussion

Our earlier work indicated that the hybridization rate between rice and weedy rice are between 0 and 0.39% (mean 0.10%) according to assays carried out under controlled experimental field conditions. However, in non-controlled field conditions gene flow may occur at lower rates. For this reason, it is necessary to optimize the evaluation with a more sensitive system of increased detection level. The *gus* gene is used herein to standardize the transgene detection. Results indicate that it is possible to clearly detect up to 1 non-transgenic individual in a bulk of 900 individuals (ratio 1:900; 0.11%) (Figure 1A). These results were confirmed by Southern blot analyses of the PCR amplified DNA fragment (Figure 1B). However, it was noted that these results may vary from experiment to another (different replicates) at ratios equal or above 1:700. Reproducible and reliable results are when analyzing up to ratio 1:600 (0.173%). This ratio is 20 fold higher than the detection sensibility ratio (1:30) obtained when using standard PCR (3.33%) as in previous reports. Although we can detect 1% hybrids per sample (1:100) by SSRs, this is only possible when using high quality gels which are not practical for large scale analysis. Next steps include determining transgene copy number and tracing weedy rice specific DNA sequences using real time PCR analysis.

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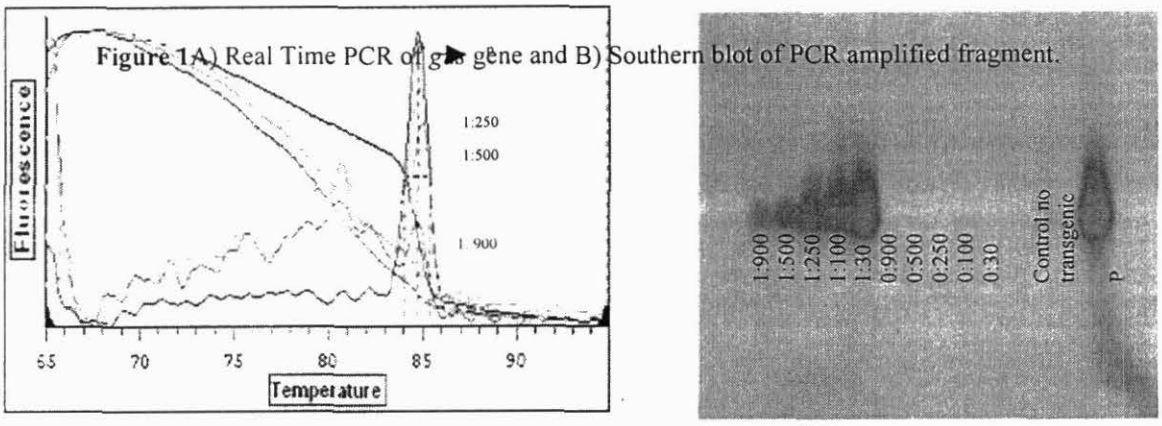

Figure 1A) Real Time PCR of gene and B) Southern blot of PCR amplified fragment.

A B

1.1.26 Use of chloroplast DNA polymorphisms for gene flow analysis in rice

L.Fory[1], M. Morales[1] and Z. Lentini[1,2]. [1]*SB2,* [2]*IP4.* *Funding from GTZ, Germany*

Introduction

The genetic information present in plant mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) complemented with RFLP analysis of PCR amplified nuclear DNA regions has been of great use in the study of phylogenetic relationship in rice and other plants (Ichikawa et al., 1986, Ge et al., 1999). Organelle plant genomes (mtDNA and cpDNA) are highly conserved in structure and the rice chloroplast genome sequence was published in 1989 (Hiratsuka et a., 1989 cited by Tang et al., 2004). Chloroplast DNA restriction patterns provide useful information to assess the phylogenetic relationships among plant species, due to the low rate of rearrangements, the relatively small size and predominantly maternal inheritance. Also the RFLP analysis of PCR amplified nuclear DNA can be used to facilitate he identification of collected wild rice germplasm, and potentially be applied to effective germplasm collection and identification of the crops (Ge *et al.*, 2001). This information will be useful for the characterization of Venezuelan and Colombian collected samples of wild/ weedy germplasm, objectives proposed for next year. In a previous work (Fory et al. 2002, SB2 Annual Report), we used 12 pairs of primers, which amplify mostly non–coding sequences. Nine cpDNA primers amplified the corresponding regions of the rice samples analyzed. These primers had been designed to amplify the complete sequences of chloroplast genome of *Oryza sativa* and *Nicotiana tabaccum* (Demuesure et al., 1995). The sizes of amplified fragments range between 1100 and 2800 pb. The digested PCR products of 8 fragment size with four restriction enzymes (PstI, Rsa I, Dra I, Hae III), revealed cpDNA polymorphisms in the region CP8 corresponding to the non-coding regions between the amino acid trnS [tRNA-Ser- (GGA)] and trnT [tRNA-Thr (UGU)]. A polymorphic fragment was observed for *O. glumaepatula* after the digestion with Dra I. 12 pairs of primer designed to

amplify the complete sequences of chloroplast (Demuesure et al., 1995) and allowed distinguishing *O. glumaepatula* from the other accessions used. This year, we used the sequences between rice plastid genes rpl16 y rpl14 which are used as cytoplasmic markers with the region ORF100. These sequences distinguish plastid subtype–identity (PSID), corresponding to differences between *indica* and *japonica* varieties, and also between tropical and temperate japonica cultivars at the cytoplasmic level (Ishii et al. 1988 Kanno et al. 1993, Chen et al. 1993, Nakamura et al., 1998). Six different plastid subtypes are found in *O. sativa* and *O. rufipogon* complex (Nakamura et al., 1998). Eight chloroplast haplotypes based on five polymorphic sites (two indels, one SPN, and a PolyC/polyA region) in the PSID and ORF100 fragments were reported using 234 rice accessions representing the geographic range of *Oryza sativa* (Garris et al., 2005). This year we are interested to study the cpDNA polymorphisms (maternal inheritance) to complement the information generated with SSRs markers (nuclear inheritance) giving a comprehensive understanding of the hybridization and introgression dynamic under field conditions. We also want to kwon if the SNP determined by Garris et al., (2005) and the 72 SNPs reported by Tang et al., (2004) can be used for the analysis of the gene flow in rice. Some SNPs are being used to evaluate flow in beans (Gonzalez et al., SB2 Annual Report 2005).

Material and methods

The materials used in this study consisted of accessions of weedy rice (6), transgenic line (1), commercial rice varieties *indica* (6) and *japonica* (9), manual crosses between a RHBV resistant transgenic Cica 8 line, non-transgenic purple variety and weedy rice (9), a cross of *O. nivara* /*O. rufipogon* (1), and wild *Oryza* species (6). Total DNA was isolated from young leaves according to the method described by McCouch et al (1988). The plastid sequence, which captures linker sequences between plastid genes rpl16 and rpl14, was amplified using the two combinations of primers reported by Sun et al. (2002) and Garris et al. (2005). The PCR was carried out in 25 µL total volume containing the following components: 120 ng of genomic DNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 2.0 U Taq polymerase, 1X PCR buffer that consisted of Tris HCl 10 mM (pH 9.0), KCl 50 mM and Triton 0.1 % 0.5 µM of each primers was used (5 GGCCATCATTTTCTTCTTTAG 3, 5 AGTCCACTCAGCCATCTC-TC 3 and 5 CAACCCACCCCATAAAATTG 3). The amplification was carried out using 1 cycle of 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 58°C, at 72°C for 5 min and one cycle of 10 min at 72°C. The PCR products were separated on agarose gel (1.4 %), stained with ethidium bromide and visualized by UV fluorescence. The regions including the ITS were amplified using a universal forward and reverse primers (ITS1 and ITS4). The PCRs included the following cycles 1 cycle of 2 min at 94°C, 30 cycles of 1min at 94°C, 1 min at 52°C, at 72°C for 1.5 min and one cycle of 10 min at 72°C. These PCR products were then digested by incubating 3µL of PCR product with Dra III and Fok I at 37°C, for 2 h. The digested amplifications were then separated on 1.4% agarose gel.

Results and Discusion

Fragments of 700 and 900 bp were amplified using the combinations of primers reported by Garris et al. (2005) and Sun et al (2002) (Figure 1 A, B, and C). A deletion of the 69 bp sequence in ORF100 region was identified in the cpDNA of all indica varieties, four weedy accessions, *O. nivara*, *O. rufipogon* and the cross between these two wild species (Figure 1). This deletion was not noted in the japonicas varieties, and two weedy type accessions, *O. latifolia*, *O. glumaepatula*, *O. alta* and *O. grandiglumis* (Figure 1 A and C). This result indicates that this deletion can be detected by PCR amplification using these primers. Weedy accessions 1-3-4 and

5-48-2 showed phenotypic and genetic similarities with *O. rufipogon*, using both morphological traits and SSRs markers (Gonzalez et al., 2003, SB2 Annual Report). In this study the *O. rufipogon* accession used (China) shows indica type cpDNA. *O. rufipogon* is considered a potential ancestor of *O.* sativa, and it is commonly associated with the *O. sativa* genome by different molecular techniques. Some accessions of *O. rufipogon* had been related with *O. sativa* indica type but others with *O. sativa* type japonica (Ge et al., 1999). Thus it will be important to include other *O. rufipogon* accessions in the analysis to have a more comprehensive diversity assayed by these types of markers. It is important to note that there are species of *O. sativa* that show the japonica nuclear and chloroplast types but they could also show an indica type of mitochondria (Sun et al., 2002). These cpDNA markers can be used when the parents are known. For example, the non deletion was inherited when the accessions 1-3-4 and 5-48-2 were used as females (pollen receptor) and the transgenic line, non transgenic or purple variety were used as the males (pollen donor) (Figure 1B). Because chloroplasts are inherited maternally, the non deletion in ORF100 region does not appear in the progeny derived when these weedy rice are used as male parent. Because this deletion may not be present in all weedy rice accessions, additionally it is necessary to use other markers at chloroplast and nuclear level to demonstrate the gene flow direction. The combined data of cytoplasm and nuclear genotype profiles would help elucidate the potential origin of weedy rice populations.

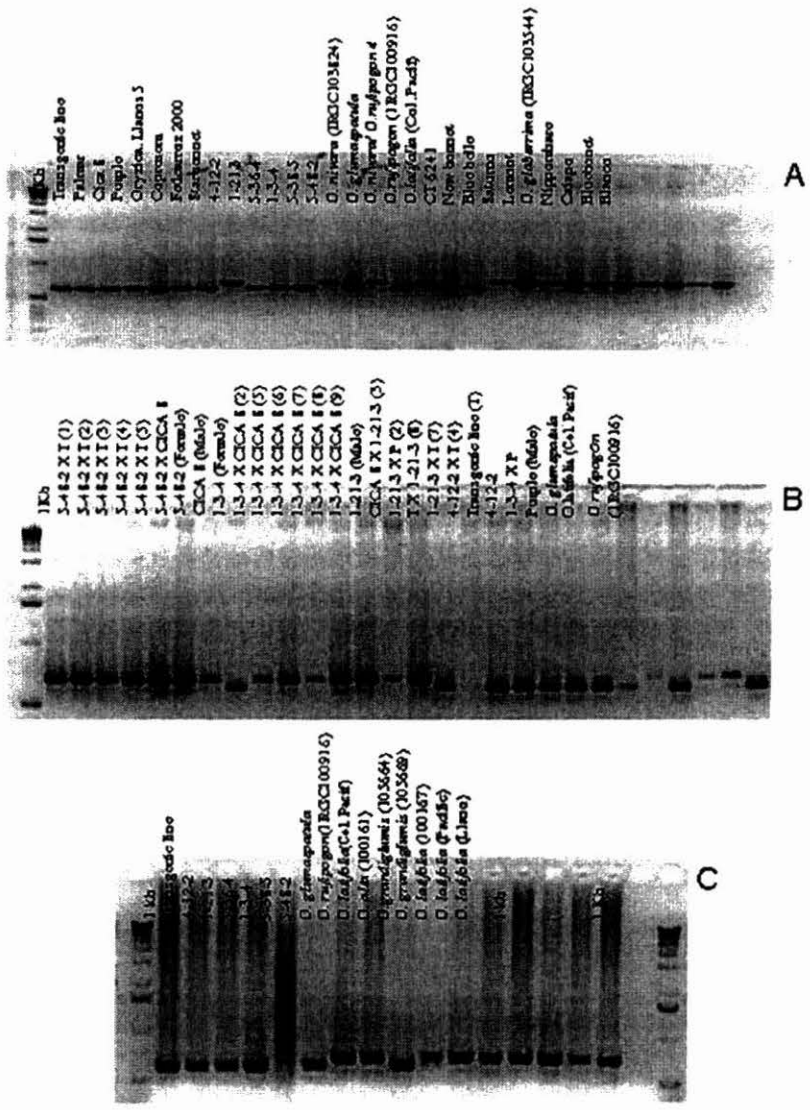

Figure 1. Analysis of cpDNA polymorphism . A) cpDNA haplotype of *japonica* and *indica* types. B) cpDNA haplotype patterns in hybrids between weedy rice and rice and C) cpDNA haplotype patterns in wild *Oryza* species

Future Activities

The amplified DNA fragment will be directly sequenced. With the sequences of cpDNA weedy rice, we will be able to establish some sites of restriction for some restriction enzymes. The evaluation of cpDNA, nuclear genome amplified product using other additional restriction enzymes in order to identify specific polymorphic patterns between varieties, weedy rice and wild *Oryza* species will follow. Some weedy rice types similar to wild *Oryza* species, similar to commercial varieties and intermediate based upon the phenotypic and molecular analyses will also be analyzed.

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1.1.27 Population structure, phenotypic information and association studies in long-generation crops

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CIAT

Funding: Generation Challenge Program (GCP)

Important Outputs

Selection of 200 varieties generated by the breeding program and compilation of their phenotypic data from cassava databases.
Genotyping of 117 varieties and 25 breeding lines with 36 SSR markers, 2 from every linkage group of cassava
Selection of 100 closely linked SSR markers from the genetic map to estimate linkage disequilibrium in the cassava genome and their use in genotyping the 200 selected varieties
The development of additional closely linked SSR markers from BAC contigs on a genome-wide basis to increase the level of resolution to detect LD in the cassava genome.

Introduction

Cassava improvement is predominantly field-based and can last 8-10 years due to long growth cycle of the crop and the need for multiple evaluations to identify superior genotypes. Cassava breeding can be more cost-effective way if a faster and more reliable way of determining the genotype, for example using molecular marker assisted selection (MAS). In the same way, the search for genetic markers can be accelerated using approaches that does not require development of mapping populations, for example association mapping. Thirty years of cassava breeding has accumulated phenotypic data for 800 improved lines. These materials have been evaluated over several years in different locations and in replicated trials for traits of agronomic importance. A subset of 117 varieties out of the 800 were selected a sample for the preliminary determination of cassava linkage disequilibrum (LD) in the cassava genome.

Methodology

Compilation of phenotypic information

Phenotypic data have been compiled for 450 lines generated by the breeding program. These materials have been evaluated over several years in different locations and replicated trials for traits of agronomic importance such as fresh and dry root yield, root dry matter content, starch content, number of roots and resistance to pests and diseases.

Selection of cassava varieties for LD determination

Initially, a set of 117 varieties (75 advance breeding lines and 42 landraces/elite) out of 450 accessions used for a pilot study to assess genetic diversity were selected for the preliminary

determination of cassava LD. The accessions included in the reference sample have been evaluated with 36 SSR markers and under the first year activities in SP1C2 of GCP (Fig 1).

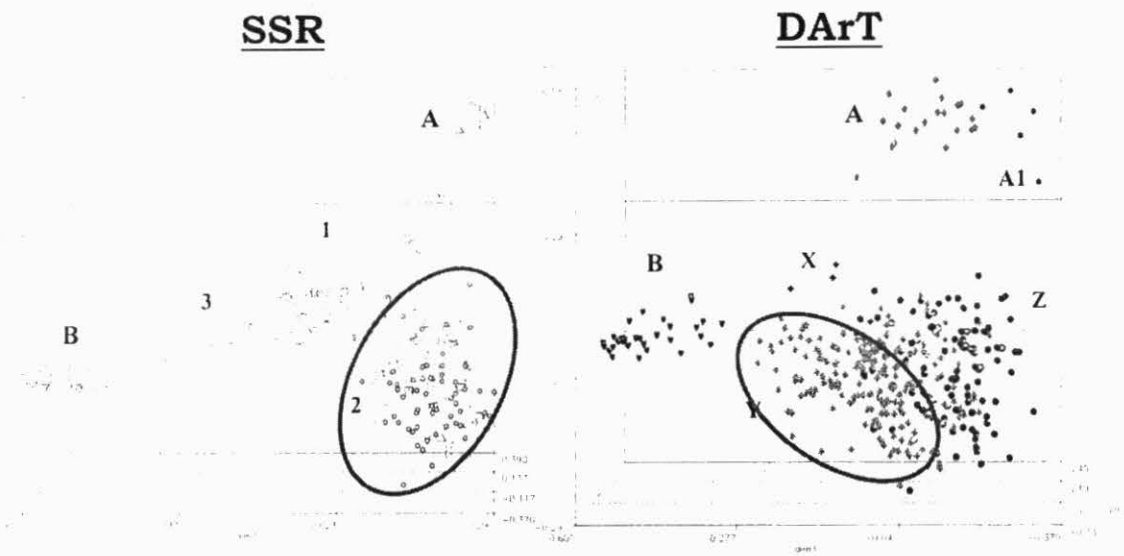


Figure 1. Analysis of 450 cassava accessions by SSR and DArT markers, in both marker systems, the cluster indicated in the pink circle is composed of 117 accessions corresponding to 75 advance breeding lines and 42 landraces/elite. These were selected just to give a rough idea of LD in cassava.

Determination of LD in Cassava

Linkage disequilibrium was calculated using the information generated by 36 SSR markers (212 alleles) in the pilot study to give a rough idea of LD in cassava. A matrix of letters was assigned to each allele and used as input file in Popgene and only significant ($P < 0.0500$) LD per allele pair was reported.

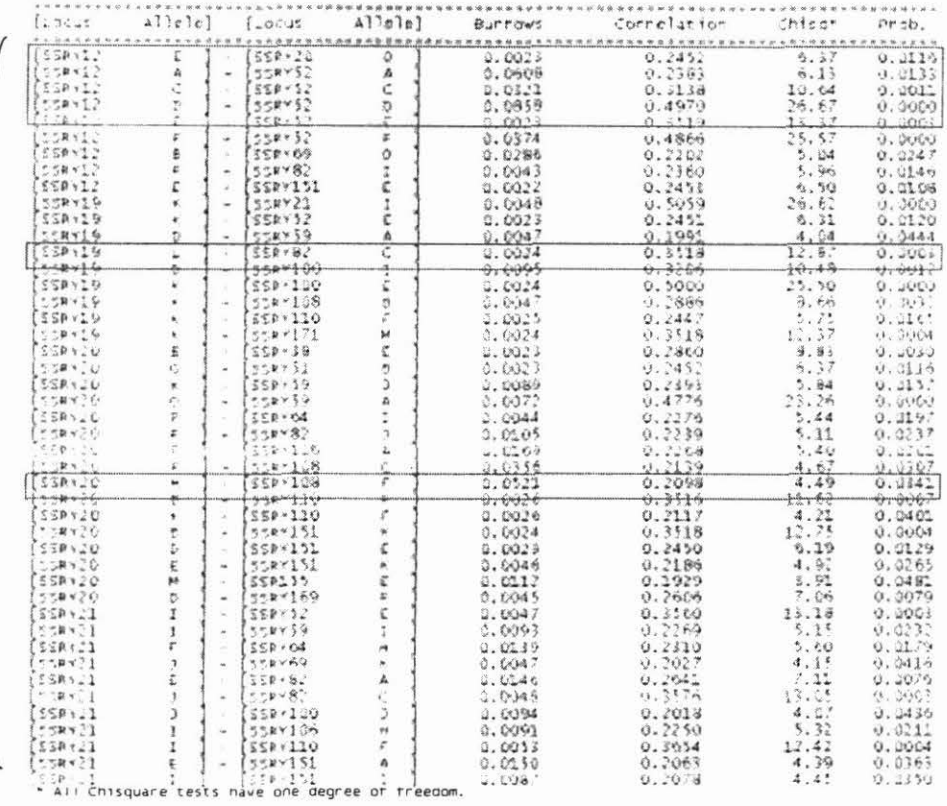
Results

Initial results using 117 varieties evaluated with 36 SSR markers revealed LD in alleles of SSR loci on different linkage groups suggesting unrelated germplasm in the 117 varieties (Fig 2), so a new sub-sampling was carried out, based on genotypic and phenotypic information, to improve LD determination in cassava. This new sampling led to the identification of 200 improved varieties to be used for the determination of LD.

Selection of closely liked SSR markers

100 closely linked SSR markers (5-10cM between markers on the same linkage group (LG) distributed on the 18 linkage groups (between 2-10 per Linkage group) of the cassava genetic map have been selected (see Figure 12.30) to estimate linkage disequilibrium in the cassava genome. A lager second set of 200 new varieties was selected out of the 800 improved lines to assess genetic diversity structure using the 100 closely linked SSR markers and to select an unstructured group for determination of LD in cassava. The new set includes varieties that have been developed for the breeding program, so they have a genetic relationship. The 200 genotypes are being evaluated with

Significant LD found in 92 allele pairs



[Locus	Allele]		[Locus	Allele]	Burrows	Correlation	Chisq*	Prob.
[SSR×12	E	-	[SSR×20	D	0.0023	0.2452	6.57	0.0119
[SSR×12	A	-	[SSR×52	A	0.0408	0.2363	6.13	0.0133
[SSR×12	C	-	[SSR×12	C	0.0121	0.5138	10.64	0.0011
[SSR×12	D	-	[SSR×52	D	0.0458	0.4979	26.67	0.0000
[SSR×12	F	-	[SSR×12	F	0.0023	0.5119	13.37	0.0003
[SSR×12	F	-	[SSR×52	F	0.0374	0.4866	25.57	0.0000
[SSR×12	B	-	[SSR×69	D	0.0286	0.2202	5.04	0.0247
[SSR×12	F	-	[SSR×82	I	0.0043	0.2360	5.96	0.0146
[SSR×12	E	-	[SSR×151	E	0.0022	0.2458	6.50	0.0108
[SSR×19	K	-	[SSR×21	I	0.0048	0.5059	26.62	0.0000
[SSR×19	K	-	[SSR×52	E	0.0023	0.2452	6.31	0.0120
[SSR×19	D	-	[SSR×59	A	0.0047	0.1985	4.04	0.0444
[SSR×19	L	-	[SSR×82	C	0.0024	0.3518	12.87	0.0003
[SSR×19	D	-	[SSR×100	I	0.0095	0.3266	10.49	0.0012
[SSR×19	K	-	[SSR×100	E	0.0024	0.5000	25.90	0.0000
[SSR×19	K	-	[SSR×108	D	0.0047	0.2886	9.66	0.0019
[SSR×19	K	-	[SSR×110	F	0.0025	0.2447	6.71	0.0161
[SSR×19	K	-	[SSR×171	M	0.0024	0.3518	12.87	0.0004
[SSR×20	E	-	[SSR×38	E	0.0023	0.2860	9.83	0.0050
[SSR×20	Q	-	[SSR×51	D	0.0023	0.2452	6.57	0.0116
[SSR×20	K	-	[SSR×19	D	0.0089	0.2393	5.84	0.0132
[SSR×20	Q	-	[SSR×59	A	0.0072	0.4776	23.26	0.0000
[SSR×20	P	-	[SSR×64	I	0.0044	0.2276	5.44	0.0197
[SSR×20	F	-	[SSR×82	D	0.0105	0.2239	5.11	0.0237
[SSR×20	F	-	[SSR×126	A	0.0269	0.2264	5.40	0.0202
[SSR×20	F	-	[SSR×148	C	0.0356	0.2139	4.67	0.0307
[SSR×20	M	-	[SSR×108	F	0.0021	0.2098	4.49	0.0341
[SSR×20	M	-	[SSR×110	F	0.0026	0.3516	12.82	0.0003
[SSR×20	K	-	[SSR×120	F	0.0026	0.2117	4.21	0.0401
[SSR×20	E	-	[SSR×151	K	0.0024	0.3518	12.75	0.0004
[SSR×20	D	-	[SSR×151	E	0.0023	0.2450	6.19	0.0129
[SSR×20	E	-	[SSR×151	K	0.0046	0.2186	4.90	0.0269
[SSR×20	M	-	[SSR239	E	0.0212	0.2929	8.91	0.0028
[SSR×20	D	-	[SSR×169	F	0.0045	0.2606	7.06	0.0079
[SSR×21	I	-	[SSR×12	E	0.0047	0.3560	13.18	0.0003
[SSR×21	I	-	[SSR×59	I	0.0093	0.2269	5.15	0.0232
[SSR×21	F	-	[SSR×64	M	0.0239	0.2310	5.60	0.0179
[SSR×21	D	-	[SSR×69	K	0.0047	0.2027	4.15	0.0416
[SSR×21	E	-	[SSR×82	A	0.0246	0.2041	4.21	0.0402
[SSR×21	I	-	[SSR×82	C	0.0046	0.3576	13.05	0.0003
[SSR×21	D	-	[SSR×100	D	0.0094	0.2018	4.07	0.0436
[SSR×21	I	-	[SSR×106	M	0.0091	0.2250	5.32	0.0211
[SSR×21	I	-	[SSR×110	F	0.0033	0.3054	12.42	0.0004
[SSR×21	E	-	[SSR×151	A	0.0250	0.2063	4.39	0.0363
[SSR×21	I	-	[SSR×171	I	0.0067	0.2078	4.41	0.0359

* All Chisquare tests have one degree of freedom.

Figure 2. Linkage Disequilibrum in 212 alleles from 117 accessions. The purple squares indicate allele pairs from SSR markers located in the same linkage group. (All Chisquare tests have one degree of freedom and single population linkage disequilibria was calculated based on Weir (1979) Biometrics 35:235-254)

100 closely linked SSR markers (5-10 cM) distributed across the 18 linkage groups (LG) of the cassava genetic map.

Conclusions and perspectives

Once genotyping is completed marker haplotypes will be defined in the 200 genotypes using the computer software Arlequin. The haplotype data will be used to calculate linkage and structural disequilibrum between alleles of the SSR loci in the selected genotypes. Based on this result a group of 64 starch biosynthetic genes will be employed to genotype the 200 accessions for association mapping of dry matter content in cassava.

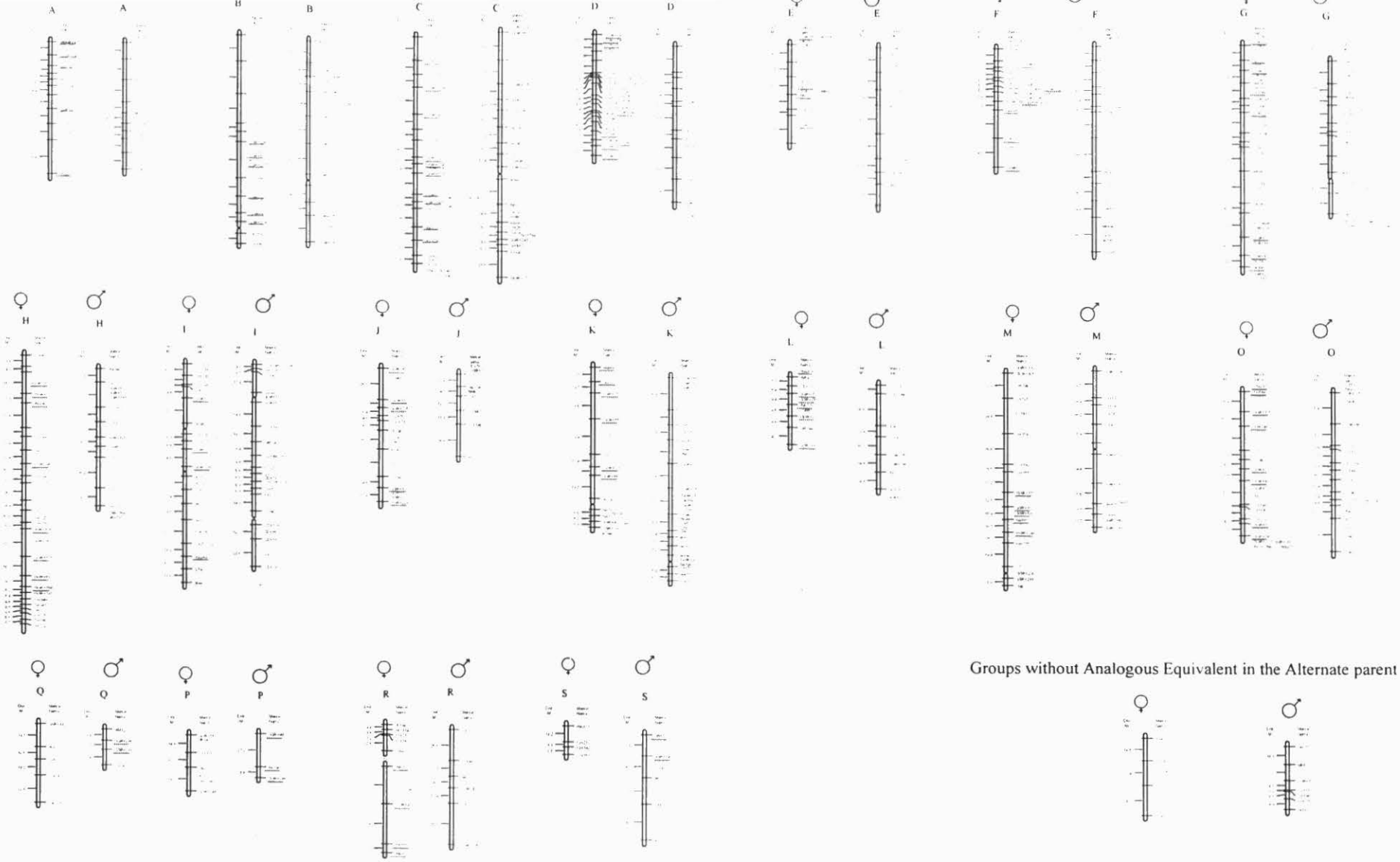


Figure 12.30 100 closely linked SSR markers distributed in the 18 linkage groups of the cassava genetic map have been selected

1.1.28 Determination of gene flow among bean species (*Phaseolus* ssp.) from Colombia y Costa Rica using microsatellites markers

R.I. González-Torres , D.G. Debouck, O. Toro, M.C. Duque.

After using microsatellite markers to successfully establish gene flow events in weedy forms of common bean, we were interested in testing the hypothesis of participation of alien species in such a gene flow. We tested six rare forms possibly resulting from interspecific hybridizations in natural conditions of Colombia (with *P. dumosus*) and Costa Rica (with *P. costaricensis* and *P. dumosus*) and the species controls (*P. costaricensis, P. coccineus, P. dumosus, P. albescens, P. vulgaris*) (Table 5). We tested microsatellites screened at 68 loci to evaluate the level of participation of nuclear genes in this natural event (Gaitán Solís et al. 2001). The atypical materials were initially selected as potential interspecific hybrids due to their phenotypic characteristics like atypical seed, infertility and other genetic abnormalities associated to the plant growing (Figure 40).

Table 5. Identification of *Phaseolus* ssp. materials used in the evaluation with microsatellites.

CIAT Identification	Species	Country	Department	Biological status
G24765 (Pop9072)	*P.* x *vulgaris*	Colombia	Boyacá	Weedy
G24666A (Pop9077)	*P.* x *vulgaris*	Colombia	Cundinamarca	Weedy
FI7031 (S34124)	*P.* x *vulgaris*	Costa Rica	Cartago	Weedy
FI7033 (S34124)	*P.* x *vulgaris*	Costa Rica	Cartago	Weedy
FI7034 (S34124)	*P.* x *vulgaris*	Costa Rica	Cartago	Weedy
FI7035 (S34124)	*P.* x *vulgaris*	Costa Rica	Cartago	Weedy
S29699	P. costaricensis	Costa Rica	San José	Wild
G36285 (Coc-1718)	*P. coccineus*	Guatemala	Quezaltenango	Wild
G36290 (Coc-1440)	*P. dumosus*	Colombia	Caldas	Cultivated
PL3592	***P. albescens***	Mexico	Jalisco	Wild
FI6846 (G23418)	*P. vulgaris*	Costa Rica	Cartago	Wild

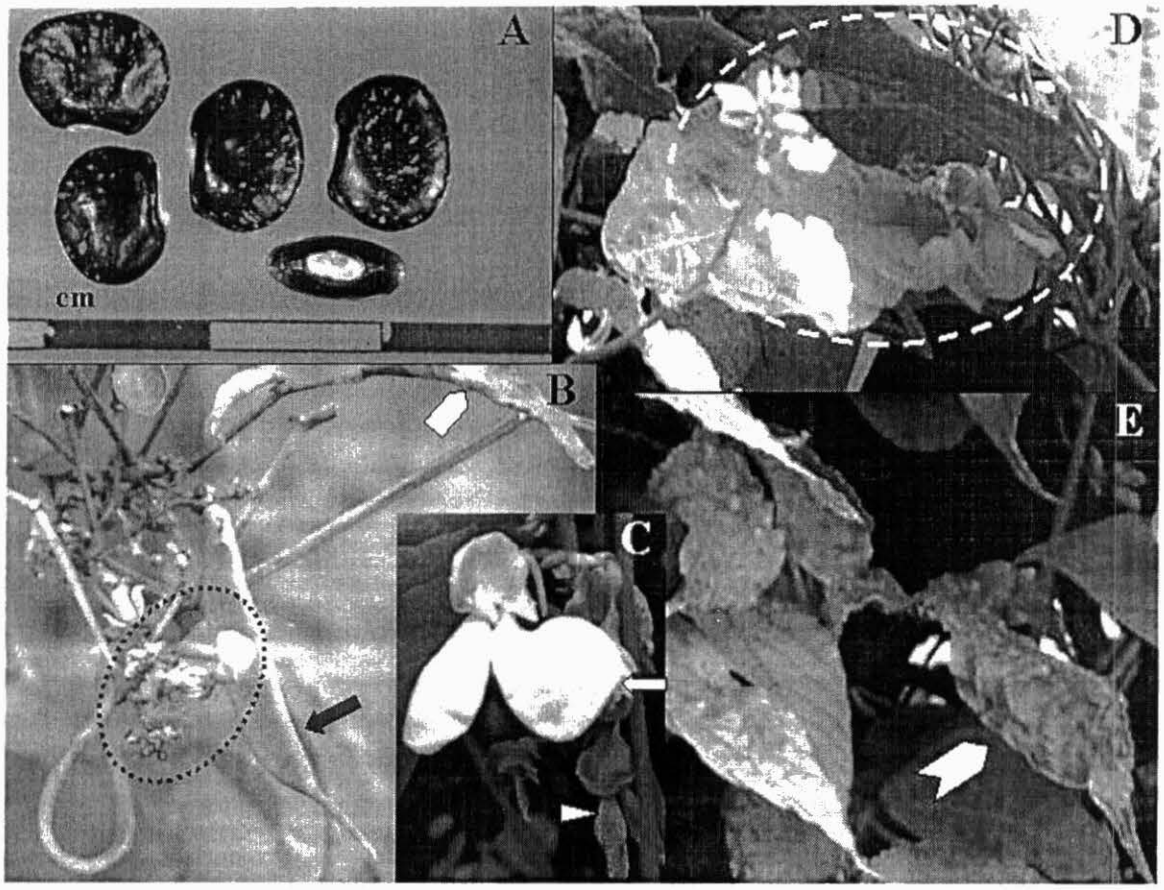


Figure 5. Phenotypic markers for interspecific hybrids.

A. Original seeds of individual FI7031
B. Individual FI7031 presents the *cripple* phenomenon and atypical floral buttons.
C. Individual G24765 shows bigger size of bracteoles, flowers and the position of wings similar to those of *P. dumosus*.
D. Two different flower colors were expressed in G24666A material.
E *Cripple* phenomenon on population G24666A.

The data analysis was carried out considering each band as an allele, using multiple correspondence analyses (MCA), also in order to understand the population structure and individual dispersion. The resulting graphic representation permitted to locate the materials according to their genetic similarity in a multidimensional plane using CORRESP module of NTSYS v. 2.10Y.

Results

The analysis of results was realized putting together specific descriptions using molecular markers and morphoagronomic characters of each species involved in possible gene flow events. These characteristics were displayed by hybrid individuals (Figure 5).

The evaluated microsatellites were of high discrimination power (PIC), so that each genotype was singled out in this evaluation, and this condition allows a detailed characterization of each species (Figure 6).

The control material of *P. costaricensis* was linked (using DICE coefficient) with a similarity coefficient of 16% with regard to the rest of individuals; *P. coccineus* and *P. albescens* had 34%, *P. vulgaris* and *P. dumosus* with similarity coefficients of 55% and 60%, respectively (Figure 42). The interspecific gene flow event among bean species has a low frequency and finding possible hybrids in the field is rare. The high differentiation obtained for the individuals studied with help of SSR permitted to attribute a gene flow event from any discrepancy and/or allelic similarity in the hybrids. The molecular characterization allowed to establish that some alleles belong to the ancestral evolutive *phylum*. The changes in the frequency of these alleles suggest generation differences, in other words, the studied individuals might not belong to the same generation (Figure 7).

The results confirm that the *loci* evaluated using microsatellites could be employed for the differentiation and characterization of the different bean species according to Gaitán-Solís *et al.* (2002), including *P. costaricensis* and *P. albescens* evaluated here for the first time (Figure 6).

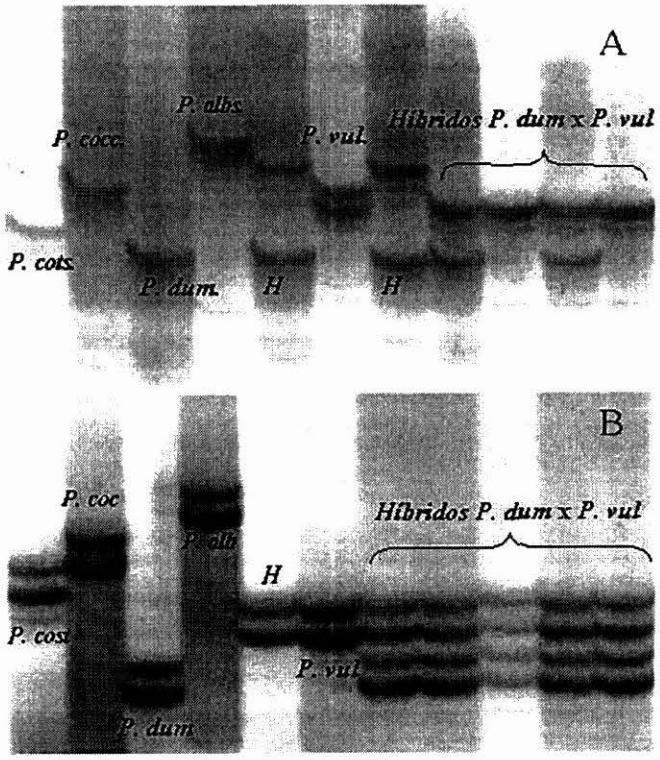


Figure 6. Specific microsatellites obtained to characterize each species and observed in the evaluated interspecific hybrids.

A. *Loci* of microsatellites GATS11 show different allelic forms in all species and the shared alleles in hybrids individuals between *P. vulgaris* and *P. dumosus*.
B. *Loci* of microsatellites BM181.

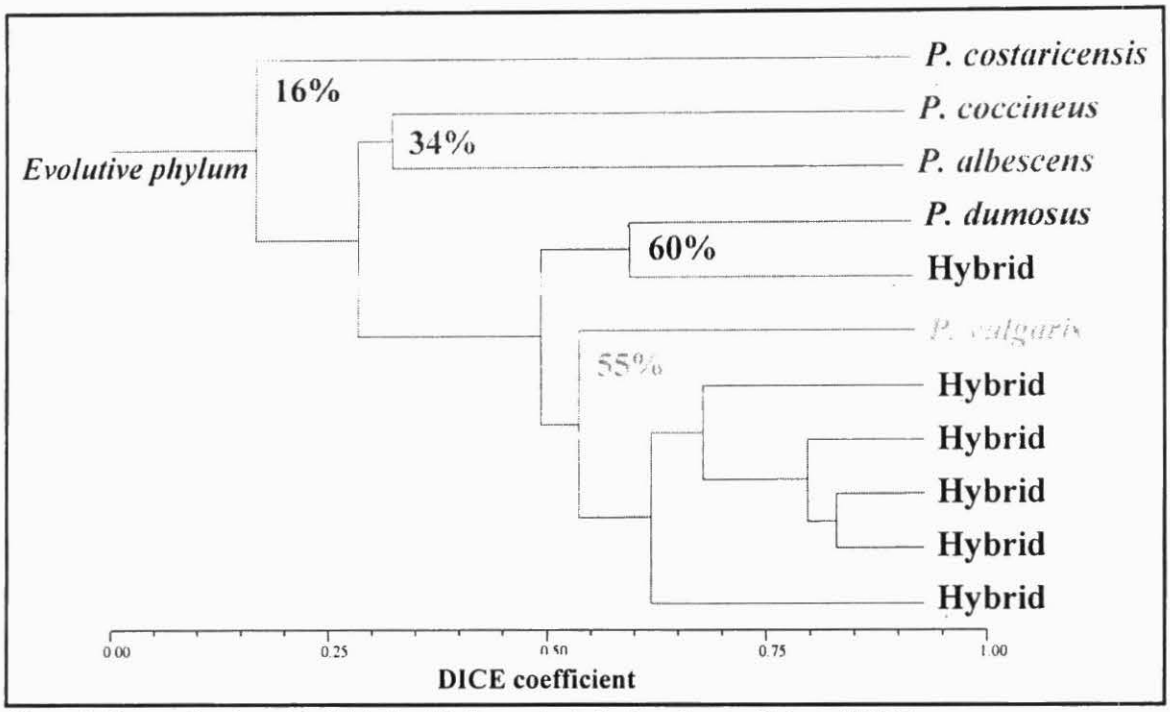


Figure 7. Cluster of evaluated individuals of their genetic similarity using coefficient of DICE.

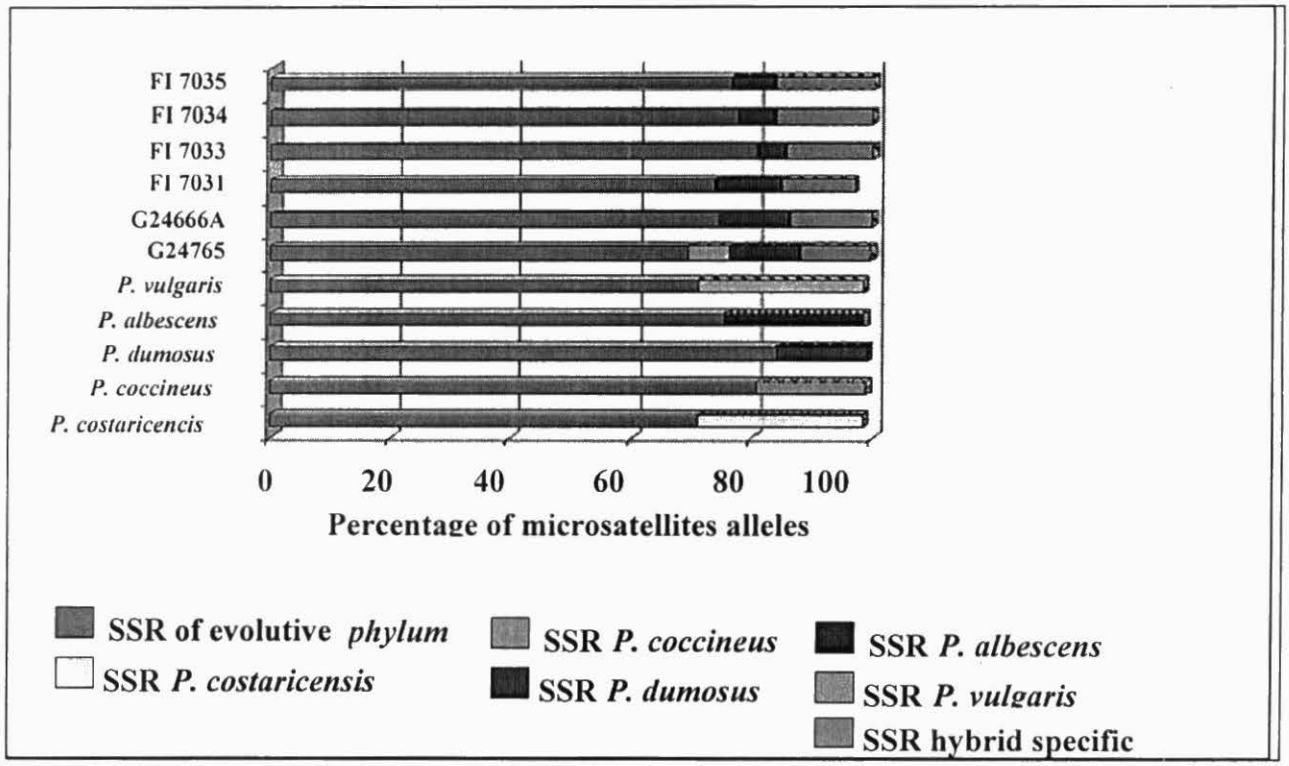


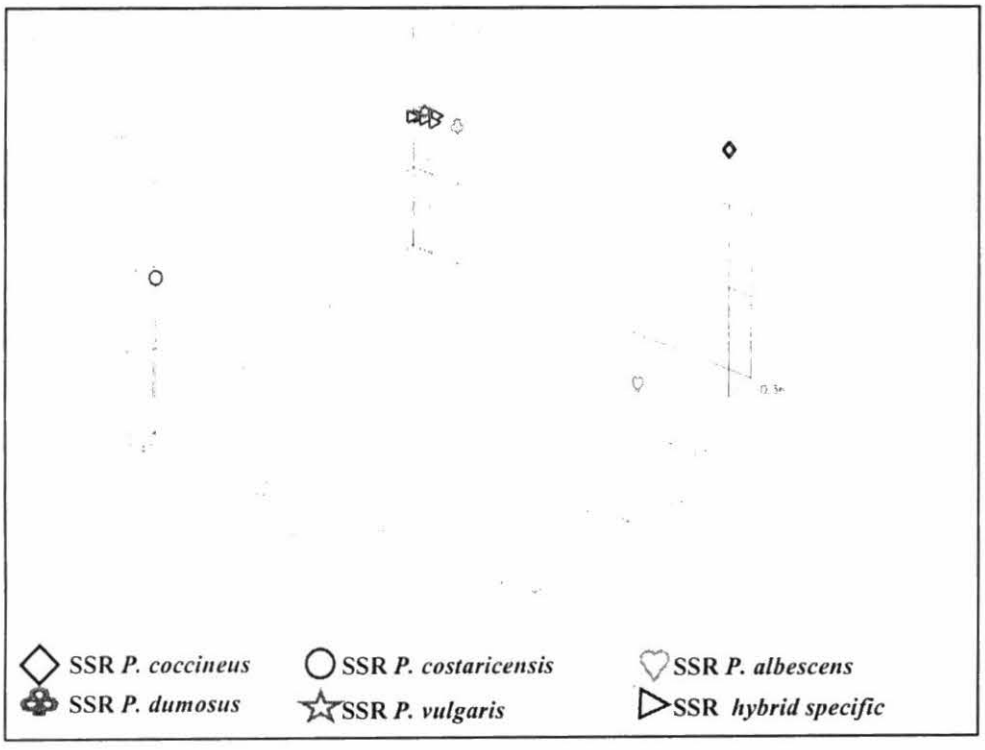
Figure 8. Graphic representation of microsatellites alleles found in the evaluated individuals.

The cluster realized with multiple correspondence analyses shows the structure of the hybrids (Figure 9). This group is spatially closed to *P. vulgaris* and *P. dumosus* suggesting that the SSR *loci* had been object of recombination by gene flow among these species. In that way, the nuclear genome of hybrids is sharing specific alleles of the evaluated species. Figure 9 displays *P. costaricensis* presenting a remote position in relation to all individuals. In an opposite position is *P. coccineus*, which has near values to *P. albescens* in the dim1 and dim2; however the last is completely contrastable in its dim3 value. Dim3 magnifies differences of *P. albescens* with reference to other individuals, as well as the dim1 increases the dissimilarity between *P.*

costaricensis and the others. Dim2 separates *P. coccineus* and facilities the discrimination of *P. albescens*. *P. vulgaris* and *P. dumosus* are closer in terms of dimensions 1 and 3 with a little divergence in dim2 (Figure 9).

The hybrid group has been sharing spatial positions nearer to *P. vulgaris*, mainly, and with *P. dumosus*; the hybrid identified as G24666A is among them signifying a recent gene flow (Figure 8). The results indicate that the evaluated hybrids are indeed the result of gene flow events between common bean and its near species *P. dumosus* in the Central Valley of Costa Rica as well as in Boyacá, Colombia. In addition, this information implies that these species could be affected by outcrossing pollination in simpatric conditions. Besides, the direction of gene flow was mainly realized from pollen of *P. dumosus* towards forms of *P. vulgaris* since the majority of specific alleles found in the hybrids were those of *P. vulgaris* as compared to the ones of *P. dumosus*.

Figure 9. Spatial distribution generated by MCA analyses of hybrid individuals with respect to control species.



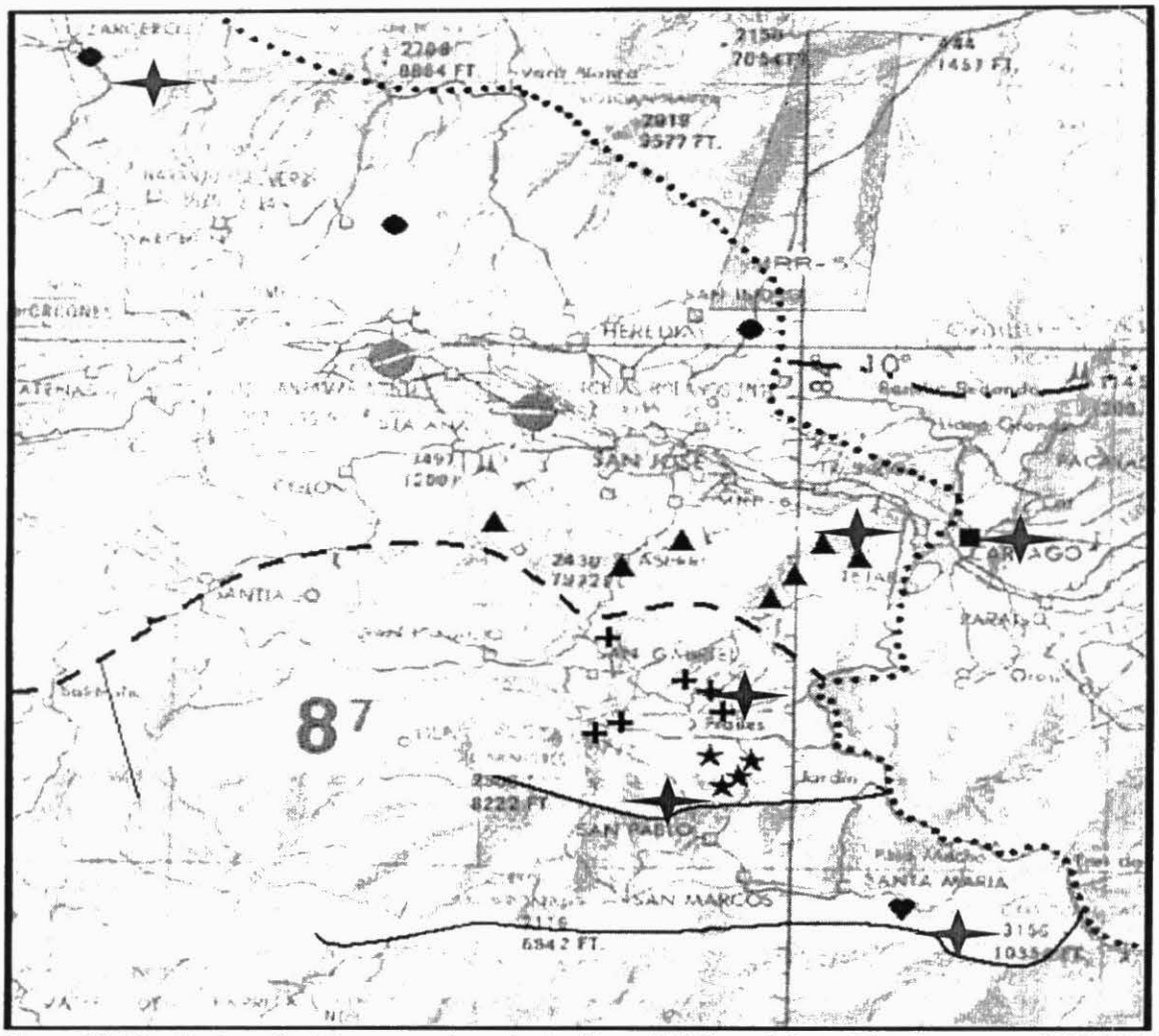


Figure 10. Distribution of wild common bean in the Central Valley of Costa Rica (base map: IGN-DGAC, 1991). Solid square: Reventazón, one population; solid heart: Pirrís, one population; closed circles: Virilla north, 3 populations; solid triangles: Virilla south, 7 populations; crosses: Candelaria north, 6 populations; and closed stars: Candelaria south, 4 populations. Dotted line represents the continental divide, while the other lines limit the different watersheds. Red closed stars: distribution of complexes of wild-weedy-cultivated.

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1.1.29 Estimation of gene flow on common bean: the case of the Central Valley of Costa Rica

The genetic compatibility between crops and their wild relatives has important implications for the conservation of genetic diversity and for the introduction and management of transgenic crops. The gene flow event, distribution of receptor populations, gene transfer effectiveness, pollination activity and long-term effects (namely in fitness) are being studied in natural populations of common bean in a center of origin and genetic diversity.

We documented the geographical distribution of each wild population as well as the biological complexes of "wild-weedy-cultivated" materials (Figure 10). Twenty-two populations of wild common bean are known for Costa Rica, and distributed in four watersheds in the central part of that country representing at least 90% of the found populations (González Torres *et al.* 2004).

Table 6. Morphological, biochemical and molecular markers used and No. individuals analyzed for each parameter

Biological form	Seed average weight (g)	Phaseolin type	Isozymes		Microsatellites		Chloroplast haplotype
			Pattern[1]	Allele[2]	Primer	Allele	
Wild	6 (2.5-7) N=1399	"S-4" "S" "M1" "S-3" N=907	DIA -1 N=229	PRX 100 N=197	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 GATS91 N=316	160 80 164 110 165 147 138 122 224	H N=540
Weedy	13 (8-21.3) N=794	"C" "CH" "H" "S" "X-7" "S-4" N=548	DIA-1 DIA-2 DIA-4 N=157	PRX 100 PRX 98 N=182	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 GATS91 N=408	160, 177 80 164, 185 110 165, 189 147, 150 138, 148 122, 136 224, 243	G, H J, K, L, F N=669
Cultivated	23 (22-46) N=225	"S" "T" "X-7" "CH" N=205	DIA -2 DIA -4 N=64	PRX 98 N=29	BM140 BM172 BM175 BM183 BM187 BM188 BM189 BM205 GATS91 N=67	180 80 183 110 189 150 148 136 243	J, K, L N=53

For the disclosure of wild populations, we applied a technique of ecogeographic surveying. For the molecular characterization, 1,232 individuals were selected based on a morphoagronomic evaluation, and 417 of them were weedy types possibly resulting from gene flow event. We used phaseolin, isozymes, and microsatellites as markers of nuclear genes. The gene flow direction was detected by RFLPs-PCR, sequencing and SNPs on chloroplast-DNA (Table 6).

The results obtained in the characterization of the populations are summarized in Table 6. The red underline fonts refer to 'wild' characteristics and the green fonts are 'cultivated' characteristics. The blue fonts are specific characteristics found only in a biological status. The data analysis showed that 98% of the putative hybrids were indeed hybrid. The direction of gene flow was mainly wild pollen towards the cultivated materials (82%). However, the other direction was observed at lower frequency but as significant percentage. The gene flow was mostly in materials belonging to the Mesoamerican gene pool (the one prevailing in the area). However, outcrossing between Mesoamerican and Andean gene pools were evidenced in 8% of the weedy individuals (Figure 11).

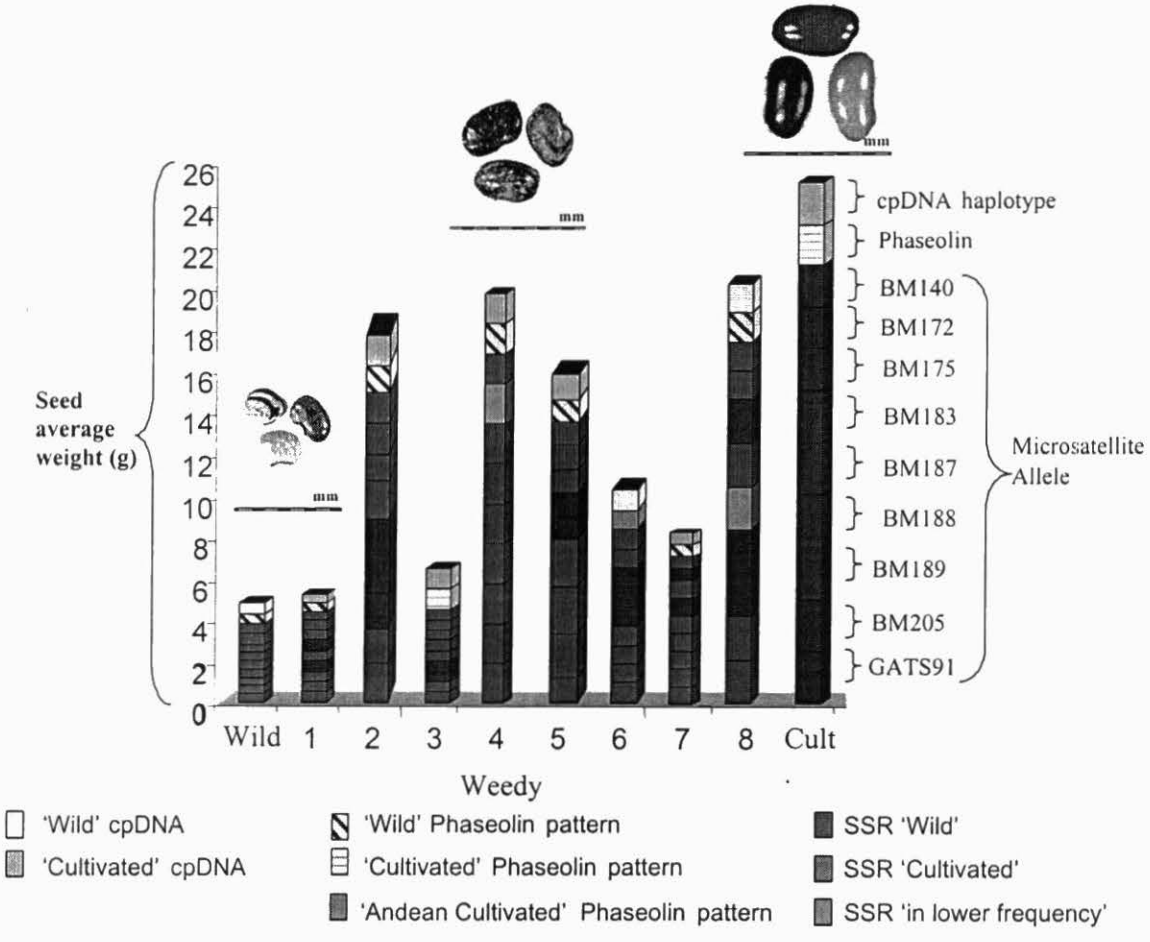

Figure 11. Graphic representation of markers used on a selection of individuals.

Figure 11 shows repeated events of gene flow of wild pollen towards cultivated forms (individuals 1 to 3). Individual 4 indicates events of repeated outcrossings resulting into a weedy form with a nuclear genome of a wild form with cytoplasmic genome of the cultivated (chloroplast capture); however, it keeps a high seed weight as in a cultivated type. Individual 6 illustrates a case of outcrossing between Andean and Mesoamerican genepools. It has a chloroplast haplotype as in the Mesoamerican genepool and an Andean phaseolin type. In contrast, its SSR alleles are 'wild' and 'cultivated'.

Our results provide an update about the distribution of wild common bean in Costa Rica, its ecology and conservation status. In addition, they allowed us to reliably establish the existence of simple or complex events of gene flow among different biological forms. Obviously, domestication has not yet altered the reproductive system of common bean up to prevent gene exchange between such forms. This in turn is also important for the production of certified seeds, or the management of genetic resources on-farm.

Contributors: R.I. González-Torres , D.G. Debouck, O. Toro, M.C. Duque.

Estimation of gene flow of complex "wild-weedy-cultivated" of common bean in its geographical range.

The complexes of forms "wild-weedy-cultivated" of common bean have been observed phenotypically in Oaxaca (México), El Progreso (Guatemala), San José (Costa Rica), Boyacá (Colombia), Azuay (Ecuador), Apurimac (Perú) and Tarija (Bolivia). The natural populations chosen from different countries and quantity of selected individuals are described in Table 7. The same methodology reported by González-Torres *et al.* (2003, 2004) was used to evaluate the participation of nuclear and chloroplast genomes in gene flow events on common bean in the range of geographical distribution.

Table 7. Identification of selected materials involved in the complex "wild-weedy-cultivated" from different countries.

	Province	Biological StatusBiological Status	Gnumber	No. of selected individuals
Bolivia	Tarija	Wild	G23445	71
		Weedy	G23445 G23446 G23447	408
		Cultivated	G23446 G23447 G23609 G24014 G24532	72
Argentina	Salta	Wild	S-512 S-513 S-514 S-515 S-515A S-516 S-517	7
		Weedy	G50015B G50015C	56
		Cultivated	G50015 G50015A G50015B G13995	105
Ecuador	Azuay	Wild	G23579 G23580	47
		Weedy	G23580	85

		Cultivated	G23580	50
Guatemala	El Progreso	Wild	G23434	26
		Weedy	G23434	77
		Cultivated	G23434 G3109	36
Perú	Apurimac	Wild	G21245 G23425	89
		Weedy	G23425	320
		Cultivated	G23425	90
Colombia	Choachi Salamina	Wild	G50879 G50983	90
		Weedy	G50879 G50983	148
		Cultivated	G50879 G50983 G51126	90

During 2005 2,849 seeds (334 seeds of 'wild' type; 1,377 seeds of 'landrace' type and 1,138 seeds of 'weedy' type) have been selected for planting in greenhouses, to obtain the phaseolin pattern and to realize the DNA extraction. In addition to this planting aprox. 150 seeds of possible interspecific hybrids were germinated. At the moment half of all materials have been processed for phaseolin and molecular markers.

Contributors R.I. González-Torres, D.G. Debouck , O. Toro, A. Hernández, C. Córdoba.

1.1.30 Estimation of gene flow of complex "wild-weedy-cultivated" of common bean in Bolivia

R.I. González-Torres, D.G. Debouck, M. Carvajal, O. Toro, M. C.Duque.

The evaluation of the complex of common bean from Bolivia using RFLP-PCR of cpDNA resulted in determining three different haplotypes as A, J, C for 233 individuals. The haplotypes A, J and C have been reported in wild and cultivated materials, respectively, from South America according to a methodology developed by Chacón (2001). This evaluation permitted a specific characterization of the each population depending on their biological status. The wild population shows only haplotype "A"; the cultivated materials exhibit hapotypes: "A", "J" and "C", and the weedy population displays shared haplotypes between wild and cultivated individuals (Table 7).

Table 7. Identification of selected materials involved in the complex "wild-weedy-cultivated" from different countries.

	Province	Biological StatusBiological Status	Gnumber	No. of selected individuals
Bolivia	Tarija	Wild	G23445	71
		Weedy	G23445 G23446 G23447	408
		Cultivated	G23446 G23447 G23609 G24014 G24532	72
Argentina	Salta	Wild	S-512 S-513 S-514 S-515 S-515A S-516 S-517	7
		Weedy	G50015B G50015C	56
		Cultivated	G50015 G50015A G50015B G13995	105
Ecuador	Azuay	Wild	G23579 G23580	47
		Weedy	G23580	85
		Cultivated	G23580	50
Guatemala	El Progreso	Wild	G23434	26
		Weedy	G23434	77
		Cultivated	G23434 G3109	36
Perú	Apurimac	Wild	G21245 G23425	89
		Weedy	G23425	320
		Cultivated	G23425	90
Colombia	Choachi Salamina	Wild	G50879 G50983	90
		Weedy	G50879 G50983	148
		Cultivated	G50879 G50983 G51126	90

References

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

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

1.2.1 Molecular mapping of genes for resistance to the bean pod weevil (*Apion godmani* Wagner) in common bean.

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[1] SB-2 and IP-1 Projects, CIAT [2] INIFAP-Sta. Lucia de Prias, Texcoco, Mexico

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) of the Jamapa x J117 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 desig*n 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 nine RAPD bands, B11400R, C1800S, F10500S, M12800S, R2012CS, U11400R, W6800R, W91300S and Z4800R that were polymorphic from the parental survey and which were significantly associated with the resistance phenotype were selected for cloning (Figure 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. All monomorphic SCARs were tested with frequently-cutting (4 bp recognition sites) restriction enzymes. 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 polymorphic with *Rsa*I or *Taq*I enzymes. The molecular markers were mapped to loci on chromosomes 2, 3, 4 and 6 (linkage groups b01, b08, b07and b11, respectively) based on genetic analysis of a the Jamapa x J-117 population and two reference mapping populations (DOR364 x G19833 and BAT93 x JaloEEP558) for which chromosome and linkage group designations are known. All the polymorphic SCAR and CAPS markers genetically mapped to the same locations as the original RAPD bands from which they were derived. In single point regression analysis, individual markers explained from 3.5 to 22.5% of the variance for the resistance trait with the most significant markers overall being F10-500S, U1-1400R, R20-1200S, W9-1300S and Z4-800S, all markers that mapped to chromosome 2 (b01). Two additional significant markers, B1-1400R and W6-800R, were mapped to chromosome 6 (b11) and explained from 4.3 to 10.2% of variance depending on the season. These two chromosomal regions are probably associated with the *Agr* and *Agm* genes although there may be additional resistance genes on chromosome 4 (b07) and chromosome 3 (b08). These are among the first specific markers developed for tagging insect resistance in common bean and are expected to be useful for evaluating the mechanism of resistance to *A. godmani*.

Future Plans

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 by conversion into polymorphic SNP markers so as to genetically map this gene in the bean genome. 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. QTL analysis will be carried out with the SCARs and a map for the Jamapa x J117 population when phenotypic data is available for a further set of RILs.

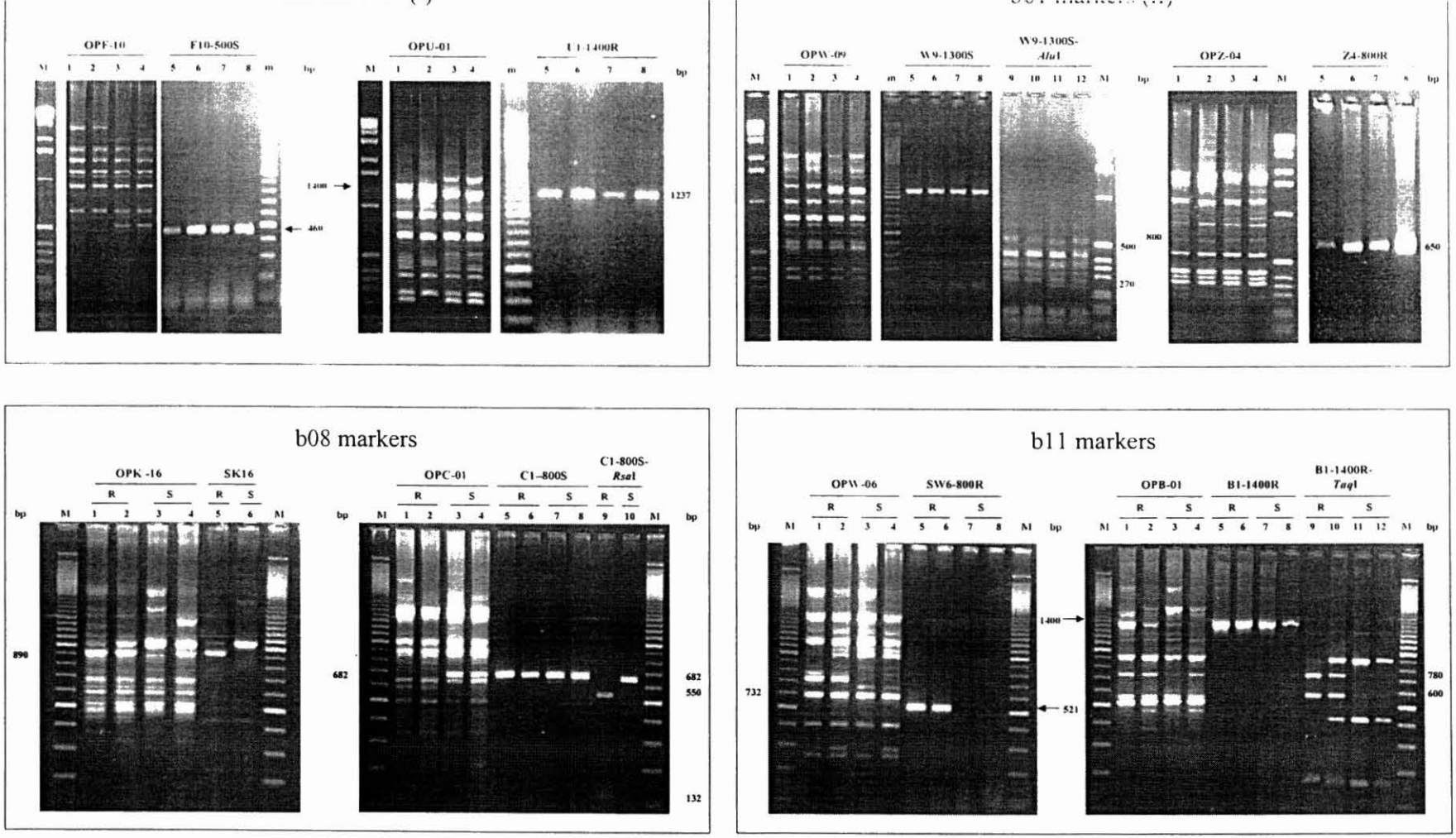

Figure 1. SCAR and CAPS markers developed from polymorphic RAPD bands found in bulked segregant analysis of *Apion godmani* resistant and susceptible parents and individuals of the Jamapa x J-117 population. SCAR markers are indicated by fragment size. CAPS markers are indicated by restriction enzyme used for cleavage. Molecular weights (in bp) are indicated for cloned band in the case of the RAPDs and expected PCR or cleavage products in the case of the SCAR and CAPS markers. Lanes 1, 5 and 9 = resistant parent (J-117); 2, 6 and 10 = resistant bulk; 3, 7 and 11 = susceptible parent (Jamapa); 4, 8 and 12 = susceptible bulk; M = molecular marker standard (1kb and 100 bp ladders).

1.2.2 Marker assisted selection for BCMV resistance in Colombian large red seeded climbing beans

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Introduction

Most of the local climbing bean varieties grown in Colombia have not been improved for BCMV resistance. We began a collaboration with Universidad Nacional - Bogotá to develop segregating populations that would contain either the dominant *I* gene and/or *bc3* resistance gene. The target seed classes has been the large red "rojo bolon" types produced in the departments of Cundinamarca and Boyacá and which are also important in Ecuador and East Africa.

Materials and Methods

Simple crosses were made to produce F1 hybrids for multiple and triple cross or back cross hybrids. A total of 15 triple crosses were made in Darien in the 2004B season (Table 1a). From separate backcrosses and double crosses crosses made in 2004A, gamete selections were advanced to the F1:3 generation by 2005A (Table 1b). Finally a set of simple crosses used to generate these double crosses was advanced to the F4 generation by 2005A (Table 1c). Among the climbing bean parents were large red seeded Colombian (LAS220) and Ecuadorian (Bolivar) varieties and landraces from Colombia (Agrario, Cargamanto and D. Moreno) and Peru (Caballero). The source of *bc3* gene resistance were a series of BCMNV resistant climbing beans described in last years annual report which have been named MBC series (MBC26, MBC 28, MBC33 and MBC34) as well as several BRC (BRC3, BRC12, BRC14, BRC31 and BRC32) and BRB (BRB153, BRB181, BRB189 and BRB211) lines. A few additional crosses contained the anthracnose resistance source G2333. The lines BRC12 and BRC14 are also expected to provide anthracnose resistance since they are derived from G2333. In parallel the parents Simijaca, Cabrerano and D. Moreno have been crossed withBRB181, BRB189, BRB211, BRC2, BRC12 and BRC14 at the Univ. Nacional to generate further populations.

Results and Discussion

The list of populations which were developed for the project and multiplied in Darien for shipment to Bogotá are shown in Table 1a, 1b and 1c. Marker evaluation was carried out on the MBC parents in preparation for the use of ROC11 marker assisted selection for the *bc3* gene and SW13 marker assisted selection for the *I* gene in subsequent generations (Figure 1). We found that some of the MBC lines also are predicted to have the *I* gene (MBC17, MBC15, MBC26, MBC28, MBC33, MBC34 and potentially MBC 38). The initial MBC lines (from 1 to 25) cannot be used for ROC11 selection due to the presence of the band that would be monomorphic in crosses with other Andean genotypes. However the latter MBC lines (from 26 onward) can be used for ROC11 selection. The explanation for this is that the initial MBC lines were derived from BRB191 which no longer had the ROC11 marker in repulsion with the bc3 resistance allele,

while the latter MBC lines were derived from BRB29 and BRB32 which do have the ROC11 marker in repulsion with the bc3 resistance allele and were selected for absence of the band.

Conclusions and Future Work

Additional populations have been planted at the Univ. Nacional Bogotá for evaluation and selection to prevalent diseases in the region of Cundinamarca/Boyacá. Screening will include virus inoculation to confirm the introgression of resistance genes.

Table 1. Populations generated with Colombian commercial dry bean genotypes in Darien.

Gamete selection:

MBC 26 X (G 2333 X AGRARIO)	(G2333 X AGRARIO)X MBC 28
(G2333 X AGRARIO)x MBC26	MBC 28X (G 2333 X AGRARIO)
MBC 26 X (G 2333 X CARGAMANTO)	MBC 28X (G 2333 X D.MORENO)
MBC 33 X (G 2333 X AGRARIO)	MBC 34X (G 2333 X AGRARIO)
(G2333 X AGRARIO) X MBC 33	(G 2333 X AGRARIO) X MBC 34
MBC 33X (G 2333 X CARGAMANTO)	MBC 34X (G 2333 X CARGAMANTO)
MBC 33X (G 2333 X D.MORENO)	MBC 34X (G 2333 X D.MORENO)
MBC 28X (G 2333 X AGRARIO)	

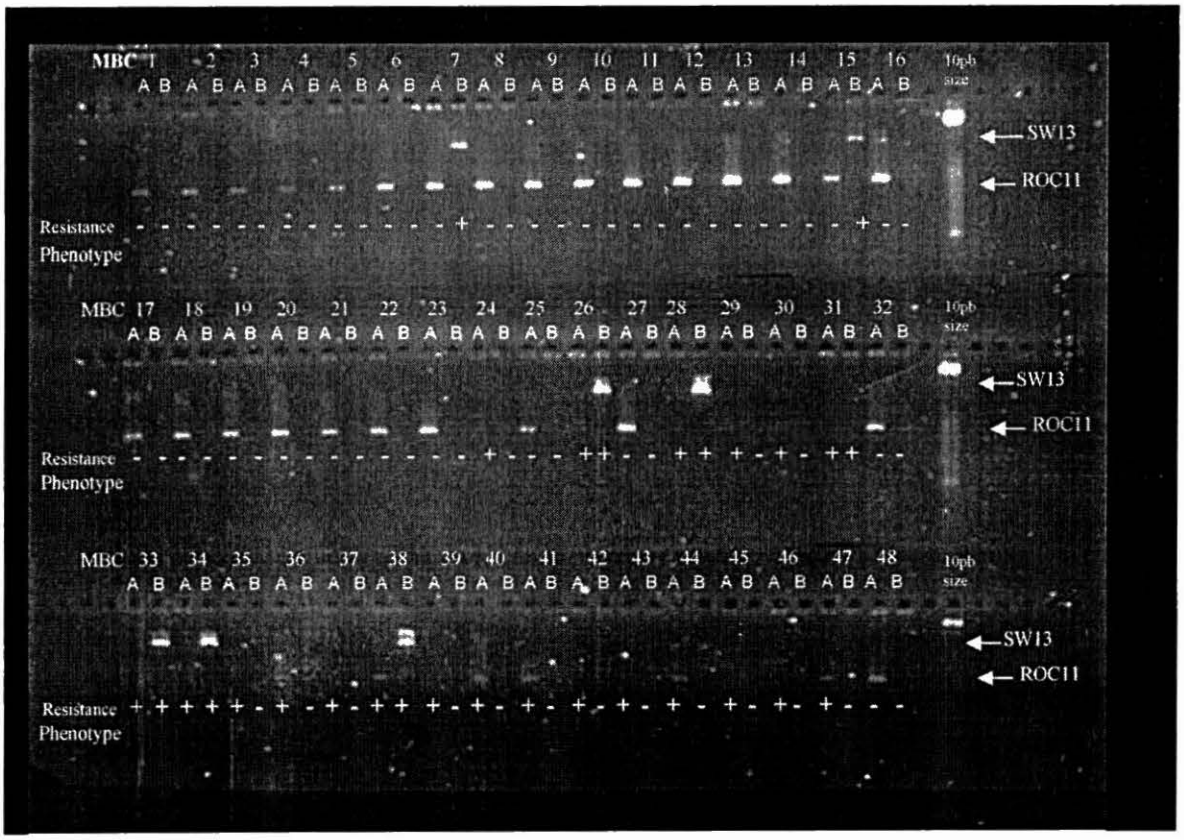
b) F3 populations

Cross code	Pedigree	No. gametes
. 22805- 1Z F2-(CM)Z	BOLIVAR X (BOLIVAR X BRB 153)	9
. 22806- 2Z F2-(CM)Z	BOLIVAR X (BOLIVAR X G 2333)	6
. 22807- 4Z F2-(CM)Z	BOLIVAR X (BOLIVAR X BRC 3)	3
. 22808- 2Z F2-(CM)Z	BOLIVAR X (BOLIVAR X BRC 12)	5
. 22809- 1Z F2-(CM)Z	BOLIVAR X (BOLIVAR X BRC 14)	1
. 22810- 1Z F2-(CM)Z	BOLIVAR X (BOLIVAR X BRC 31)	6
. 22811- 4Z F2-(CM)Z	BOLIVAR X (BRC 34 X BOLIVAR)	4
. 22815-10Z F2-(CM)Z	(LAS 220 X G 2333) X (LAS 220 X BRB 189)	2
. 22836- 1Z F2-(CM)Z	CAB 19 X (CABALLERO X BRB 197)	7
. 22837-10Z F2-(CM)Z	CAB 19 X (CABALLERO X PERRY MARROW)	1
. 22838- 3Z F2-(CM)Z	CAB 19 X (CABALLERO X G 2333)	1
. 22839- 1Z F2-(CM)Z	CAB 19 X (BRC 14 X CABALLERO)	10
. 22839- 9Z F2-(CM)Z	CAB 19 X (BRC 14 X CABALLERO)	2
. 22840- 1Z F2-(CM)Z	CAB 19 X (CABALLERO X BRC 30)	9
. 22841- 1Z F2-(CM)Z	CAB 19 X (CABALLERO X BRC 31)	3

c) F4 populations

Cross code	Pedigree
. 22600-(M)Z F2-CM(15)Z-(CM)Z	G 2333 x BRB 153
. 22539-(M)Z F2-CM(13)Z-(CM)Z	G 2333 x BRB 181
. 22540-(M)Z F2-CM(10)Z-(CM)Z	G 2333 x BRB 189
. 22541-(M)Z F2-CM(12)Z-(CM)Z	G 2333 x BRB 211
. 22543-(M)Z F2-CM(9)Z-(CM)Z	BRC 32 x G 2333
. 22546-(M)Z F2-CM(9)Z-(CM)Z	BOLIVAR x BRC 12
. 22547-(M)Z F2-CM(11)Z-(CM)Z	BOLIVAR x BRC 14
. 22548-(M)Z F2-CM(3)Z-(CM)Z	BOLIVAR x BRC 31
. 22550-(M)Z F2-CM(22)Z-(CM)Z	BOLIVAR x BRB 153
. 22551-(M)Z F2-CM(15)Z-(CM)Z	BOLIVAR x BRB 181
. 22552-(M)Z F2-CM(25)Z-(CM)Z	BOLIVAR x BRB 189
. 22553-(M)Z F2-CM(18)Z-(CM)Z	BOLIVAR x BRB 211
. 22554-(M)Z F2-CM(16)Z-(CM)Z	BRC 32 x BOLIVAR
. 22587-(M)Z F2-CM(23)Z-(CM)Z	LAS 220 x BRB 189

Figure 1. SCAR markers and predicted resistance gene phenotype (+/-) of MBC lines for A) SW13 (marker specific for the dominant I gene) and B) ROC11 (marker specific for the dominant susceptibility allele of the recessive resistance gene bc3).



1.2.3 Genetic mapping and use of single nucleotide polymorphisms (snps) for the characterization of common bean (*phaseolus vulgaris* l.) germplasm

C. Quintero, E. Gaitán-Solís, C. Quigley, P. Cregan and J. Tohme

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 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. The purpose of this project is to add to the CIAT's principal mapping population, *P. vulgaris* SNP markers developed at CIAT (Gaitán and Tohme, 2002) together with soybean SNPs (developed at BARC-USDA); study their potential for characterizing genetic diversity of wild *P. vulgaris* core collection, and to explore the association either of single SNPs or haplotypes blocks in cultivated varieties with well known response to some of the most important biotic production problems of the crop, such as bean golden mosaic virus (BGYMV), anthracnose (ANT), common bacterial blight (CBB) and angular leaf spot (ALS).

Materials and Methods

A population of F_9 plants from 87 recombinant inbred lines (RILs) developed at CIAT from the cross between G19833 (Andean genetic pool) and DOR364 (Mesoamerican genetic pool) was used to a linkage map of common bean (Beebe *et.al.*, 1998). SNP markers were added to this linkage map using MAPMAKER/EXP (version 3.0) (Lander *et.al.*, 1987). RFLP markers shared with the core mapping population (Freyre *et.al.*, 1998) were used as anchors in each of the 11 linkage groups defined for *P. vulgaris*. The "group" command, with a LOD threshold of 4.0, and a recombination fraction of 0.3, were used to assign SNP markers to the existing linkage groups, and the "order" command was used to find the most likely interval in which to place the new marker on the linkage groups. Single marker analysis for QTL detection was carried out using WinQTLCartographer (version 2.5) software (Wang et. al., 2005). Interval mapping and composite interval mapping analysis are in process.

Bean genotypes that are being used in this study include most of the wild *P. vulgaris* core collection selected by Tohme *et. al.* (1996), some genotypes evaluated by Chacón (2001) and 10 weedy *P. vulgaris* accessions from Colombian origin not evaluated before with molecular markers. A total of 108 wild and weedy accessions are included in this study representing the geographic distribution of the species.

A set of 165 Andean and Mesoamerican varieties belonging to the known bean market classes was 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 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 *et.al.* (2004).

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

Microsphere fluorescence was measured using a Luminex-100 flow cytometer equipped with a Luminex XY Platform plate reader. The fluorescence on the surface of the microspheres was measured and converted to a mean fluorescence intensity (MFI) value, based on a minimum of 100 microspheres of each type, in a 50μl sample size. Multi-SBE was achieved and a maximum number of nine alleles were extended in the same reaction.

Allele calling was done using Masterplex GT software (MiraiBio). The background MFI values were low (less than 60), regardless of the SNP. Although differences in signal intensity among the SNPs were evident, all of the SNPs were genotyped correctly according to sequence data for eight *P.vulgaris* genotypes used as checks in all the experiments. The MFI values for 9 SNPs in G19833 and DOR364 are shown in Figure 1.

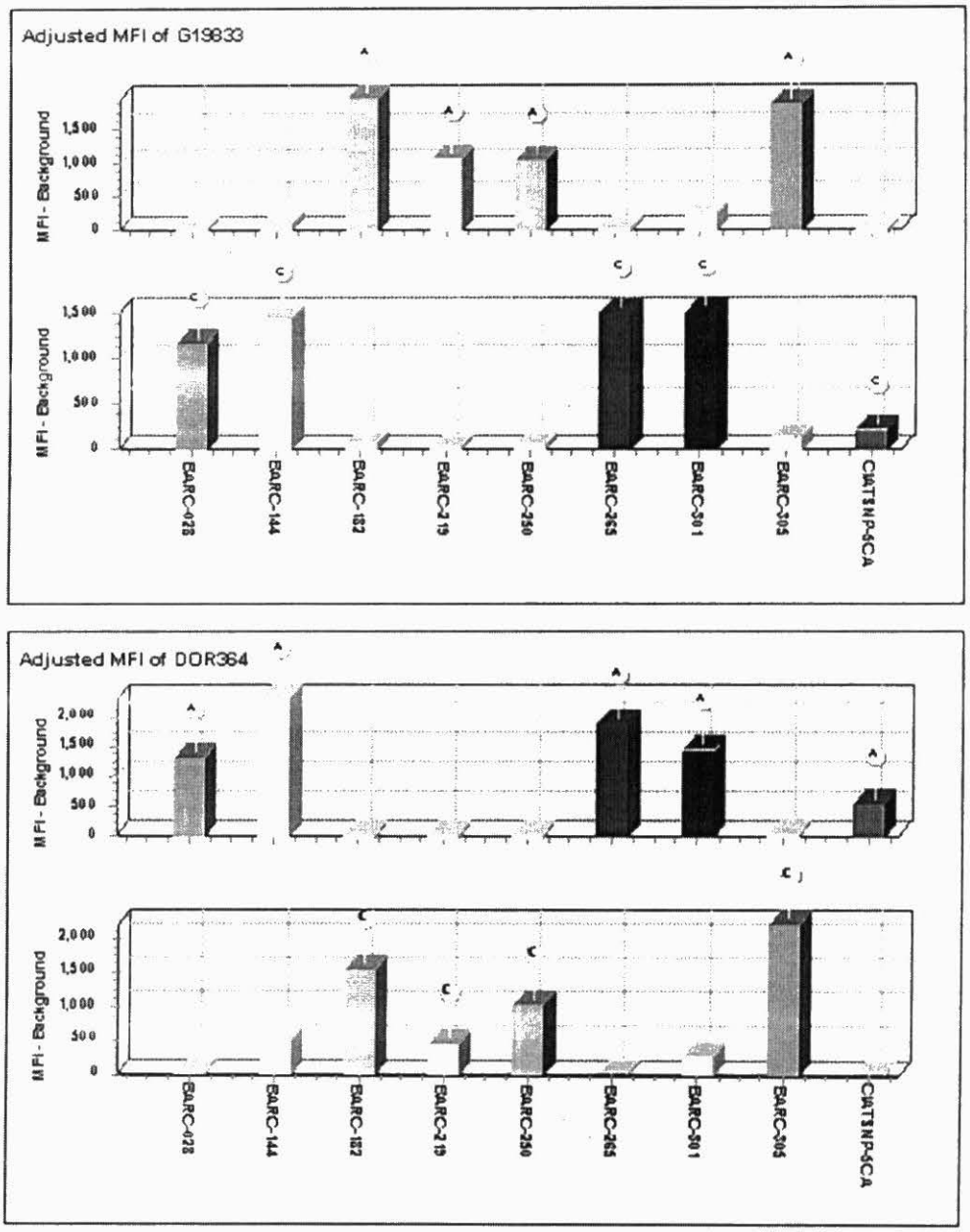


Figure 1. SNP genotyping of mapping population parents, G19833 and DOR364, using SBE method for SNP detection. Allele calling based on mean fluorescence intensity measurements for nine SNPs analyzed with Masterplex GT.

44 soybean SNPs designed by Quigley (P. Cregan's Lab., BARC-USDA) and 19 *P. vulgaris* SNPs (Gaitán-Solís, *et.al.*, 2004) were screened in 87 RILs of CIAT's principal mapping population (DOR364 x G19833).

The genetic map was constructed and all the markers were assigned to linkage groups at a minimum LOD score of 4.0. A total of 58 SNP markers (39 from soybean and 19 from *P. vulgaris*) were added to the linkage map previously developed at CIAT (Beebe *et.al.*, 1998). Five soybean SNPs remained unlinked. The resulting map has 396 markers including AFLPs, RAPDs, SCARs and RFLPs described in Beebe *et.al.* (1998); RGA sequences described in López *et.al.* (2003) and 58 SNPs placed in this study. The total cumulative length was 2000cM (Kosambi units) and the average chromosome length was 181.7cM.

Each of the eleven *P. vulgaris* linkage groups had at least two SNP markers attached. The average number of SNPs per linkage group was five with B03 and B07 having the highest number of SNPs placed on them (nine) while B05 had only two. The distribution of the SNPs along linkage groups was variable and the average distance between them in this map was 13.1cM.

The single marker analysis showed QTLs for ALS in chromosome B10, for ANT in chromosome B03 and B11, and presumably for BGYMV in chromosome B03. Some SNPs markers seem to be in or surrounding these QTL regions, but more accurate analysis such as interval mapping is being done to finally declare QTLs and associated SNPs.

The information about the order of SNP markers along the genome is essential for haplotype prediction, block partition (Halperin and Eskin, 2004) and for association of SNP haplotypes and phenotypic traits (Zaykin *et.al.*, 2002). The number of SNPs mapped in this study is still low to pursue these approaches. Then, the validation of a second batch of 84 soybean SNPs also developed at BARC-USDA, in 10 Andean and Mesoamerican bean genotypes has been initiated. Hopefully after placing these markers in the linkage map, haplotype-phenotype association would be accomplished and representative SNPs determined.

Although more SNP analysis needs to be done, genome-wide scan of *P. vulgaris* germplasm has been initiated. All the 108 wild and weedy accessions and the 165 cultivated varieties have been genotyped with 21 *P. vulgaris* SNPs discovered by Gaitán and Tohme (2002). These 21 SNPs clearly separated the accessions in the two major *P. vulgaris* genepools, Andean and Mesoamerican.

All *P.vulgaris* SNPs were polymorphic in wild germplasm. In cultivated varieties, only CIATSNP-13TC was found to be monomorphic. Codominant natures of SNPs allow to distinguish between homozygotes and hetezygotes individuals for almost all loci. No heterozygote individuals were identified in cultivated varieties with CIATSNP-22TA, and in wild germplasm with CIATSNP-30CT and CIATSNP-13TC.

The PIC value for each of the 21 SNPs was calculated using the following formula:

$$PIC = 1 - \Sigma p_n^2$$

where p_n is the frequency of allele n. The SNPs reported here are all biallelic, thus a PIC value of 0.50 is the maximum achievable.

The range of values of PIC founded in cultivated varieties was between 0.04 (CIATSNP-7GC) to 0.36 (CIATSNP-17TC), with 8/21 (38%) of the primer pairs having PIC values >0.30. In wild germplasm, PIC values ranged from 0.11 (CIATSNP-17TC) to 0.37 (CIATSNP-22TA) with 9/21 (43%) of the SNPs having PIC values >0.30 (Table 1). These data are comparable to those reported in wheat by Sommers *et.al.* (2003) who suggest that SNPs can be very useful in genetic diversity studies, given their high abundance, their amenability to high throughput detection platforms, and because of the reasonable PIC values observed in a large proportion of the SNPs assayed.

Table 1. Polymorphism information content and chromosome location for 21 SNP loci in *P. vulgaris* germplasm.

	PIC value in germplasm type		
MARKER	Cultivated	Wild/Weedy	Chromosome
CIATSNP-11GT	0.30	0.29	B11
CIATSNP-13TC	0.00	0.11	-
CIATSNP-15GT	0.28	0.37	B01
CIATSNP-16AC	0.35	0.30	B09
CIATSNP-17TC	0.36	0.22	B07
CIATSNP-19CA	0.28	0.33	B04
CIATSNP-20GT	0.31	0.34	B10
CIATSNP-21CT	0.28	0.30	B03
CIATSNP-22TA	0.06	0.37	-
CIATSNP-23TC	0.35	0.36	B09
CIATSNP-24CT	0.19	0.13	B07
CIATSNP-26TC	0.35	0.33	B06
CIATSNP-27GA	0.28	0.33	B11
CIATSNP-28AG	0.35	0.30	B03
CIATSNP-29AT	0.29	0.33	B03
CIATSNP-29GA	0.31	0.22	B03
CIATSNP-30CT	0.25	0.25	B05
CIATSNP-4GC	0.18	0.17	B05
CIATSNP-5CA	0.34	0.23	B07
CIATSNP-7GC	0.04	0.27	-
CIATSNP-8GC	0.23	0.34	B06

Once mapping analysis is completed and representative SNPs selected, genotyping will continue in order to achieve haplotype-trait association. We are exploring a regression approach developed by Zaykin, et.al. (2002) to pursues this objective since, it has been proposed that 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 genetic mapping of 124 SNP markers in *P.vulgaris* DOR364 x G19833 population.
- Continue the characterization of genetic diversity in *P. vulgaris* with single nucleotide polymorphisms.
- Study the association of SNP markers with ALS, ANT, CBB, BGYMV.

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1.2.4 Molecular marker-assisted selection (MAS) for breeding resistance to the cassava mosaic disease (CMD) in Latin American cassava gene pools

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Funding: Rockefeller Foundation, CIAT

Important outputs

Field evaluation of CMD resistant selections developed by MAS at CIAT and confirmation of the value of MAS for breeding CMD resistance at CIAT
New crosses between CMD donor parents and CIAT elite parental lines based upon the performance of previous crosses in the field in Africa and MAS for CMD resistance

Introduction

Molecular marker-assisted selection (MAS) for CMD resistance at CIAT is a pre-emptive measure should in case the disease is accidentally introduced in Latin America, and a dynamic measure to enable a true evaluation of the value of CIAT improved germplasm in India and Africa. MAS was been utilized in 2003 and 2004 to transfer CMD resistance, conferred by a dominant gene, CMD2, to elite parental lines at CIAT. The CMD resistant germplasm has been shared with NARs partners in Tanzania, Uganda, Nigeria, South Africa, Ethiopia, Kenya, and India. We describe here results of field evaluation of the CIAT lines bred for resistance to CMD in Tanzania and Nigeria. We also present in this report the processing of a new batch of crosses

made between CMD resistance donor parents and CIAT elite parental lines based upon the performance of previous crosses in the field in Africa

Methodology

A total of 508 genotypes derived from the AR, BC_2 derivatives from *M. esculenta* sub spp *flabellifolia* having resistance to CMD and CGM, and CR, cassava only hybrids with resistance to CMD, families, were shipped to Tanzania and Nigeria between November 2004 and March 2005, an average of 8 plantlets per genotype were shipped. These genotypes were evaluated for CMD and CGM symptom severity in the field at 6 and 9 months after planting according a standard symptom severity scale of 1 (no disease or pest symptoms) to 5 (very severe stunting due to CMD or CGM). Molecular diagnostic for presence of the virus via PCR was also performed on genotypes that remained free from the disease at harvest to reduce the possibility of 'escapes'. Plant material was harvested from each plant of the 151 genotypes that had no visual symptom and from a single infected genotype as positive PCR amplification control. About 0.2mg of leaf tissue was crushed with 700ul of extraction buffer (Dellaporta et al. 1983) and DNA extraction carried out as described by Ndunguru et al. (2005) and re-suspended in 200ul of sterile water. PCR, using EACMV, the predominant strain of the virus in the Eastern coast of Tanzania, or ACMV was carried out using 2ul of the DNA extraction as described by Ndunguru et al. (2005) and visualized on ethidium bromide stained 1.5% agarose gels. The CMD resistant lines introduced to Tanzania and Nigeria in the 2003/2004 planting season were harvested at 10 months after planting. Based upon the performance of the CMD resistant introductions in Africa, new crosses were made between parents showing good specific or general combining ability as demonstrated by progenies that showed superior agronomic performance and the CMD resistance donor parents. Particular emphasis was placed on crosses between CM523-7 and C4, parents of the CR14 family, progenies from this cross yielded progenies with excellent agronomic performance. Over 4000 sexual seeds were obtained from new the crosses made, the seeds were tested for viability and then established *in vitro* from embryo axes according to standard protocols at CIAT (CIAT 2003). DNA was isolated from leaf tissue harvested from one-month old tissue culture plants and analyzed with 2 markers, NS158 and RME1 according to standard methods (CIAT 2003).

Results

Evaluations of 503 genotypes at 6 and 9 months after planting revealed 224 genotypes with no visible foliar symptoms for CMD and176 genotypes that did not show any visible foliar symptoms for CMD and CGM (Table 1). The large number of susceptible varieties in the CR and AR genotypes that had been selected with markers for resistance to CMD conferred by the *CMD2* gene was unexpected. Further analysis of the results by family revealed that a number of large families had over 90% of genotypes susceptible, these families had the parent C127 in common (Fig 1). These results suggest that the C127 parent sent to CIAT from IITA along with 17 other F_1 progeny of TME3, the source of *CMD2*, as donor parents for CMD resistance is a susceptible genotype. When families having this parent were removed from the analysis, the percentage of resistant genotypes was 70%, which is the expected percentage, given that *CMD2* controls 70% of CMD resistance. Molecular diagnostics of CMD infection in genotypes with no visible symptoms revealed amplification in only the control and a single genotype, suggesting a low percentage of 'escapes' in the field evaluation. A summary of evaluations of agronomic traits of the CIAT introductions at harvest (10 months after planting) are shown in table 2. Performance of some the introductions in Nigeria are shown in Table 3. At harvest all roots from the genotypes harvested were visually inspected for root symptoms of cassava frog skin disease (CFSD), and no symptoms were found confirming earlier results of CFSD diagnostics that showed the introductions were free from CFSD.

Table 1. CMD and CGM evaluations conducted at 6 months after planting in the CIAT introductions at the Alavi estate close to Dar es Salaam, Tanzania

		Nr. Planted	No CMD & CGM		No CMD but with CGM		With CMD but no CGM		With both CMD & CGM	
			Nr.	%	Nr.	%	Nr.	%	Nr.	%
AR Series	Genotypes	325	127	39.1	1	0.3	191	58.8	6	1.8
	Plants	2,282	837	36.7	9	0.4	1,388	60.8	48	2.1
CR Series	Genotypes	178	49	27.4	66	36.9	33	18.4	31	17.3
	Plants	750	210	28.0	231	30.8	152	20.3	157	20.9
Both Series	Genotypes	503	176	34.9	67	13.3	224	44.4	37	7.3
	Plants	3,032	1,047	34.5	240	7.9	1,540	50.8	205	6.8

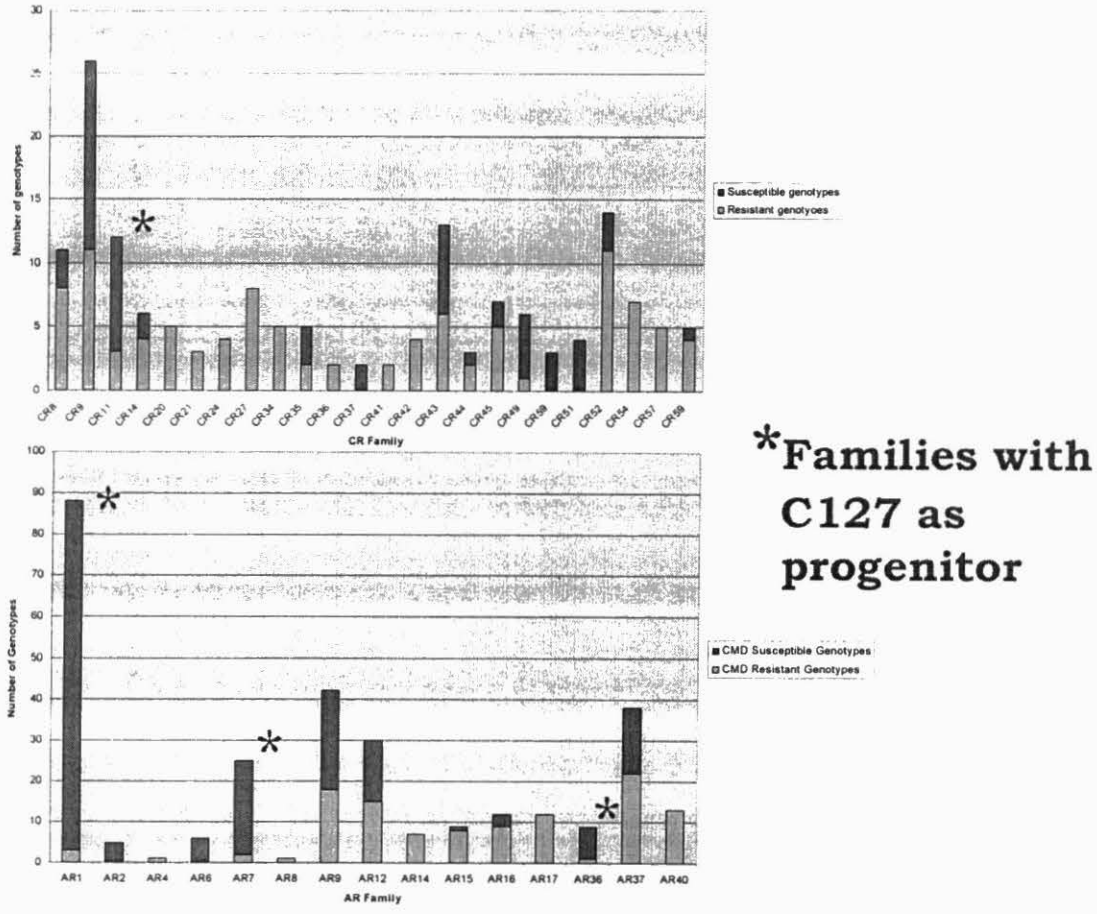

Figure 1. Response to CMD by family in the CR and AR genotypes of the CIAT introductions

Table 2. Simple statistics of agronomic traits evaluated in the CIAT introductions harvested at 10 months after planting in the Alavi estate in Tanzania.

	Plants/clone in field	No. roots/plants	Fr root wt/plant (kg)	Foil. Wt/plant (kg)	Biomass/plant (kg)	Harvest Index
Maximum	13.00	20.50	3.20	1.60	8.80	0.67
Minimum	1.00	1.50	0.39	0.18	1.18	0.19
Average	6.04	4.21	0.93	0.50	2.72	0.34
Std Dev.	2.34	2.23	0.49	0.28	1.14	0.10

Table 3. Performance of some CMD resistant lines from CIAT in Nigeria in the 2004/2005 planting season

Genotype	No. of Roots per plant	Root Weight per plant (Kg)	Tons/ha	CBB symptom score	CMD symptom score	No. of Stakes available for Clonal trial
CR 14A-1	20	15	150	1	1	300
AR 38-3	10	12	120	2	1	300
CR 41-10	18	10	100	3	1	100
CR 36-2	15	8.5	85	2	1	100
CR 52A-41	14	7	70	4	2	100
AR 15-5	10	6	60	2	1	60
CR 26-1	10	5	50	2	1	56
CR 52A-25	8	4	40	3	1	140
CR 52A-22	12	3.5	35	4	2	17
CR 42-4	7	3	30	2	1	150
AR 37-108	6	3	30	2	1	200

Based upon the performance of the CIAT introductions, new crosses were made early in 2005 between parents with good specific or general combining ability. A total of 4,335 seeds were obtained. A sub-set of 1759 seeds were tested for viability and 1,141 planted in vitro using embryo axes (Table 4). Of this number 511 or 45% formed plants, the low percentage recovery of plantlets this time around was traced to the unusually large volume of seeds, >15,000, from multiple experiments that had to be processed this year by the cassava tissue culture facility which may have led to a trade-off in efficiency of embryo axes culture. Selection for CMD resistance by MAS led to the identification of 225 resistant genotypes, they are being multiplied for shipment to Brazil, Uganda, Nigeria, and Ghana for use as parents in a MAS breeding program under the auspices of the Generation challenge program (GCP) project 'Development of low cost markers for the pyramiding of useful genes from wild relatives into elite progenitors'.

Table 4. Summary of embryo rescue of new crosses generated for MAS for CMD resistance in 2005.

CODE	Female Parent	Male Parent	No. of Seeds received	Non-viable seeds.	No. Seeds planted	No. Plants of plantlets obtained	% Recovery of Plantlets
CR 9TH	C-4	TAI 8	218	18	200	103	52%
CR 9B	TAI 8	C-4	110	65	45	0	0%
CR 14B	C-4	CM 523-7	604	202	402	234	58%
CR 15B	C-33	CM 523-7	18	0	18	9	50%
CR 24	CM 7951-5	C-18	7	1	6	6	100%
CR 39	C-4	SM 1219-9	238	31	207	89	43%
CR 54B	TAI 8	C-243	182	134	48	16	33%
CR 60B	C-18	TAI 8	83	45	38	15	39%
CR 61ST	TAI 8	C-33	113	101	12	4	33%
CR 97	OW 181-2	C-18	65	6	59	8	14%
CR 99	OW 181-2	C-33	12	3	9	2	22%
CR 100	OW 181-2	C-377	63	3	60	23	38%
CR 101	OW 181-2	C-413	4	3	1	0	0%
CR 103	OW 230-6	C-19	19	4	15	2	13%
CR 104	OW 230-6	TAI 8	23	2	21	0	0%
TOTAL			1759	618	1141	511	45%

Conclusions and perspectives

Selections made using markers associated with CMD resistance at CIAT were tested in the field to confirm the utility of MAS for breeding CMD resistance at CIAT. Results of field evaluation confirmed the efficacy of MAS but also revealed that one of the CMD resistance donor parents from IITA being used to breed resistance is a susceptible parent. Based upon preliminary agronomic performance of the CIAT introductions additional crosses were made from parental cross combinations having progenies with good agronomic performance in the field. The new crosses were established from embryo axes and evaluated with markers to select CMD resistant progeny these are being multiplied for shipment to NARs partners. Perspectives of the MAS work is to evaluate the CIAT CMD resistant lines in all 7 countries they have been sent to date.

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1.2.5 Molecular marker-assisted selection (MAS) for the improvement of local cassava germplasm in Tanzania for pest and disease resistance

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Funding: Rockefeller Foundation, CIAT

Important outputs:

Selection of CIAT introductions and local varieties and setting up of a controlled and open pollinated crossing block to generate breeding populations for improvement of local cassava varieties
Generation of over 20,000 crosses between CIAT introductions and local varieties

Introduction

Centralized breeding cassava programs in International Agricultural Research Centers (IARCs) and National Programs is a multi-stage evaluation scheme lasting 8-10 years with farmers being introduced at the very end of the scheme. The low adoption of improved cassava genotypes coming from centralized breeding programs in many African countries have led to the proposal of a decentralized breeding scheme involving molecular marker-assisted selection (MAS), to quickly reduce the size of breeding populations derived from crossing local varieties to improved introductions, and PPB, to introduce farmer early in the breeding cycle. A project to test this idea was initiated in October 2003 with support from the Rockefeller foundation and has completed its second year. Breeding populations for MAS and PPB have been developed and will be planted in the third year followed by MAS and a single evaluation on-station before the introduction of farmer evaluation. The project is expected to produce improved cassava varieties acceptable to farmers within the next 4 years but more importantly it is expected to provide a proof of concept for the MAS-PPB paradigm in cassava breeding capable of increasing the productivity and stability of farmer varieties.

Methodology

Tissue culture plantlets from 508 genotypes selected with markers for CMD resistance at CIAT (CIAT 2003) and having resistance to CGM and local Tanzanian varieties collected from the three major cassava growing region of the country, namely the Eastern, Southern, and Lake region, were evaluated for resistance to CMD and CGM, and agronomic traits in the field at Alavi estate in February 2005 at 10 months after planting. The principal selection criteria in the introductions were resistant to CMD and CGM, little emphasis was therefore put on fresh root yield, number of roots and other traits that vary between tissue culture and clonally produced plants. Rather harvest index, a highly heritable trait, and a low selection intensity, about 53% of all genotypes were selected, was employed to select genotypes for the crossing block. The introductions were also evaluated for resistance to the cassava brown streak disease (CBSD) in the expectation that a novel source of resistance to the disease could be found. For the local varieties the principal selection criteria was resistance to CBSD, a major production problem in

the Eastern and Southern zones, and on farmer preferred characteristics, farmers were invited to participate in the culinary and agronomic evaluations of the local varieties.

Results

A total of 27 and 24 varieties were selected from varieties from the Southern and Eastern zones respectively (Table 1). From the CIAT introductions a total of 80 genotypes were selected (Table1). The cassava crossing blocks were established at Chambezi experimental station situated about 60km North-west of Dar es Salaam. Three crossing blocks were established consisting of one controlled crossing block and two polycross b blocks, a latin square design that maximizes the possibility of pollination between the local varieties and introduction under conditions of natural open pollination. The controlled crossing block was planted on 22.02.05 and the two polycross blocks on 23.02.2005. The controlled crossing block consists of 46 local cassava genotypes and 60 improved CIAT genotypes, the fifth block was planted with 25 genotypes as a back up. All genotypes were planted at 1.8m x 2.0m spacing in 10-plant rows and a total of five blocks with each block having 30 genotypes. To increase chances of getting enough flowers, some of the commonly grown cassava genotypes for example Namikonga, Kalolo, Kitumbua, and Nanchinyaya, Kiroba Naliendele Kibaha were respectively replicated four or three times. The two polycross blocks, one for the Eastern zone and one for the Southern contained 23 and 19 local cassava genotypes respectively along with 20 CIAT genotypes each. Chemical fertilizer NPK 20:10:10 was applied in split application, at one month after planting (MAP) and 2MAP respectively. To encourage flowering foliar fertilizer (COLJAP FLORESCENCIA) consisting of 10%N: 28%P:19% was applied on 12.7.05, 27.7.05, 31.8.05, and every two weeks after that. The crossing block was evaluated for CMD and CBSD resistance at 3 and 6 months after planting. Flowering started on 13.7.05 at 5 months after planting in the following cultivars: Kalolo, Bwana Mrefu, Muzege, AR42-4, AR32-1, AR9-14, AR12-14 AND AR30-3. Pollinations started on 26.7.05 on the above mentioned cultivars with an emphasis on crosses between CBSD resistant locals and the CIAT introductions. This emphasis is based on combining resistance to CMD and CGM in the CIAT introductions with CBSD resistance in the local varieties to produce genotypes with good resistance to the principal abiotic constraints in the Eastern and Southern regions. To date over 20,000 crosses have been made and at least 40,000 sexual seeds, assuming an average of 2 sexual seeds per cross, are expected. Pollination is still on going to achieve a target of 60,000 sexual seeds in total.

Results of CBSD evaluations of the CIAT introductions revealed that 9 genotypes had a symptom score of 3 (Fig 1) on a scale of 1 (no necrosis) to 5 (100% root necrosis), 72 genotypes a score of 2, and 32 genotypes a score of 1. Twenty three other genotypes had a score of 1 but results were not conclusive due to the insufficient number of roots for these genotypes. A total of 60% of all genotypes showed some disease symptoms (score of 2 or 3).

Table 1. List of local cassava varieties and CIAT introductions for genetic crosses

Eastern Zone	Southern Zone	CIAT	CIAT	CIAT
1. Kitingisha 2	1. Kabinda	1. AR 21-2	28. AR 14-14	55. AR 16-16
2. Cheusi mwangia 1	2. Albert	2. CR 45-3	29. AR 16-1	56. AR 37-73
3. Kibangameno	3. Limbanga	3. CR 27-9	30. AR 38-6	57. AR 17-8
4. Kigoma mtoto	4. Sheria 1	4. CR44-6	31. CR 44-8	58. CR 21-10
5. Pamba	5. Mzungu	5. CR 45-10	32. CR20A-1	Back up genotypes
6. Guzo	6. Kalolo (Mtwara)	6. AR 37-6	33. CR 45-9	1. AR 40-10
7. Kibwegere	7. Lipukalyene	7. CR 35A-9	34. CR 54B-44	2. AR 40-12
8. Muarusha	8. Kigoma red	8. AR 30-4	35. AR 35-1	3. AR 30-3
9. Mshelisheli	9. Saranga	9. CR 27-24	36. AR 16-3	4. CR 52A-19
10. Kalolo	10. Kiroba	10. CR 11A-20	37. AR 15-3	5. AR 17-5
11. Dide	11. Nanchinyaya	11. CR 45-1	38. AR 17-27	6. AR 16-5
12. Cheusi	12. Kalinda	12. CR 20B-2	39. AR 32-3	7. AR 38-3
13. Moshi wa taa	13. Mreteta	13. CR 43-13	40. AR 37-81	8. AR 43-12
14. Mahiza	14. Chimaji1	14. AR 17-18	41. AR 15-9	9. AR 17-23
15. Bwana mrefu	15. Namikonga	15. CR 11A-25	42. AR 9-44	10. AR 14-2
16. Kiroba	16. Kitumbua	16. AR 37-96	43. AR 40-11	11. AR 37-54
17. Mfaransa	17. Mreteta	17. AR 17-3	44. AR 12-14	12. AR 14-5
18. Mkiwa	18. Kalombe	18. AR 17-25	45. CR 52A-41	13. AR 37-38
19. Kibaha	19. NDL 90/34	19. AR 17-16	46. AR 42-3	14. AR 37-92
20. Kaniki	20. Toa pesa	20. AR 37-89	47. CR 20A-6	15. AR 9-54
21. Kasumoni	Back up	21. AR 11-13	48. AR 15-10	16. AR 14-1
22. Kikombe	21. Tukuyu	22. CR 25-4	49. AR 9-18	17. AR 9-6
23. Bora kupata	22. Usilie chumbani	23. CR 52A-40	50. AR 12-11	18. AR 9-48
24. Muzege	23. Kichoko	24. AR 16-8	51. AR 41-2	19. AR 9-2
25. Jaribu tena	24. Bilali	25. AR 42-4	52. CR43-AR 9-22	20. AR 37-1
26. Kilokote		26. AR 9-25	53. AR 9-14	21. AR 30-5
27. Kifumulo		27. AR 11-12	54. AR 32-1	22. CR 8A-5
				23. AR 37-48
				24. AR 40-3
				25. AR 37-99

The high frequency of CBSD in these materials made us shelve the intended transfer to Maruku for genetic crosses with materials from the Eastern Zone, CBSD is not known in the Lake zone and extra care needs to be taken that it is not introduced, crosses with genotypes from the lake zone will be done via transfer of pollen from Chambezi to Maruku. It must be mentioned that the high incidence of CBSD in these genotypes is expected as the disease is not known in South America and therefore there has not been any breeding for resistance to CBSD. But the genotypes (40%) that failed to show symptoms need to be re-evaluated again, as these genotypes have introgression (for resistance to CGM) from a wild *Manihot* relative of cassava. The high incidence of CBSD in the CIAT materials also led to the decision to make crosses only to local varieties having resistance to CBSD.

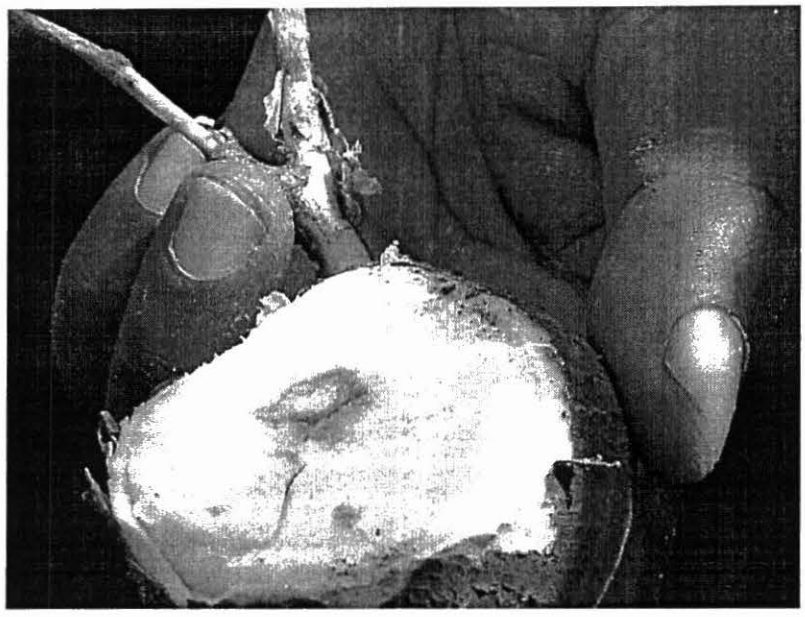

Figure 1. Root necrosis class 3 symptom of the cassava brown streak disease (CBSD) (CBSD) in a root of the CIAT introduction CR27-9

Conclusion and perspectives

Selections of CIAT introductions and local varieties, and the setting up of a crossing block have been successfully achieved in Tanzania. Over 20,000 crosses have been made and 40,000 seeds are expected. Perspectives include planting of the seeds and transfer of the seedling nursery to the field early next year. At 6 months after planting molecular markers will be used to identify CMD resistant plants in the breeding populations, this would be the only selection criteria. A much reduced clonal evaluation will be planted and evaluated on station for traits of agronomic interest after which subsequent evaluations will be performed in parallel on station and by farmers.

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1.2.6 Development of molecular marker-assisted selection (MAS) programs for breeding pest and disease resistance in NARS of Brazil, Nigeria, Ghana, and Uganda

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Funding: The Generation Challenge Program (GCP)

Important Outputs

Deployment of CMD, CGM, Whiteflies, hornworm resistant materials, and delayed PPD materials to participating NARs for marker-assisted improvement of the above traits
Establishment of simple molecular marker laboratories at NRCRI, Nigeria, and CRI, Ghana, for conducting MAS in the NARs

Introduction

The main objectives of the Generation Challenge Program (GCP) project 'Development of Low-cost Marker Technologies for Pyramiding Useful Genes from Wild Relatives of Cassava into Elite Progenitors' is to develop new varieties with delayed post-harvest physiological deterioration (PPD), resistance to cassava mosaic disease (CMD) (resistance to the hornworm and white flies for Brazil), and cassava green mite (CGM) through marker-assisted introgression of exotic genes from wild relatives from the center of origin and diversity into elite cassava progenitors. Cassava progenies with excellent resistance to CMD and CGM developed by MAS and the molecular markers associated with genes for the above traits will be shipped to NARs in Uganda, Ghana and Nigeria in the first year of the project for a MAS project similar to that ongoing in Tanzania. Back cross derivatives with delayed PPD and resistance to whiteflies and hornworm will also be shipped. In the second year these lines will be crossed to widely grown local varieties collected from all over the country. Also in the second year NARs partners should have simple marker laboratories, supplies, training and technical backstopping for the implementation of MAS. In the third year, populations generated will be selected using low cost markers and a small set of progeny sent for further evaluations in the regular breeding scheme with farmers. NARs partners include the National Root Crop Research Institute (NRCRI), Umudike, Nigeria, Crop Research Institute (CRI), Kumasi, Ghana, the National Animal and Agricultural Research Institute (NAARI), Namulonge, Uganda, and EMBRAPA Fruits and Cassava center (EMBRAPA-CNPMF), Cruz das Alma.

Shipment of Germplasm with useful traits

CIAT has started using the Advanced Backcross QTL scheme to map and transfer genes progenitors from wild relatives into cassava. Initial traits include resistance to CGM but has been expanded to delayed PPD, and resistance to hornworm, CGM, and white fly. CIAT has developed BC_1 and BC_2 and S_1 populations with the above mentioned traits and they are being multiplied *in vitro* at CIAT for distribution to participating NARS. The first shipment of 500 *in vitro* plants representing 100 genotypes was sent to Nigeria in May 2005. The *in vitro* materials include CMD resistant (CR series) and mite resistant (AR series) plants. The materials have undergone the hardening process in the screen house, over 80% of the introduced materials successfully went through the hardening process. Plants in the screen house were inspected by officials of the Nigerian Plant Quarantine Services (PQS) in the first week of August and approval given for their transfer to the field. The 440 individuals successfully hardened have been transplanted to the field for evaluation on monthly basis for their disease and pest resistance. Initial results obtained indicate that over 95% of the transplanted plants are showing resistance after two months in the field. Farmer-preferred varieties (twenty-three in number) have been planted and are also been evaluated for pests and disease on monthly basis. After harvest, selected introductions will be used for crosses with farmer-preferred local varieties.

Additional *in vitro* materials having resistance to CMD, CGM, hornworm, white flies and delayed PPD are being multiplied in CIAT and will be shipped before the end of the year to Brazil Nigeria, Ghana and Uganda (see Activity 1.2. No. 11 for details). In addition to *in vitro* materials, CIAT has also shipped F_1 seeds to participating NARs to accelerate the introgression of desirable traits from wild relatives into the breeding scheme of participating NARs. The seeds were derived from three types of crosses: 1. crosses between *Manihot esculenta*; 2. crosses between *Manihot esculenta* and wild *Manihot* species; and 3. crosses between wild *Manihot* species). A total of 12,000 seeds were sent (i.e about 8,000 for NRCRI, 3,000 each for CRI and NAARI). At the moment about 750 seedlings have been transplanted to the field at the NRCRI, Nigeria after plant quarantine inspection.

Molecular Marker Facilities

For efficient transfer of MAS technology to NARs, it is important that the necessary facilities are established and put in place at participating NARs institution for easy application and use of molecular marker technology in plant breeding. EMBRAPA-CNPMF already has a molecular marker facility while the three African NARs (NAARI, CRI and NRCRI) in the projected have taken steps to set up a simple molecular markers laboratory with assistance from CIAT. A positive impact from this project is that NARs institutions have been challenged to invest considerably in molecular marker technology in plant breeding for the development of improved varieties. While GCP funds have been committed to the procurement of laboratory equipment and accessories, NARs institutions have spent almost equal funds to support the establishment of MAS laboratory through the provision of laboratory space and renovation, provision of staff personnel, cabinet fittings and some lab equipment. A brief description of the MAS laboratory for each NARs partner is described as follows:

(a) NRCRI - The National Root Crops Research Institute. Umudike, Nigeria, until the commencement of this project did not have a biotechnology laboratory. Through this project, the institute has established a molecular biology laboratory. NRCRI has provided a laboratory space for the lab and has made great strides in providing the laboratory with cabinet fittings and

refrigerators. CIAT assisted the institute in procuring laboratory equipment such as PCR machine, centrifuge, gel rigs. power pack, stirrers, glass plates, micro pipette, and laboratory consumables such as chemicals, eppendorfs, etc.using funds from the GCP. NRCRI purchased with its own funds fume cupboard/laminar flow hood, pH meter, oven, water bath, and sensitive weighing balance and vortex due to be delivered in weeks. The MAS laboratory for the institute is about 85% ready. It is anticipated that NRCRI will commence activities in the MAS laboratory this year. The first DNA extraction will be done in early December. The institute has also employed five staff for the GCP MAS laboratory. The institute has a tissue culture laboratory and some of its facilities like the autoclave machine will be shared with the newly established MAS laboratory.

(b) CRI – The Crops Research Institute, Kumasi Ghana has also set up a MAS laboratory. The laboratory was set up at the commencement of the project. The institute used GCP funds as well as its own funds to buy equipment, and laboratory consumables. The laboratory has all the necessary equipment for MAS. Equipment and consumables available include PCR machine and accessories, gel rigs, camera, refrigerators, glass plates, power pack, fume cupboard, autoclave machine, eppendorfs and laboratory safety facilities. The MAS laboratory in Ghana is about 90% ready.

(c) NAARI – The Namulonge Agricultural & Animal Production Research Institute, Uganda is working on the establishment of MAS laboratory. GCP funds are being used for this purpose. The institute has also provided funds to support the initiative.

Following the commissioning of the project, CIAT appointed a visiting scientist, Dr. Emmanuel Okogbenin, a breeder with experience in molecular genetics to technically backstop project activities in the NARs. The visiting scientist is placed at the NRCRI, Nigeria but oversees project activities at the African NARs as well as serving as liaison officer between CIAT headquarters (PIs) and NARs partners (collaborating scientists). Activities so far supervised by the visiting scientists include the shipment of germplasm from CIAT to Africa and supervision of the post-flask management and transplant to the field. The visiting Scientist has also been responsible in arranging for plant quarantine inspection and evaluation of the materials on the field together with collaborating scientists. The visiting scientist has successfully assisted the NRCRI, Nigeria to establish a MAS laboratory which is nearing completion in addition to backstopping the institute in its cassava breeding activities. In June, the visiting scientist in company of CIAT cassava geneticist visited CRI, Ghana to inspect the MAS laboratory and to deliver seeds from CIAT to the Institute. A similar visit was paid to NAARI in June 2005.

Conclusion

In summary, the initiatives at the African NARS in the GCP project have been to introduce germplasm from CIAT, technical support in tissue culture handling, and establishment of molecular marker laboratories in preparation for MAS activities due to commence next year according to schedule. In this process, a visiting Scientist has been appointed to ensure smooth operation of activities, assist in training of laboratory technicians, and establishment of crossing blocks to pyramid genes toward the development of new varieties combining useful traits (PPD, pest and disease resistance) via MAS at the NARs.

1.2.7 Quantitative trait loci for root architecture traits correlated with phosphorus acquisition in common bean

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Introduction

Eighty-two percent of the soils in Latin America are deficient in phosphorus. Phosphorus deficiency is widespread in East Africa as well, possibly affecting over 50% of soils there. Studies at CIAT have identified several sources of tolerance to low phosphorus conditions including G19833 the parent of the central CIAT mapping population (DOR364 x G19833). This genotype is thought to take up and use phosphorus more efficiently. In the breeding programs at CIAT, one of our long-term objectives is to increase the capacity of new breeding lines to grow under low P conditions. The objectives of this study were to identify quantitative trait loci (QTL) for P accumulation and associated root architectural traits, to facilitate genetic improvement and to reveal physiological relationships.

Materials and Methods: Eighty-six F5.7 Recombinant Inbred Lines (RIL) were developed from a cross between G19833, an Andean landrace with high total P accumulation, and DOR 364, a Mesoamerican cultivar with low total P accumulation in low P conditions. A genetic map constructed with RFLP, microsatellites and PCR-based markers covering 1703 cM total genetic distance and all eleven linkage groups was used for QTL analysis. Seventy-one RILs were evaluated in the field in Darien at high P (fertilization of 300 kg/ha TSP (45 kg P2O5)) and low P (7.5 kg P2O5)) levels for P accumulation, total root length, specific root length and plant dry weight, while all 86 RIL were evaluated in a hydroponic system in the greenhouse for tap, basal and total root length, specific root length and plant dry weight.

Results and Discussion: Phosphorus accumulation in the field correlated with root parameters measured in the greenhouse. A total of 20 individual QTL were identified for P accumulation and associated root characters using composite interval mapping analysis (Table 1). Phosphorus accumulation QTL often coincided with those for basal root development, thus, basal roots appear to be important in P acquisition. Independent QTL were identified for basal and tap root development, and for specific root length. Distinct QTL for greater specific root length had positive, null and negative effects on P accumulation. Our results confirm the importance of root structure for low P adaptation and highlight the need for a more detailed understanding of root architectural traits for phenotypic as well as marker aided selection of more P efficient crops.

Table 4. Quantitative trait loci (QTL) revealed by composite interval mapping analysis of indvidual traits from the field and greenhouse evaluations of the DOR364 x G19833 RIL population. Values represent QTL significance (LOD) and determination coefficients explained by each QTL (R^2 and T R^2). Linkage group location and nearest marker to the peak LOD value are given for each QTL.

Trait (unit)	Experiment	QTL name	LG[2]	LOD[3]	R2[4]	TR2[5]	Increased effect
P accumulation (mg/pl)	Field-low P	Pup4.1	B4	3.15	0.1341	0.4929	G19833
	Field-low P	*Pup10.1*	B10	3.16	0.1405	0.5019	DOR364
Root length (m/plant)	Field-low P	*Rlf4.1*	B4	4.17	0.2060	0.6121	G19833
	Field-low P	*Rlf7.1*	B7	2.68	0.1033	0.6111	G19833
	Field-low P	*Rlf8.1*	B8	3.81	0.2500	0.6464	DOR364
	Field-low P	*Rlf8.2*	B8	5.61	0.2141	0.6105	DOR364
Specific root length (m/g root)	Field–low P	*Srl8.1*	B8	4.34	0.2023	0.5832	DOR364
	Field–low P	*Srl10.1*	B10	4.23	0.1913	0.4181	G19833
P accumulation (mg/pl)	Field-high P	Pup2.1	B2	6.58	0.5133	0.7158	G19833
Root length (m/plant)	Field-high P	*Rlf4.2*	B4	3.37	0.1242	0.4918	G19833
	Field-high P	*Rlf7.2*	B7	4.70	0.3742	0.6976	G19833
	Field-high P	*Rlf11.1*	B11	4.78	0.3050	0.6949	G19833
Specific root length (m/g root)	Field–high P	*Srl3.1*	B3	3.81	0.1730	0.4295	G19833
	Field–high P	*Srl7.2*	B7	3.52	0.1594	0.4298	G19833
Basal root length (cm/pl)	Greenhouse	*Brl3.1*	B3	5.87	0.1997	0.4691	DOR364
	Greenhouse	*Brl10.1*	B10	3.80	0.1197	0.4696	G19833
Basal root DW[1] (g/pl)	Greenhouse	*Brd3.1*	B3	3.66	0.1138	0.4811	DOR364
	Greenhouse	*Brd7.1*	B7	2.91	0.0885	0.4823	G19833
	Greenhouse	*Brd10.1*	B10	2.99	0.0899	0.4825	G19833
Tap root length (cm/plant)	Greenhouse	*Trl3.1*	B3	4.86	0.2530	0.4829	G19833
Tap root DW[1] (g/pl)	Greenhouse	*Trd8.1*	B8	4.34	0.1463	0.4424	G19833
	Greenhouse	*Trd9.1*	B9	4.75	0.2242	0.5118	G19833
	Greenhouse	*Trd11.1*	B11	2.86	0.1392	0.5095	G19833
Specific root length (m/g root)	Greenhouse	*Srl*1.1	B1	4.15	0.2462	0.4801	DOR364
	Greenhouse	*Srl7.1*	B7	2.59	0.0944	0.3649	G19833
	Greenhouse	*Srl10.2*	B10	3.24	0.1202	0.3649	G19833
Seed weight (g/100seed)	NA	*Swf3.1*	B3	3.94	0.1023	0.5768	DOR364
	NA	*Swf4.1*	B4	3.08	0.0878	0.6598	G19833
	NA	*Swf11.1*	B11	7.50	0.2163	0.5814	G19833

[1] DW = dry weight; [2] LG = linkage group as defined by Freyre et al (1998); [3] LOD threshold of 2.5 used for QTL detection; empirical LOD thresholds based on 1000 permutations as recommended by Churchill and Doerge (1994)

1.2.8 Genetic mapping of beta-carotene content from multiple sources in cassava

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CIAT

Funding: Harvest Plus

Important Outputs

Markers earlier identified to be associated with beta carotene content in a S_1 family (AM320) of a yellow variety MTAI8 were also found to be associated with the trait in 3 F_1 progenies generated from crosses between 3 other yellow cassava varieties

Introduction

Beta-carotene is the precursor of vitamin A, an important micronutrient in human diet. Deficiency of Vitamin A in Developing countries is the leading cause of child blindness in the developing world. Beta-carotene content was earlier studied in S_1 progeny of MTAI8 a yellow cassava variety, bulked segregant analysis, and QTL mapping was used to identify 2 loci (SSRY313 and SSRY 251) that together explain over 80% of phenotypic variance for color of root parenchyma. The 2 loci are situated on linkage group 1 of the AM320-derived genetic map of cassava. This opens up the possibility of improving beta-carotene content beyond current levels by crossing high beta-carotene lines with complementary favorable alleles. To test if the hypothesis of multiple alleles at 2 loci controlling yellow root color is true, the above markers and others in the same region were evaluated in 6 F_1 progenies generated from crosses of yellow fleshed cassava varieties crossed to either other yellow or white varieties. Preliminary results of association between markers for beta-carotene content and color of root parenchyma in these lines are presented below.

Methodology

Plant materials for the genetic analysis of beta-carotene color were GM 708,GM 705, GM 734, CM 893, GM 734, CM 9816 F_1 families with progeny sizes ranging from 10 to 65 individuals. The F_1 progeny were established in a non-replicated trial at CIAT headquarters in Palmira, Colombia, and harvested after 10 months. Root parenchyma was visually measured using a scale of 1 (white parenchyma) to 9 (pinkish parenchyma) based on a color chart. Root parenchyma color is known to be highly correlated to beta-carotene content ($r=0.85$ at $P<0.05$). A total of 7 markers, namely SSRY313, NS980, SSRY92, SSRY251, SSRY9, and SSRY66, and NS717 found in the region of the cassava genome associated with root parenchyma color were used. The use of all 7 markers rather than just the 2, SSRY313 and SSRY251, tightly linked to beta carotene content is to increase the chances of finding polymorphism in the parents of all six crosses for genetic analysis of the trait. Parental lines of the 6 F_1 crosses were first evaluated for polymorphism in the 7 markers and polymorphic markers evaluated in the progenies. Association with color of root parenchyma was determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the

Microsoft Excel. The amount of phenotypic variance explained by each marker was obtained from the R^2 value.

Results

All 7 SSR markers were polymorphic in the cross GM708, other crosses had between 2 and 5 polymorphic markers each (Fig1). A simple regression of root parenchyma color in individuals of the GM708 cross on marker classes of the 7 markers revealed markers SSRY313 and SSRY92 each explained between 27 and 36% of phenotypic variance (Table 1). A similar result was found for crosses GM705 and GM734, where markers SSRY313 and SSRY9 explained between 25 and 30% of phenotypic variance. Analysis of marker association in the other crosses is yet to be completed. The differences in the variance explained for root parenchyma color in the different families suggests the presence of multiple alleles with different effects on the trait.

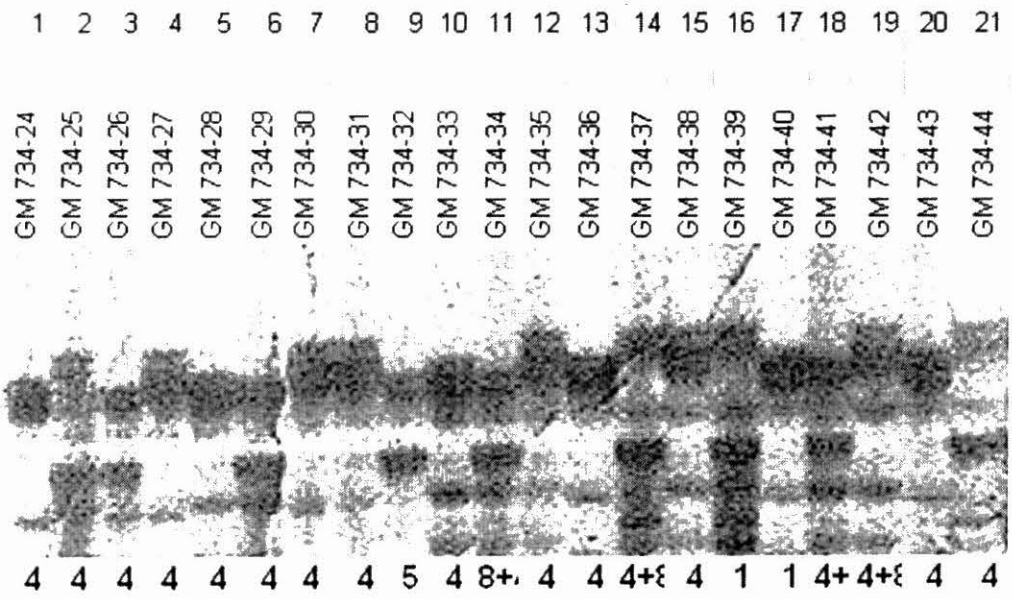

Figure 1. Silver stained polyacrylamide gel of PCR amplification product of marker SSRY313 in individuals of the F1 family GM734, the figures under the gel are root parenchyma color scores.

Table 1. Correlation (r-value) between SSR markers and root parenchyma color in 3 F_1 families obtained from crossing 2 yellow or a yellow and white variety

	SSRY-9	SSRY66	SSRY92	SSRY251	SSRY313	NS717	NS980
GM708	0.098282	0.109114	0.368828	0.12627	0.270877	0.117881	0.086813
GM705	0.212666	0.14199	0.089803	0.044901	0.237595	0.14199	0.08086
GM734	0.230383	0.505165	0	N/A	0.587795	N/A	0.016392

Conclusion and Perspectives

SSR markers earlier identified to be associated with beta carotene content with the trait in a S1 cross were revealed to also be associated with the trait in 3 F_1 progenies generated from crosses between yellow or yellow and white fleshed cassava varieties. This result suggests multiple alleles for yellow root parenchyma color in cassava. We intend to carry out a test of allelism to

estimate the effect of different alleles as a first step towards increasing beta-carotene content via combination of favorable alleles.

1.2.9 Genetic mapping of multiple sources of resistance to the cassava mosaic disease (CMD)

Alfred Dixon, Jonathan Mkumbira (IITA), Martin Fregene (CIAT)
Funding: Rockefeller foundation

Important Outputs

Establishment of crossing blocks for the development of mapping populations for multiple sources of CMD resistance

Introduction

Many land races of cassava from several African countries have been shown to possess very high levels of resistance to CMD, a total of seven sources of natural resistance have been described (Dixon et al. 2004). These sources of natural resistance represent a very important resource in the struggle to contain cassava mosaic begomoviruses that have shown a remarkable ability to recombine into more virulent forms. It is important to remain one step ahead of the virus by the continuous identification and pyramiding of resistance genes thereby making it harder for the virus to overcome host plant resistance. A project involving IITA and CIAT to tag all sources of natural resistance to CMD and pyramid CMD resistant genes in cassava germplasm was recently funded by the Rockefeller foundation. Markers developed by the project will be indispensable in combining different sources of genes that are undistinguishable by their phenotypes alone. This project will also initiate a molecular marker-assisted pyramiding of resistance genes into elite parental lines for use in different MAS projects working on the development of CMD resistance ongoing in several African countries.

Methodology

Plant materials for the identification of molecular markers for the different sources of CMD resistances are F_1 segregating populations developed from crossing local cassava varieties that represent the 7 different sources of resistance and susceptible improved varieties. The local varieties will be crossed extensively to both as pollen and staminate parents to produce F_1 populations, at least 250 seeds per family will be produced. All crosses produced will be established *in vitro* from embryo axes at the tissue culture facility of IITA. In the case of destructive screening for CMD disease reaction it is essential that copies of the mapping progenies are maintained *in vitro* in a healthy state throughout the project duration. Putting the mapping populations *in vitro* also permits facilitates sharing of the mapping populations and propagation for rapid progress to replicated multi-locational trials. Fifteen copies of each genotype of the mapping population will be produced, 3 will be kept in vitro while 12 will be sent to the screen house for hardening and eventual transfer to the field at IITA. Concurrently the parental materials of the above crosses will be assessed for marker polymorphism using all available 850 cassava SSR markers.

Results

A crossing block involving the above mentioned local and improved varieties have been planted at IITA (Table 1) The crossing block was planted in June/July 2005 and genetic crosses will be initiated in 2006 beginning April. The F_1 populations obtained will be established from embryo axes, multiplied and planted in a single row trial (SRT) or clonal evaluation of six plants and 1 replication at IITA, Ibadan. IITA is in the derived savannah belt of Nigeria and experiences very heavy CMD disease pressure all year round. At 3, 6, and 9 months after planting the mapping populations will be evaluated for resistance to CMD as follows:

(i) Field resistance (a visual assessment of symptom intensity ranked according to the IITA scale ranged from 0, for no observable symptom to 5 for very severe chlorosis and reduction in leaf area).

(ii) Virus incidence (percentage of shoots infected by the virus)

(iii) Virus diffusion resistance (development of symptoms with time)

Table 1. Line x tester mating design for allelism to CMD resistance

Source	Female parents	Male Parents
SN1	TME478, TME572, TME638, TME1	TME117,TMS30572,TMS30555,TMS30001
SN2	TME11, TME258, TME419, TME568	TME117,TMS30572,TMS30555,TMS30001
SN3	TME6, TME3, TME7, TME240	TME117,TMS30572,TMS30555,TMS30001
SN4	TME498, TME278, TME287, TME461, TME279	TME117,TMS30572,TMS30555,TMS30001
SN5	TME232	TME117,TMS30572,TMS30555,TMS30001
SN6	TME230, TME479, TME431	TME117,TMS30572,TMS30555,TMS30001
SN7	TME225, TME236	TME117,TMS30572,TMS30555,TMS30001

The following year progenies will be re-established in a SRT trial with 3 replications at two locations, IITA high forest station in Onne and at the IITA station in Ibadan, to enable an analysis of genotype by environment (GXE) interactions of identified CMD resistance genes. Evaluations of the mapping populations for host plant resistance to CMD will be as described above. Based upon results of the first year phenotypic evaluations, phenotypes showing highly resistant and highly susceptible response to CMD will be built for bulk segregant analysis (BSA) (Michelmore *et al.* 1991) using the polymorphic SSR markers from the parental survey and protocols described by Akano *et al.* (2002). Polymorphic markers will be evaluated in individuals of the segregating populations and strength of association measured by simple regression as described for cassava (Okogbenin and Fregene 2003; Akano et al. 2002). Should BSA fail to identify markers, then a standard QTL procedure, including development of a genetic map with SSR markers and a modified interval mapping approach (Lincoln et al. 1992; Liu 1998), will be followed in only the S_1 populations as described earlier for cassava (Okogbenin and Fregene 2001).

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1.2.10 Genetic mapping of cyanogenic glucoside content in cassava

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Funding: BIOEARN, CIAT

Important outputs
Identification of QTLs controlling cyanogenic glucoside content in cassava

Introduction

Cassava produces cyanogenic glucosides which are often rightly or wrongly seen as a health hazard to consumers, particularly in the very poor segment of the population that depend on cassava as a staple. Two cyanogenic glucosides, linamarin and lotaustralin occur in all parts of the plant. Cyanogenic glucosides are synthesized in the leaves and translocated to all other parts of the plant including the storage roots. Environmental factors during the growing season contribute significantly to the variation in CNP among genotypes, within genotypes and in various parts of the plant. Hydrogen cyanide (HCN) in cassava tissues has been medically proven to be a potential health hazard for consumers if the plant is inadequately processed (Mlingi et al.1998). An important objective in cassava improvement programmes is therefore breeding for low cyanogenic potential (CNP) and high DM content. Conventional breeding for low CNP in cassava has been fraught with the confounding effect of the environment on its expression and the long growth cycle of cassava. Since the biosynthesis of the cyanogenic glucosides is complex and probably affected by many genes with influence from the environment, marker assisted selection (MAS) has the potential to make more efficient breeding for low cyanogenic potential. This study uses a linkage map of cassava previously constructed using a S_1 to identify molecular markers associated with CNP in cassava.

Methodology

The S_1 mapping population (AM320) of 199 individuals used in this study is from a cross between a local Colombian variety, (MCol1684), and a Thai variety, Rayong 1. The grandparental clones were selected for contrasting levels of CNP, with MCol1684 having high CNP while Rayong1 has a low CNP. Dry matter content (DM) among other traits has also been shown to segregate in this S_1 population. The field experiment was established in August 2003 at CIAT headquarters in Palmira (Valle del Cauca department). The experimental design was a completely randomized block design with 8 blocks, with a single plant plots. Border rows of

plants from a different variety were included. Selected mature stem cuttings of 199 genotypes were planted vertically on ridges at a spacing of 1 x 1 m.

The experiment was harvested in January 2005 at 5 months after planting (MAP) and roots immediately taken to the laboratory for the trait measurements. All storage roots per plant in each plot were bulked and used for measurements of CNP. Cyanogenic potential (CNP)] was measured using the modified method by Essers (1993). Fresh root sample was then cut into 1 cm cubes and mixed; 40g of cassava fresh root cubes were homogenized in 100mL cold 0.1 M orthophosphoric acid in a blender for 15 seconds at low speed, followed by 60 seconds at high speed, 60 seconds of rest and 60 seconds full speed again. Extraction in the acid stops linamarase activity and stabilizes cyanohydrins. The homogenate was centrifuged in closed tubes at 4,000g for 10 minutes and the supernatant used as extract for further analysis. Cyanogens were then assayed in triplicate as described by Essers (1993)

Cyanogen levels were calculated in mg HCN equivalent per kg sample on a dry weight basis (mg HCN equivalent kg-1, DWB) as follows:

$$[\text{HCN equivalent}] = \frac{x\,(v + s.\,m/100)}{s\,(1 - m/100) * d} * 0.027$$

where s = sample weight (g)

v = volume of extraction medium (mL)

d = Volume of extract assayed (mL)

m = moisture content (%)

x = quantity of cyanogen (nmol) in the tube; x is calculated from the calibration curve as $x = \frac{(A_{605} - a)}{\text{slope}}$

where A_{605} is the absorbance measured at 605nm. Both slope and intercept, a are derived by linear regression of the calibration points by means of a calculator. For very low values of a (< 0.005) and higher values of A_{605} (< 0.200) the intercept can be neglected.

Data analyses include the calculation of mean values, determination of statistical significance of the sources of variation, calculation of estimates of variance components, and determination of broad sense heritability from the ANOVA. Distribution analyses as well as analyses of variance were performed for each trait using JMP programme Version 3 (SAS INSTITUTE, 1994). Analyses of variance for the mapping population were conducted on untransformed data. Sixteen different genotypes had establishment problems arising from poor vigor of the mother plants from which the cuttings were made, and some few plots corresponding to these genotypes resulted in some missing data points. The final data were analyzed as a completely randomized block experiment on 160 individuals with the genotype effect considered random. Phenotypic data were subject to the QTL analysis using untransformed data and marker genotypic data from the cassava genetic map (CIAT 2003). The cassava genome was scanned for the presence of the QTL effect at 2.0-cM intervals using the computer package QTL Cartographer by composite interval mapping (CIM) using model 6.

Results

Phenotypic evaluation of the traits CNP content at 5 MAP in the F_2 population revealed continuous variation, typical of quantitative traits (Fig.1). Broad sense heritability estimates based on the means of the genotypes was 77.8% for CNP.

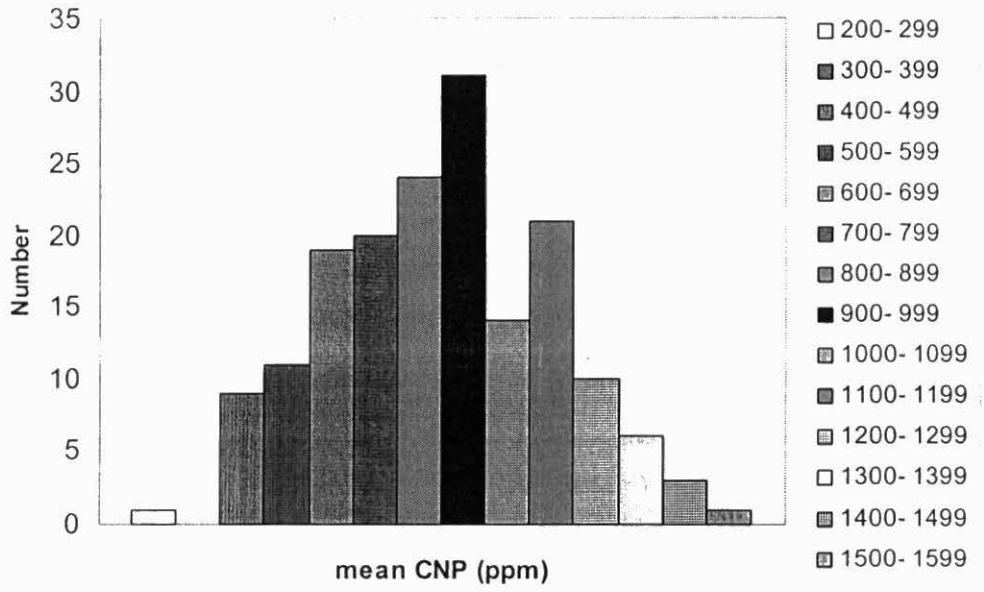

Figure 1. Distributions of the mean percentage DM and mean CNP in the AM320 mapping population.

We detected 2 QTLs, 1 each on chromosome 10 and chromosome 23 (Table 1). The two QTLs explained between 7 and 20% of the phenotypic variation in this S_1 population. The QTL on chromosome 10 had significant LOD scores (with a threshold of 4.0 with 1000 permutations). At this locus genotypes with the homozygous allele A showed the highest mean CNP score while with the QTL on chromosome 23 the genotypes with homozygous B allele showed the highest mean CNP score. This might suggest that the

Table 1. Quantitative trait loci (QTL) as determined by composite interval mapping for the traits CNP, their chromosomal (Chr), nearest flanking molecular marker, LOD scores, additive and dominance effects, dominance/ additive ratio (d/a) and gene action. *** QTL significant at $p<0.001$, * QTL significant at $p<0.05$.

Trait	Chr	Marker	LOD	a	d	d/a	Gene action
CNP	10***	SSRY105***	8.4	-161.96	4.46	-0.03	additive
	23	SSRY242	3.2	98.96	-10.35	-0.10	additive

Mcol1684 allele of one QTL of each pair had a positive effect on the trait, whereas for the other QTL the *Rayong 1* allele contributed negatively to the trait.

Conclusion and perspectives

Results of QTL mapping of CNP in a F2 cross led to the identification of 2 QTLs for the trait, perspectives include evaluation of the QTLs in additional sites and other crosses involving the MTAI8 parent to test genotype by environment and genotype by genotype interactions in preparation for its use in molecular marker-assisted selection (MAS) for CNP.

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1.2.11 Progress in the Genetic Mapping of dry matter content in cassava

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CIAT
Funding:
Rockefeller Foundation, CIAT

Important Outputs

Establishment of 2 large gene tagging population in a replicated trial for evaluation of dry matter content
Identification of putative markers linked to dry matter content in an inter-specific cross between cassava and its wild progenitors

Introduction

In 2002 a project was initiated to develop molecular markers for dry matter content (DMC) based upon initial quantitative genetic analysis of the trait. SCA and GCA estimates from an earlier genetic analysis diallel experiment (CIAT, 2003) were used to select families for bulked segregant analysis (BSA) of dry matter content (DMC). A marker was found to be associated with DMC in the selected families having SM 1741-1 as parent. Based upon the above results larger populations were generated for QTL analysis. More than 1500 F_1 seedlings from 8 families (CIAT, 2004) were established in the field and evaluated for DMC. Cuttings from the seedlings were used to establish a clonal trial. DMC data from the seedling evaluation was used to initiate bulked segregant analysis (BSA) in the larger population. BSA analysis on an inter-specific family CW 208, a cross between MTAI 8 and *Manihot tristis* with wide segregation for DMC was also initiated in 2004 (CIAT, 2004). We describe here the evaluation of the seedling nursery and establishment of a replicated trial, initiation of BSA of the large population, continuation of the BSA of CW 208 cross and testing of promising marker SSRY 160 on the larger population.

Methodology

A seedling nursery consisting of 8 crosses established from specific crosses made in 2004 was evaluated in May, 2005. Crosses were derived from parents selected from the three major cassava growing areas- mid-altitude, low land semi-arid and acid savannah for dry matter and yield related agronomic traits (CIAT, 2004). Individual plants were harvested, the biomass divided into vegetative and economic, and used to calculate yield related parameters. Dry matter content (DMC) of the tubers was estimated using the standard CIAT procedure. The stems from individual plants were stored separately to provide the planting material for the F_1 clonal evaluation. In July 2005, the F_1 clonal evaluation trial was established from the stored stems. Plants were planted on ridges on spacing of 1.6 by 0.7 m, the spacing being mainly to reduce

inter-genotype and increase intra-genotype competition. Of the 8 families available, three families (GM 252, GM 901 and CM 9953) with genotypes greater than 200 were replicated, the rest were planted as single row plots. Of the replicated families only genotypes with enough planting material to provide 9 or more stakes were replicated. Genotypes with 12 or more cuttings were planted on 1 x 4 plots with 3 reps, while those with less than 12, but with at least 9 cuttings were planted on 1 x 3 plots with 3 reps, and the rest as 1 row plots of 6 plants or whatever number could be obtained.

To initiate marker development for dry matter, bulk segregant analysis (BSA) was performed on families with more than 200 genotypes. Two F_1 families GM 901 with SM 1741-1 and MPER 183 as parents and CM 9953 with SM 1741-1 and SM 1219-9 as parents were initially used (third set GM 252 was later included). Using data from the seedling evaluation, the extreme low and high DMC genotypes were selected for each cross and group to form low and high bulks respectively. DNA samples were prepared by drying young leaves in the oven at 55^0 C overnight, then grinding 100mg in an eppendorf containing acid washed sand using a power drill fitted with a small pestle until a fine powder was obtained. DNA was then extracted according to a modified Dellaporta *et al.* (1983) protocol. Between 50µg to 100µg of high quality DNA was obtained from each extraction and quantified using flourometor. The samples where then diluted to 10 ng/µl. Bulks and parents were evaluated with 850 simple sequence repeat (SSR) markers available at CIAT. PCR product was denatured and electrophoresed on 4 % polyacrylamide gels. Polymorphic markers will be analyzed in individuals of the bulks.
Crosses between elite varieties and accessions of the wild progenitor of cassava *Mannihot esculenta* sub *spp flabellifolia* were made in 2000. The F_1 were back-crossed to the elite cassava lines to form Backcross 1 generations (BC_1). The BC_1 were evaluated for dry matter and family, CW 208 with high standard deviation for dry matter was selected for bulk segregant analysis. Segregation of dry matter content in this family is maybe the highest found to date (CIAT, 2004). DNA was extracted and bulks made as described earlier. The available 850 SSR markers were used to screen the bulks. The polymorphic ones were used to screen individuals within the bulks and the promising used to screen the whole population. The candidate will be used to screen individuals from other families in the population.

Following the establishment of association between SSRY 160 and DMC in families selected from the diallel experiment and having SM 1741-1 as a parent, DNA was extracted from samples of the large families (GM 901 and CM 9953) having SM 1741-1 as parent. A total of 335 and 175 samples respectively were extracted and analyzed with SSRY 160.

Results

Only seedlings with measurable biomass were evaluated, the rest were just cloned to provide planting material. A relatively high total number of storage roots for seedlings was obtained, average 6.67; with clone GM 901-192 having the highest number of 19. Clones with the highest number of roots, were from families GM 901 and CM 9953B both of which have SM 1741-1 as a common parent. Dry matter content ranged from 16.3% in GM 252-307 and CM 9953B-030 to 69.1% in GM 901-270. Highest yield was recorded in GM 901-263 (8.8 kg) followed by GM 901-263 (8.6 kg), while highest dry matter yield was recorded in GM 901-263 (2.60 kg) and GM 9953B-075 (2.56 kg).

Data was averaged per family to give an indication of a family's performance. Family GM 256 registered the highest number of tubers (7.62) and GM 853 the lowest (5.87) (Table 1). Families

GM 901 and GM 9464 had the highest average harvest index estimates, 52.7 and 50.3% respectively while GM 256 had the lowest (37.0%). Highest percent dry matter content was found in GM 901 (33.5%), GM 256 (33.3%) and the lowest in GM853 (29.2%). Highest dry matter yield was observed in family GM 536 with 0.65kg, while the lowest was GM 9953 (0.39 kg). Mean performance of offsprings derived from a parent was used to estimate the parents breeding value. Best parent for DMC was MPER 183 (33.6%) followed by SM 1741-1, confirming previous diallel analysis, that SM 1741-1 was a good parent for DMC. CM 4574-7, CM 6754-8 and CM 8027-3 had the best breeding value for number of commercial roots, tuber weight, yield and dry matter yield, while MPER 183 and SM 1741-1 ranked as the best for DMC and harvest index were ranged low. Jaramillo *et al.,* 2005, reporting on the diallel experiment where these parents were selected from, indicated that there was a negative association between DMC and the yield potential in a number of parents.

Table 1. Means and Standard Deviations of root quality characteristics of 8 families evaluated at harvest in CIAT-ICA, Palmira in April, 2005.

FAMILY	PARENTAGE	V A R I A B L E					
		TlRt	ComRt	HI	TBWt	DMC	Yield
GM252	SM1665-2 x SM805-15	6.51±3.14	2.10±1.66	48.0±14.11	0.252±0.18	32.2±4.36	1.64±1.9
GM901	SM1741-1 x MPER183	6.65±3.04	2.18±1.73	52.7±12.79	0.246±0.13	33.5±5.04	1.61±1.1
GM9953	SM1219-9 x SM1741-1	6.27±2.73	1.73±1.53	48.1±15.89	0.225±0.14	31.7±5.50	1.42±1.1
GM536	SM1565-15 x CM4574-7	7.08±3.34	2.89±2.37	46.6±13.14	0.281±0.18	30.4±3.84	2.01±1.6
GM9958	SM1411-5 x MTAI-8	6.65±2.87	1.49±1.21	45.5±16.84	0.200±0.11	28.6±6.69	1.31±0.9
GM853	CM8027-3 x CM6754-8	5.87±2.77	2.26±1.48	46.3±16.55	0.345±0.21	29.2±3.13	1.90±1.2
GM256	SM1219-9 x SM1565-15	7.62±3.16	2.05±1.53	37.0±14.70	0.197±0.12	33.3±3.58	1.45±0.9
GM9464	CM4574-7 x SM1411-5	7.17±1.94	1.67±1.63	50.3±18.25	0.241±0.23	33.2±3.74	1.58±1.3
Total		*6.66±3.09*	*2.17±1.80*	*48.1±14.57*	*0.248±0.16*	*32.0±4.81*	*1.64±1.5*

Dry matter and harvest index ranked the parents the same and in opposite direction with other yield characteristics. Jaramillo *et al.,* (2005), also noted that harvest index had the highest correlation with DMC (r=0.45) and that in some parents there was a negative association between DMC and yield potential. Overall ranking placed CM 4574-7 as the best parent followed by MPER 183 and SM 1565-15. A total of 220, 147 and 91 genotypes from families GM 252, GM 901 and CM 9953 were established as a replicated trial (4reps, 4 plant plots) and 41, 64 and 49 as a 3 rep, 4 plant plots trials respectively. Overall 261, 211 and 150 genotypes of GM 252, GM 901 and CM 9953 respectively were established as replicated trials.

Of the 400 primers run so far on the large families, 42 were polymorphic between the parents and bulks of family, GM 901, 5 between those of CM 9953 and 3 in both families. From observation so far it appears there is little polymorphism in family CM 9953, the parental combination SM 1741-1 and SM 1219-9 are monomorphic in almost all the primers. BSA of GM 252 has just been initiated.

Fifty-five SSR markers were polymorphic between the bulks in the inter-specific crosses , of these 17 were polymorphic among the genotypes of the bulks (Fig 1). To determine promising markers for dry matter content, phenotypic data in individuals of the CW208 family was regressed on marker data classes of the 17 markers. However due to apparent difference between the 2003 (seedling data) and the 2004 (clonal data), both data was used (Table 2), (the bulks were

made using 2004 data because it was from several plants as opposed to the 2003 which was from a single plant). The 2003 data identified SSRY 99, SSRY 141 and NS 169 with R^2 values of 22.68, 35.89 and 20.01 respectively while the 2004 data identified SSRY 11 (R^2=26.85). The phenotypic variance explained by these markers based on their regression coefficient, is enough to consider them as markers for marker-assisted selection (MAS). These are to be used to screen progenies derived from this family.

Running of SSRY 160 on the large family GM 901 is still ongoing, there were initial problems with adequate separation of alleles on 4% PAGE gels which led to wrong reading of the bands, the electrophoresis is being repeated with 6% gels in increase band resolution.

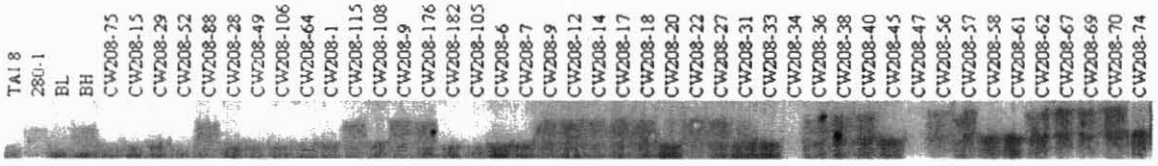

Figure 1. Acrylamide gel showing PCR amplification of marker SSRY 11 of parents bulks and individuals constituting the wild cross CW 208.

Table 2. Regression analysis results of markers polymorphic in the open bulks when used for screen the whole population.

Marker	R^2 values (2003 data)	R^2 value (2004)
SSRY 298	0.34	7.42
SSRY 306	2.7	7.74
C306R	0.6	7.64
SSRY 69	5.72	3.02
SSRY 99	22.68	0.07
SSRY 141	35.89	0.09
SSRY 11	1.19	26.85
SSRY 23	10.22	0
SSRY 27	7.1	16.6
SSRY 36	10.52	5.15
SSRY 47	1.91	9.97
SSRY 49	2.89	3.18
SSRY 54	6.9	15.37
SSRY 57	5.7	0.11
SSRY 60	0	6.66
SSRY 66	6.7	4.2
SSRY 75	1.5	7.9
NS 169	20.01	7.40

Conclusion

1453 seedlings from families generated for QTL analysis were evaluated for yield related traits. Best parent for DMC was MPER 183 followed by SM 1741-1 (the parents for GM 901) confirming previous diallel analysis results that SM 1741-1 is a good parent for DMC. Genotypes from families GM 901 and CM 9953 both having SM 1741-1 as a common parent

showed superior performance in most traits evaluated, indicating that SM 1741-1 has a good breeding value for most agronomic traits. Forty two (42) primers were found to be polymorphic in bulks of family GM 901 and 5 in CM 9553. Four SSR markers:- NS 169, SSRY 99, SSRY 11, SSRY 141 with R^2 values of 20.01, 22.68, 26.85 and 35.89 were found to be putative markers for DMC in the inter-specific crosses between MTAI 8 and *Manihot esculenta* sub spp *flabellifolia*.

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1.2.12 Genetic mapping of high protein content in first back cross derivatives of M. *esculenta* sub spp

Olalekan Akinbo, Luis Guillermo Santos, Cesar Ospina, Edgar Barrera, Janneth Guttierrez, Martin Fregene

Funding: The Rockefeller Foundation

Important Outputs

Establishment of 3 BC_1 mapping populations *in vitro* and in the field for QTL mapping of high protein content in inter-specific hybrids between cassava and its close wild relatives

Introduction

Several accessions of *M.esculenta* sub *spp flabelifolia*, *M.esculenta* sub spp *peruviana* and *M.tristis* collected in Brazil were found to have between 10-18% (dry weight basis) protein in the storage roots. These protein rich genotypes were crossed to elite parents of CIAT's cassava gene pools and a number of F_1s identified that retained the high protein content trait. The inter-specific F_1 parents were backcrossed to the elite parents to transfer the trait to the cultivated gene pool. This is the second step in a modified advanced back crossing QTL (ABC-QTL) mapping scheme being used to accelerate the introgression of useful traits from wild relatives into cassava. We describe here the establishment of the BC_1 protein mapping populations in vitro, screen house hardening, and transfer to the field. .

Methodology

Selected inter-specific F_1 hyrbids between cassava *M.esculenta* sub *spp flabelifolia*, and *M.tristis* were back crossed to the elite cassava parents (Table 1), the selection criteria used was a high protein value of the F_1 individual and family. A total of 1,575 crosses were made from April 2004 to December 2004 and 1,821 seeds were collected from crosses of CW 198 – 11, CW 205 –

2, CW 201 – 2 with MTAI 8. Of this number, 1,113 seeds were viable and germinated in vitro from embryo axes according to standard procedures in the cassava tissue culture laboratory at CIAT (CIAT 2003). The plantlets derived from embryo axes were multiplied, over 2-3 cycles, to produce 8-10 plants for screen house hardening. The plantlets were hardened in the screen house according to standard methods (CIAT 2003) and then transferred to a single row trial in the field at ICA-Corpoica.

Results

A total of 1113 sexual seeds from 3 families were germinated, of these 675 genotypes, 225 per family were multiplied and transferred to the screen house for hardening. The rest was kept *in-vitro* against any failure of the selected genotypes during hardening or field transfer. Between 4 and 6 plants of the 675 genotypes were successfully hardened

Table 1. BC_1 Families established from embryo axes, hardened in the screen house and transferred to the field in the screen house and 4,932 plants were transplanted to the field at Corpoica (Fig. 1 and Fig 2).

No	BC1 Family	Donor parent	Female parent of donor	Male parent of donor	Recurrent Parent	% protein of donor parent	No of plants
1	B1P2	CW 198 - 11	OW 230 - 3	CW 30 - 65	MTAI 8	11.28	225
3	B1P5	CW 205 - 2	OW 231 - 3	MTAI 8	MTAI 8	10.54	225
4	B1P6	CW 201 - 2	OW 230 - 3	CW 56 - 5	MTAI 8	10.2	225

Fig 1. Plants of the BC1 protein content mapping population being hardened in the screen house

Fig 2. The BC1 protein content mapping populations in the field at ICA-CORPOICA, Palmira

Conclusion and Perspectives

Three BC1 populations for mapping of high protein content have been successfully established from embryo axes and established in the field at ICA-CORPOICA, Palmira. The parental lines of these populations are being evaluated with all 850 cassava SSR markers to identify polymorphic markers in preparation for bulk segregant analysis (BSA) and quantitative trait loci (QTL) mapping of the trait.

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1.2.13 Genetic mapping of physiological traits related to drought tolerance in cassava

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Funding: Generation Challenge Program (GCP)

Important Outputs

Multiplication and distribution of 28 drought tolerant and 12 susceptible genotypes, 10-20 plants per genotype, to participating institutions in the drought project

Establishment of a trial to evaluate the above germplasm in Tolima and Huila departments of Colombia and a crossing block to generate segregating populations for the genetic mapping of drought tolerance.

Introduction

Cassava is considered one of the most drought tolerant crops. Cassava's photosynthesis and growth decrease to near zero during episodes of water deficit and it achieves most of its growth after rainfall resumes. This suggests that a key to cassava's success is its ability to regulate numerous plant processes to rapidly change course between episodes of favorable and unfavorable weather. A project was funded by the Generation challenge program (GCP) to study the physiological and genetic basis of cassava's remarkable tolerance to drought to determine the best traits and develop markers for use in breeding programs for drought tolerance. Contrasting genotypes for several traits related to drought tolerance were selected for evaluation and the development of segregating progenies for genetic studies. Segregating progenies will be evaluated in semi-arid environments and analyzed using a set of genome-wide molecular markers and candidate genes to identify quantitative trait loci (QTL) of component traits of drought tolerance.

Methodology

Based on the outcomes from an earlier breeding program for drought carried out by Embrapa (Brazil) and CIAT (Colombia), in the last 15 years, 40 cassava varieties were identified as contrasting for drought tolerance attributes, 28 tolerant and 12 susceptible (Table 1). Experimental sites to evaluate drought tolerance were also selected in Brazil, Colombia, Tanzania, and Ghana to evaluate the contrasting genotypes. The semiarid representative sites for the replicated field trails at each country will be: 1) Petrolina, Northeastern Brazil; 2) Tolima, Central-West Colombia; 3) Dodoma, Central Tanzania; and 4) Tamale, Northern Ghana. In Colombia two sites were selected in semi-arid areas the departments of Tolima and Huila

respectively. To identify traits related to drought tolerance, the responses of these genotypes to water deficit and control conditions will be evaluated for the selected traits related to probable mechanisms for drought tolerance. These traits include:

1. Leaf gas exchange (leaf conductance, transpiration, and photosynthesis)
2. Osmotic adjustment (accumulation of certain compatible solutes, such as proline and sugars)
3. Accumulation of specific low molecular weight proteins (Late Embryogenesis Abundant, LEA proteins), that may confer osmoprotection to cellular membrane and protein systems.
4. Abscisic acid (ABA) accumulation and, as a time-integrated measure of ABA synthesis during drought, the accumulation of biologically inactive catabolites phaseic acid (PA) and sugar esters of ABA and PA.
5. Accumulation and utilization of nonstructural carbohydrate reserves (sugars and starch) in leaves, stems, and roots, during stress and recovery episodes.

Table 1. Contrasting genotypes to drought tolerance selected for development of segregating population for genetic mapping of drought tolerance.

No.	Accession (CIAT code)	No. of plants	Country origin	of Reaction to Drought
1	BRA 114	70	Brazil	Susceptible
2	BRA 116	70	Brazil	Tolerant
3	BRA 134	70	Brazil	Tolerant
4	BRA 165	70	Brazil	Tolerant
5	BRA 179	70	Brazil	Tolerant
6	BRA 200	70	Brazil	Tolerant
7	BRA 201	70	Brazil	Susceptible
8	BRA 209	70	Brazil	Tolerant
9	BRA 216	70	Brazil	Tolerant
10	BRA 249	70	Brazil	Tolerant
11	BRA 253	70	Brazil	Susceptible
12	BRA 255	70	Brazil	Tolerant
13	BRA 264	70	Brazil	Tolerant
14	BRA 293	70	Brazil	Tolerant
15	BRA 346	70	Brazil	Susceptible
16	BRA 534	70	Brazil	Tolerant
17	BRA 835	70	Brazil	Susceptible
18	BRA 846	70	Brazil	Susceptible
19	BRA 878	70	Brazil	Tolerant
20	BRA 879	70	Brazil	Susceptible
21	BRA 886	70	Brazil	Tolerant
22	BRA 969	70	Brazil	Tolerant
23	BRA 974	70	Brazil	Tolerant
24	BRA 997	70	Brazil	Susceptible
25	BRA 1142	70	Brazil	Tolerant
26	BRA 1204	70	Brazil	Tolerant
27	BRA 1342	70	Brazil	Tolerant
28	BRA 1346	70	Brazil	Tolerant
29	BRA 1356	70	Brazil	Susceptible
30	BRA 1394	70	Brazil	Tolerant
31	BRA 1400	70	Brazil	Tolerant
32	CM 3306-9	70	Colombia	Tolerant
33	COL 948D	70	Colombia	Tolerant
34	COL 1468	70	Colombia	Susceptible
35	COL 1522	70	Colombia	Susceptible
36	COL 1719	70	Colombia	Tolerant
37	COL 1725	70	Colombia	Tolerant
38	COL 1734	70	Colombia	Tolerant
39	COL 2066	70	Colombia	Susceptible
40	COL 2215	70	Colombia	Tolerant

Photosynthesis and stomatal conductance will be measured using field-portable gas exchange equipment, which will be available at each of selected sites. Osmolyte accumulation will be measured with freezing point osmometers following re-hydration of tissue to a common reference relative water content (RWC), and freeze-thaw/centrifugation to release cell sap (Babu et al. 1999). LEA proteins will be quantified by an ELISA procedure that employs antibody directed against a conserved dehydrin amino acid sequence (Close et al. 1993), which will be validated with immunoblot methods. ABA and cytokinins will be purified by preparative chromatography and quantified by ELISA, as described by Setter et al

Results

Forty drought tolerant contrasting cassava varieties contrasting varieties were multiplied by *in vitro* micro-propagation to produce around 70 individual copies of each contrasting genotype. Twenty plants of each genotypes have already been shipped to EMBRAPA-CNPMF while the same set of genotypes will soon be shipped to Cornell University (10 plant per genotype, IITA-Ibadan (20 plants per genotype), SARI, Tamale, Ghana (10 plants per genotype), and ARI-Naliendele, Mtwara, Tanzania, (10 plants per genotype). An initial constraint to the distribution of contrasting drought tolerant materials was bureaucratic delays in obtaining import permit to ship the cassava genotypes (*in vitro* plants), this has now been resolved for Brazil while we await import permits to ship the plants to the USA. The contrasting genotypes have been planted in a crossing block at CIAT for pair-wise crosses to produce segregating populations, they will be planted in Brazil in Dec 2005/Jan 2006. Due to lack of planting material for all contrasting genotypes, a sub-set was established in replicated trials in Tolima and Huila in November 2005 for the evaluation of the drought trait next year.

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1.2.14 Quantitative trait loci (QTL) mapping of traits associated with domestication in first back cross generation derivatives of wild progenitor of cassava

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Funding: SIDA-FORMAS

Important Outputs

Development, *in vitro* establishment and transfer to the field of a S1 population derived from an inter-specific cross between MCOL1734 and *M. esculenta* sub spp *flabellifolia*

Introduction

Strong selection on specific traits during the domestication of cassava has gradually decreased the genetic variability in the crop and has lead to loss of traits which are not under artificial selection. Some of these traits may be of importance for cultivation of modern cassava varieties such as pest and disease resistance and high contents of protein in roots. Such lost genotypes and traits can be recovered only by reintroducing the allelic variations of genes preserved in the wild ancestor by crossing the crop with its wild ancestor. It is of course very important to retain the "good" traits which have been selected upon during domestication such as large root tubers and thickened stems with enlarged nodes for vegetative propagation. Accordingly, it is of major interest for future breeding to have knowledge about the genetic basis of quantitative traits controlling the differences between cassava and its wild ancestor.

QTL mapping is a powerful method for determining the genetic control of trait differences between lines and species (Lander and Botstein 1989, Zeng 1994, Kao et al. 1999). This method is built upon (1) the ability to construct saturated linkage maps using molecular markers and (2) statistical methods for mapping and characterizing QTLs. QTL mapping enables one to determine the minimum number of QTL controlling a trait, the relative magnitudes of their individual effect, their chromosomal location and their mode of gene action. This method can be used to study the genetic changes that have occurred during domestication (e.g. Paterson et al 1988, Doebley et al. 1990) and the evolution of wild relatives of crops (Westerbergh and Doebley 2002, 2004, Lauter et al. 2004) and other natural species (Kim and Rieseberg 1999).

Methodology

The genetic analyses of traits that differentiate cassava from its wild relatives will be studied in an S_1 population derived from an inter-specific cross between cassava and wild *M. esculenta* ssp. *fllabellifolia* (the ancestor to cassava). Selected parental genotypes differ in a number of traits such as root number and root size, protein, nutrient and cyanogenic glucoside content in leaves and roots and pathogen resistance. The study will focus on plant morphology and root quality. Several F_1 inter-specific hybrids from the family CW188, derived from crossing the cassava genotype MCOL1734 and a *M. esculenta* ssp. *Fllabellifolia* OW181-2, were selfed to produce

between 200 and 300 seeds per S_1 family. The seeds of were established *in vitro* from embryo axes and multiplied to produce 4-6 plants per genotype. A single S_1 cross was selected for screen house hardening and establishment in the field at ICA-CORPOICA, Palmira.

The development of a molecular linkage map for cassava and its wild ancestor *Manihot esculenta* ssp. *fabellifolia* and the QTL mapping analysis will be carried out by a student registered at the Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences (SLU), Uppsala and supervised by Dr Anna Westerbergh. This department has a lot of experience in molecular biology and marker work and Anna Westerbergh is well experienced in quantitative genetics and QTL mapping analysis from work on maize and its wild relatives and lately on cassava.

Results

Six S_1 families from the genotypes CW188-1, CW188-4, CW188-9, CW188-12, CW188-14, and CW188-19 were established *in vitro* from embryo axes (Table 1). The family CW188-1 had the highest number of *in vitro* plants and was selected for screen house hardening and transfer to the field. Between 2 and 5 plants per genotype of 186 genotype of the CW188-1 family was planted in a replicated trial (one plant per replicate) in the field in ICA-CORPOICA in November. Spacing between plants was 1.0m x 1.6m to enable proper morphological evaluation of the S_1 hybrids. The plants will be evaluated in March 2006 for plant morphology and root quality. Parental and grand parental lines of the S_1 family are also being evaluated with 300 SSR markers, selected from the 3 genetic maps of cassava constructed to date, for identification of polymorphic markers for QTL mapping.

Table 1. S_1 crosses developed for genetic analyses of traits that differentiate cassava from its close wild relatives

CODE	MOTHER	FATHER	Delivered No. Seeds	No. Sem. Shallow/Perd.	Planted No. Seeds	Formed No. Plants	% Formation of Plants
CW188-1	CW 188-1	CW 188-1	514	89	425	212	50%
CW188-12	CW 188-12	CW 188-12	545	144	401	138	34%
CW188-14	CW 188-14	CW 188-14	369	213	156	59	38%
CW188-18	CW 188-18	CW 188-18	306	45	261	53	20%
CW188-9	CW 188-9	CW 188-9	73	30	43	19	44%
CW184-4	CW 184-4	CW 184-4	94	44	50	15	30%

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1.2.15 Development of mapping populations for identification of markers associated with genes for resistance to whiteflies, hornworm and delayed post-harvest deterioration from wild relatives of cassava

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Funding: Generation Challenge Program (GCP)

Important Outputs

In vitro establishment of BC_1 and S_1 mapping populations for QTL mapping of post-harvest deterioration (PHD), resistance to whiteflies and hornworm and screen house hardening

Introduction

Wild *Manihot* germplasm are a wealth of useful genes for the cultivated species *M. esculenta* but their use in regular breeding programs is limited due the long reproductive breeding cycle of cassava and linkage drag associated with the use of wild relatives in crop improvement. Dramatically delayed PPD has been identified in an inter-specific hybrid between cassava and *Manihot walkerae*, a unique source of resistance to the cassava hornworm was also identified in 4th backcross derivatives of M. glaziovii. Moderate to high levels of resistance to white flies have been found in inter-specific hybrids of M. esculenta sub spp flabellifolia. This project seeks to transfer genes controlling useful traits already discovered in wild relatives, namely resistance to the whitefly and hornworm, and post-harvest deterioration to cassava. The project seeks to accelerate the process via the development and deployment of low cost marker tools associated with the above mentioned genes. During the first twelve months of the project the following outputs were obtained

Methodology

Back cross and selfed mapping populations for the identification of molecular markers for genes controlling delayed PPD, resistance to the cassava hornworm and white were developed last year. They include open pollinated and controlled crosses between CW429-1 (F1 hybrid of M.

walkerae) and several elite lines (BC1), a cross between MNG11 (BC4 derivative of M. glaziovii) and CMD resistant donor parents, and a cross between CW67-7 (F1 hybrid of M. esculenta sub spp flabellifolia) and several elite lines. Over 1,000 crosses were made from April 2004 to December 2004 and 1,243 seeds were collected from crosses of CW429-1 and MNG11. Of this number, 900 seeds were viable and germinated in vitro from embryo axes according to standard procedures in the cassava tissue culture laboratory at CIAT (CIAT 2003). The plantlets derived from embryo axes were multiplied over 2-3 cycles to produce 8-10 plants for screen house hardening. The plantlets were hardened in the screen house according to standard methods (CIAT 2003) in preparation for transplanting to a single row trial in the field at ICA-Corpoica.

Results

A total of 605 genotypes of back cross and selfed populations have been obtained from CW429-1 and MNG11 (Table 1), crosses from CW67-7 are currently being processed. Between 4 and 8 plants of each of the above genotypes have been successfully hardened in the screen house in preparation for transfer to the field. They will be transferred to the field in January for the first cycle of evaluation in replicated trails. The parental lines of these mapping populations are also being evaluated with over 850 SSR markers to identify polymorphic markers for bulked segregant analysis and QTL mapping.

Table 1. BC_1 and S_1 mapping populations for QTL mapping of post-harvest deterioration (PHD), resistance to whiteflies and hornworm

Family	Female Parent	Male Parent	No. of seeds received	No. of non-viable seeds	No. of seeds planted	No. of plants obtained	% recovery of plants
B1PD280	CW 429-1		569	152	417	304	73%
B1PD284 A	TAI 8	CW 429-1	108	28	80	21	26%
B1PD284 B	CW 429-1	TAI 8	187	50	137	79	58%
B1PD289	CW 429-1	SM909-25	103	28	75	57	76%
S1CW	CW 429-1	CW 429-1	2	0	2	2	100%
S1CH	NGA 11	NGA 11	43	30	13	10	77%
B5RC1	C-19	NGA 11	52	12	40	34	85%
B5RC2	C-243	NGA 11	16	2	14	12	86%
B5RC3	C-6	NGA 11	113	35	78	50	64%
B5RC4	C-18	NGA 11	24	2	22	19	86%
B5RC5	C-413	K150-309	26	4	22	18	82%
TOTAL			1243	343	900	605	67%

Conclusion and Perspectives

Several populations have been developed for genetic mapping of genes controlling resistance to whiteflies and hornworm and delayed post harvest deterioration. The plants are being hardened in the screen house at the moment and will be transferred to the field early next year. The populations will be established in a replicated trial and evaluated for the target traits.

1.2.16 Resistance to the cassava green mites in first backcross derivatives of *M. esculenta* sub spp *flabellifolia*

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Funding: Generation Challenge Program (GCP), CIAT

Important Outputs

Evaluation of BC_1 derivatives of *Manihot esculenta sub spp Fabellifolia* for resistance to cassava green mites (CGM) over a period of 2 years in replicated trials
Development of markers for resistance to CGM using BC_2 derived from selected BC_1 progenies

Introduction

Cassava green mites (CGM) is one of the most important pests of cassava in regions with seasonal dry spells in causing damages in the foliage and considerable losses in the production in Africa and South America. There are about 40 species of mites that attack cassava but the most economically important one are species of the genus *Mononychellus*. Evaluation of the CIAT cassava germplasm bank has led to the identification of about 300 accessions or 6% with a moderate level of resistance to *Mononychellus tanajoa*. More recently F_1 inter-specific hybrids with wild progenitors of cassava, *Manihot esculenta sub spp fabellifolia*, have led to the identification of additional sources of resistance, these new sources of resistance provides the possibility of breeding for high levels of resistance to cassava green mites in the cultivated gene pool. The F_1 inter-specific hybrids were back crossed to cassava and evaluated over 2 seasons in the field, at the same time selected BC_1 individuals with moderate to high levels of green mites resistance were backcrossed to cassava to generate 43 families BC2 (CIAT 2003). The BC_2 families are being evaluated in the field this year and are also the basis for a search of molecular markers associated with resistance to green mites.

Methodology

The green mite pest tends to attack in a localized manner and homogenous pest pressure in the field is uncommon therefore there is a need to evaluate plant materials over several years to avoid escapes due to low pest pressure. Figure 1 describes the scheme for the generation of inter-specific hybrids and backcross populations and the multi-stage evaluation of resistance to cassava green mites as well as perspectives for the development of molecular markers associated with resistance

The evaluation of green mite resistance is based on a severity of the damage scale with values between 1 - 6 where 1 is total absence of damage and 6 severe damage with complete leaf necrosis and apical defoliation. Evaluation was conducted by the cassava entomology group directed by the Jose Maria Guerrero. In order to identify markers associated with resistance to CGM two sets of plant materials were employed: the BC_1 families, that have been evaluated over three cycles, and the BC_2 (AR) families derived from crossing resistant BC_1 individuals to CGM susceptible CIAT elite parental lines, the resistant BC_1 individuals and AR families were planted

in a single row or advanced yield trial (replicated) this year and will be evaluated during the dry spell early in 2006.

Genotypes with symptom damage of less than 3 will be bulked, resistant bulk, and those with 4-6 will be constituted into the susceptible bulks, bulks will be made on a family basis. The bulks will be evaluated with the 850 SSR markers available for cassava to select polymorphic markers which will then be evaluated in the progeny to identify markers associated with CGM resistance.

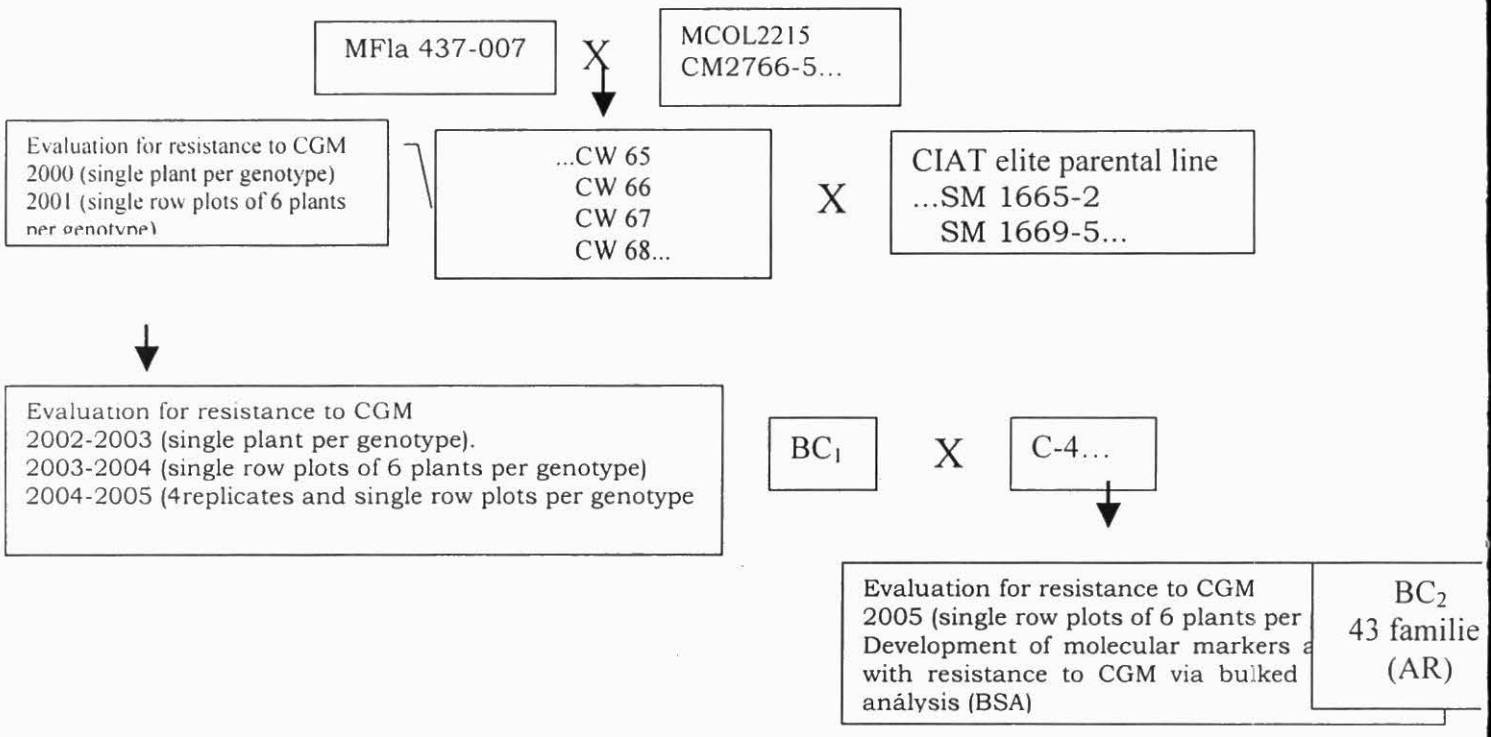

Figure 1. Pedigree of plant materials used for the introgression of CGM resistance from wild progenitors of cassava and evaluation of resistance

Results

Of the 429 BC_1 genotypes evaluated in the period 2002 (seedling trial), 2003 (clonal evaluation), and in 2004 (preliminary yield trial), 27 genotypes showed consistent high levels of resistance to the cassava green mites (Table 1). A major problem with these BC1 genotypes was the gradual loss of genotypes due to poor germination after storage of woody stems before planting as required by phyto-sanitary measures to control white flies of a one month period of cassava-free fields. Of the 429 genotypes that were used to initiate evaluations in 2002 only 268 genotypes (62.8%) are left. Another trait of interest found in these backcross derivatives as very high dry matter content, a selection index based on resistance to green mites, dry matter, and yield was used to select 30 genotypes for establishment in the crossing block, a selection pressure was of 12%. The BC_1 genotypes observed to be resistant to cassava green mites and BC_2 progenies (AR families) derived from some of the BC_1 individuals is appropriate material to begin looking for markers associated with resistance to CGM. Resistant BC_1 individuals and AR families were planted in a single row trial this year and will be evaluated during the dry spell early in 2006

Conclusion

Search for resistance to cassava green mites (CGM) in wild progenitors have led to the identification of 27 BC_1 progeny with high levels of mites resistance. These lines and their BC2 progenies are being used for the development of markers associated with resistance to CGM.

Table 1. Selections of BC_1 progenies showing moderate to high resistance to CGM used, some of the BCI individuals were used to generate the BC_2 families (AR)

Genotype	Female Parent	Male Parent	1st Objective	2nd Objective	Evaluation 2004	Evaluation 2004	Average 2003-2004
CW 215- 1	SM 909- 25	CW 66- 60	Z02	Green Mites	2	3	2.5
CW 220- 9	SM 1219- 9	CW 67- 123	Z02	Green Mites	1	3	2
CW 224- 6	SM 1460- 1	CW 66- 60	Z02	Green Mites	3	3	3
CW 225- 6	SM 1460- 1	CW 66- 62	Z02	Green Mites	1	3	2
CW 226- 10	SM 1460- 1	CW 66- 73	Z02	Green Mites	1	3	2
CW 226- 5	SM 1460- 1	CW 66- 73	Z02	Green Mites	2	3	2.5
CW 229- 7	SM 1511- 6	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 231- 19	SM 1565- 15	CW 66- 60	Z02	Green Mites	2	3	2.5
CW 235- 10	SM 1665- 2	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 235- 23	SM 1665- 2	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 235- 46	SM 1665- 2	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 235- 82	SM 1665- 2	CW 67- 87	Z01	Green Mites	1	3	2
CW 236- 15	SM 1669- 5	CW 66- 19	Z01	Green Mites	1	3	2
CW 236- 16	SM 1669- 5	CW 66- 19	Z01	Green Mites	1	3	2
CW 236- 17	SM 1669- 5	CW 66- 19	Z01	Green Mites	1	3	2
CW 242- 2	SM 1669- 7	CW 67- 87	Z01	Green Mites	1	3	2
CW 246- 5	SM 1741- 1	CW 67- 91	Z02	Green Mites	4	3	3.5
CW 248- 2	SM 1778- 45	CW 67- 45	Z02	Green Mites	1	3	2
CW 258- 3	MTAI 8	CW 66- 60	Z01	Green Mites	2	2	2
CW 259- 21	MTAI 8	CW 66- 73	Z01	Green Mites	2	3	2.5
CW 259- 40	MTAI 8	CW 66- 73	Z01	Green Mites	2	3	2.5
CW 260- 5	MTAI 8	CW 66- 74	Z01	Green Mites	1	3	2
CW 76- 1	CM 3306- 4	CW 68- 3	Z01	Green Mites	2	3	2.5
CW 76- 5	CM 3306- 4	CW 68- 3	Z01	Green Mites	1	3	2
CW 76- 7	CM 3306- 4	CW 68- 3	Z01	Green Mites	1	3	2
CW 80- 1	CM 7951- 5	CW 67- 42	Z02	Green Mites	1	3	2
CW 81- 2	CM 7951- 5	CW 67- 98	Z02	Green Mites	1	2	1.5

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1.2.17 Evaluations of wild relatives of cassava for resistance to the cassava bacterial blight (CBB) in the screen house and association of resistance with Resistance Gene Analogs (RGAs)

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CIAT

Funding: Generation Challenge Program (GCP)

Important Outputs

Evaluation in the greenhouse of BC_2 derivatives of M. esculenta sub spp flabellifolia for reaction to cassava bacterial blight (CBB)

Detection of candidates for analogous genes of resistance to CBB.

Introduction

Several BC_2 derivatives of *M. esculenta* sub spp *flabellifolia* designated as AR and developed to transfer resistance to mites from the wild progenitor to cassava were evaluated for reaction to different isolates of *Xantomonas axonopodis* p.v. *manihotis* causative agent of the cassava bacterial blight. The parents of these BC_2 families have not been scored for reaction to CBB but the wild *Manihot* parent comes from the Amazon basin of Brazil where the disease is endemic.

Methodology

Evaluation for reaction to CBB was carried out in the greenhouse using 78 genotypes of the AR families (AR) and three insolates of *Xantomonas axonopodis p.v. manihotis*. The isolates used were CC3 from Codazzi located in the Department of César, northern area of Colombia, and two insolates identified as VM12 and VM14 from Villavicencio in the Eastern Savannah plains of Colombia. Plant materials were *in vitro* plantlets of the AR genotypes that have been hardened in the screen house for 3 to 4 months, four plants were used per genotype or three in some cases depending on the availability of plant material. One plant each was inoculated with the three aforementioned isolates separately by puncturing the stems with a needle and syringe filled with a suspension of the bacteria. Positive control of inoculation was carried out using MCol 1505 (susceptible) and Chiroza (tolerant) and a negative control with one plant per AR genotype injected him sterile distilled water. Evaluations were made 12, 19, and 26 days after inoculation respectively. A symptom severity scale with 0.5 units graduation from 1.0, asymptomatic plants and 5.0, completely wilted plants (die-back) was used to evaluate disease reaction after inoculation.

Results

The evaluation of the genotypes of the AR families in the greenhouse showed clear cut differences in response to infection by *Xantomonas axonopodis p.v. manihotis*. A frequency of distribution of reaction types to the 3 isolates of the pathogen is shown in Figure 1. Response to

the strain CC3 from Codazzi revealed 28% were susceptible individuals (score of 4-5), 30% of intermediate response (score of 3), and 18% resistant individuals (score of 1-2)

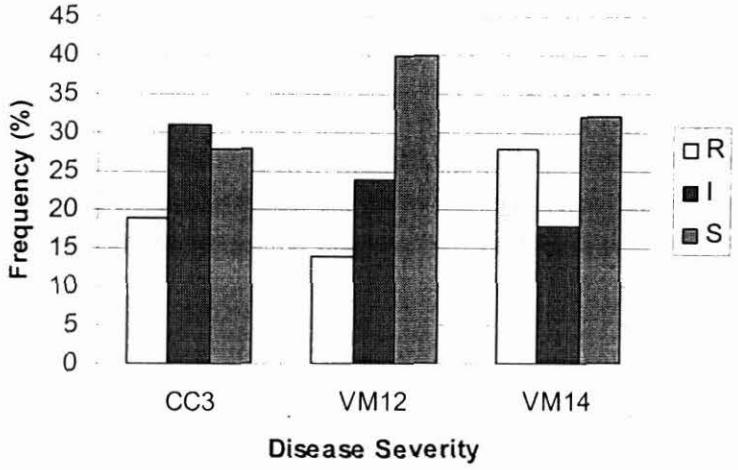

Figure 1. Distribution of individuals of the AR families in accordance with its resistance to three insulations of *Xanthomonas axonopodis* pv. *manihotis*. Strains: CC3, isolated from Codazzi (César); VM12 and VM 14, isolated from Villavicencio (Goal).R= resistentes; I= intermedios; S = susceptibles.

The strain (VM12) was the most virulent of all the 3 strains used, 40% of the individuals were susceptible to this strain, while only 21% of the individuals evaluated with this strain showed resistance. The third evaluated insulation (VM14) showed 32% of susceptible individuals, 17% of intermediate affected individuals, and 27% of resistant individuals.

With regard to the genotypes, the highest levels of resistance was observed in the family AR-9 obtained from crossing CW-257-12, a BC_1 derivative of *M. esculenta* sub spp *flabellifolia*, as male parent to C-243, a CMD donor parent, as mother. Genotypes AR-9-24, AR 9-44 and AR 9-51 from this family were resistant against all three insolates of the pathogen. The genotype AR 7-14 showed no symptoms with just the CC3 strain, while 5 genotypes AR 6-7, AR 6-3, AR 6-14, AR 1-123, and AR 8-8 did not show symptoms with VM12 and VM14, both isolates of plant samples from Villavicencio, but were infected by CC3. With regard to the distribution of the disease response, the isolates CC3 and VM12 showed a normal distribution, with the majority of genotypes having an intermediate response, while the isolate VM14 has a distribution that was skewed to the right.

Conclusion and perspectives

The response of a number of genotypes of BC_2 derivatives of *M. esculenta* sub spp *flabellifolia* to different isolates of *Xantomonas axonopodis* p.v. *manihotis* suggests that there may be a useful source of resistance to cassava bacterial blight (CBB) in the wild progenitor of cassava. The results obtained will be further tested using inoculation of screen house plants raised from woody stems and also evaluations under natural pathogen infection conditions.

1.2.18 Standardization of the SDS-PAGE methodology for Characterization of Proteins in Cassava Roots during introgression of High Root Protein from Accessions of *Manihot esculenta* sub spp *Fabellifolia* and *Manihot Tristis* into Cassava

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Important outputs

Standardization of a SDS-PAGE analysis method towards a rapid characterization of proteins contained in high protein content Inter-specific hybrids from wild relatives and cassava genotypes

Introduction

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 fourth year at CIAT. Development of a tool to rapidly assess protein in large segregating populations was initiated last year as a way of accelerating the development of markers for high protein content. The suitability of the SDS-PAGE methodology for determination of total protein profile was assessed and several protocols tested (CIAT 2004). We describe here identification of an appropriate protocol for reproducible assessment of proteins in dried root flour samples.

Methodology

Samples for SDS-PAGE gel analysis of protein content were root flour from some inter-specific hybrids (Table 1) with 6-8% of crude protein from evaluations conducted in August 2003 and May 2004. The samples were evaluated using a saline buffer extraction protocol described by Jorgensen (2005, personal communication) with some modifications. Six different saline buffers were used (Table 2), a range of sample sizes between 40 mg and 500 mg; and two loading buffer; blue juice (CIAT 1996) and cracking buffer (Laemmli, 1970), were selected to identify the most suitable. Samples were suspended in of 200 µl saline buffer, spun for 30 min at 15000 rpm in a table top centrifuge, and the supernatant carefully transferred to new tubes while avoiding the pellet as much as possible. The supernatant was spun for 10 min at 15000 rpm and the appropriate loading buffer (blue juice or cracking buffer), in different concentrations –1:1, 2:1, and 3:1 was added. Proteins were completely denatured by immersing the sample tubes in a boiling water bath for 5 minutes, the samples were briefly centrifuged at 14000 rpm to pellet cellular debris and 12, 15 or 20 µl. loaded on the SDS-PAGE gel. SDS – PAGE denaturing electrophoresis was according to Laemmli (1970) as modified by CIAT (2003). Constant voltage of 180 V, 80 mA and 50 W, at 10 ◦C were applied until the tracking dye reached the bottom of the gel. A *Phaseolus vulgaris* seed protein sample was used as positive control in every gel assay.

After electrophoresis, gels were stained in a solution containing 50% Methanol, 10% Acetic acid and 0.25% Coomassie

Table 1. Percent protein content of inter-specific lines selected for SDS-PAGE analysis

	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	61	CW 177-28	OW 132-2	CM 1585-13	6.7
18	70	CW 177-37	OW 132-2	CM 1585-13	7.73
19	84	CW 177-51	OW 132-2	CM 1585-13	7.05
20	87	CW 177-54	OW 132-2	CM 1585-13	7.17
21	89	CW 177-56	OW 132-3	CM 1585-14	6.77

Table 2. Different saline buffers for isolation of proteins in cassava root.

Saline buffers	Reference
NaCl 0.5 M pH 3.2, cracking 30 mg	Laemmli, 1970
Na_3PO_4 0.05M pH 7.0, PVP 2%	Jasso et al. 2002 (modificated)
KCl 0.1M, cysteine, pH 7.3	Bourdon, 1988
SDS 5%, glycerol 10%, Tris 80mM, DTT 25mM, pH 6.8	Carvalho. Et al, 2004
Na_3PO_4 0.05 M	Carvalho, 1992
Na_3PO_4 0.05M, PVP 2%, acetone wash, pH 7.0.	Rodriguez et al, 2002

Brilliant Blue R, over night at RT. Gels were washed with water and de-stained by two changes of de-staining solution, first with 50% Methanol and 10% Acetic acid, and secondly with 5% Methanol and 7% Acetic acid.

Results

SDS-PAGE analysis of the protein was conducted using different samples sizes and different saline buffers of which 300 mg of sample and the saline extraction buffer Na_3PO_4 0.05M (Table 2), gave the best results of band resolution and intensity (Fig. 1).

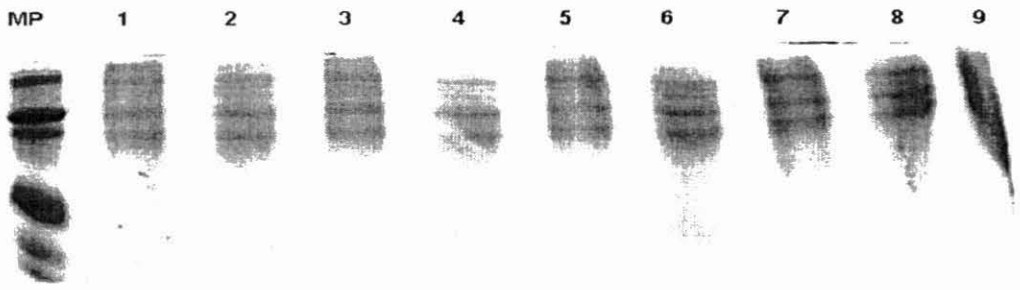

Figure 1. Visualization of bands in SDS-PAGE denaturing electrophoresis gel using several samples sizes prepared with Na_3PO_4 0.05M saline buffer of accessions number 31. 1) 200 mg of sample + Blue Juice; 2) 250 mg of sample + 2β- mercaptoethanol; 3) 250 mg of sample + Blue Juice; 4) 300 mg of sample + 2β- mercaptoethanol; 5) 300 mg of sample + Blue Juice; 6) 400 mg of sample + 2β- mercaptoethanol; 7) 400 mg of sample + Blue Juice; 8) 500 mg of sample + 2β- mercaptoethanol; 9) 500 mg + Blue Juice.

The best loading buffer fwas the blue juice and the best dilution was 2:1. With respect to sample volume, 50 µl enhanced resolution of bands in the gel (Fig. 2).

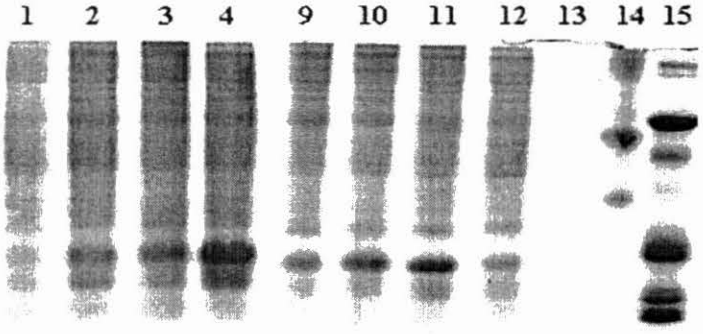

Figure 2. Visualization of bands in SDS-PAGE denaturing electrophoresis gel of several accessions of cassava with different loading sample volume. 1)Accession 33, 80µl; 2) Accession 1, 100µl; 3) Accession 9, 100µl; 4) Accession 15,100µl; 9) Accession 3, 50µl; 10) Accession 7, 50µl; 11) Accession 11, 50µl; 12) Accession 16, 50µl; 13) Accession 19, 50µl; 14) Sample control (*phaseolus*); 15) Molecular marker.

Conclusions and Perspectives

Generation of backcross families for QTL mapping and development of improved varieties with high protein content was continued this year. Standardization of SDS-PAGE protein analysis made possible the identification of a good extraction protocol for rapid evaluation of breeding populations to identify high protein content varieties. Future perspectives include the establishment and evaluation of the BC_1 QTL mapping populations for high protein content using both the total protein method (Kjedhal method) and the SDS-PAGE methodology.

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1.2.19 New molecular markers generation (scars) for apomictic plant selection in *brachiaria decumbens*

Mauricio Quimbaya, Gerardo Gallego and Joe Tohme

Introduction

Apomixis is a desirable character for breeders in specific forage improvement programs, but the suitable selection of genotypes and individuals with this character is most of the times a slow, complicate and time-consuming labor (Grimanelli, 2001), for this reason, molecular markers as SCARS and MAS procedures (Molecular Marker-Assisted selection) play a basic role for apomictic genotypes identification (Mohan, 1997).
According previous investigations, SCAR N-14 marker has a strong association with apomictic behavior of *Brachiaria decumbens*; beside this, during MAS procedures mediated by this marker a not enough association (less than 100%) has been evidenced; because of that, a molecular searching for new markers genetically close to SCAR N-14 and therefore close to the gene or genes that regulate the apomictic character of *B. decumbens* is necessary with the purpose to establish a direct link between this generated markers and the apomictic behavior of this specie. So in this way and taking in count the linkage map for *B. decumbens* that was built by Rosero *et al* (2005), the AFLP markers that were mapped within the same linkage group of SCAR N-14 were eluted, re-amplified, cloned and sequenced to generate new molecular markers (SCARs). In this way five new SCARs markers were generated; two of them (ScC6b2 y ScC6b6), were mapped in population; ScC6b2 were mapped at 35cM of SCAR N-14 and ScC6b6 does not mapped within molecular map; one Scar (ScC7b6) does not produce any amplification, another (ScC6b4) was amplified in *B. decumbens* and in *B. ruziziensis* also, and the last one (ScC3b2) is being standardized. On further investigations a stronger association between these new markers and the apomictic behavior of *B. decumbens* should be demonstrated to increase the effectiveness of MAS procedures.

1. Materials and Methods

1.1. Bands selection and elution

According to *Brachiaria decumbens* linkage map (Rosero, *etal*, 2005), five AFLP markers originated from 3 AFLP+3 combinations and one SSR (table 1) were segregated in the same linkage group with SCAR N-14 (Linkage group 8). The AFLP combinations that produced those AFLP markers were PCR amplified and visualized on 6 % acrylamide gels, afterwards bands were directly cut from gel and placed into a ephendorf tube with 20 µl of PCR water. The process was done by triplicate for each band. Tubes with bands were placed into a boiling water bath during 10 minutes for DNA recovering and storage at 4°C.

Table 1. Markers mapped inside linkage group 8.

Marker	Type of Marker	Combination	Distance from N-14	Originated SCAR
C7b6	AFLP	E-AAC/M-CAA	44.5 cM	ScC7b6
C6b2	AFLP	E-AGG/M-CAG	30.8 cM	ScC6b2
C6b4	AFLP	E-AGG/M-CAG	26.6 cM	ScC6b4
C3b2	AFLP	E-ACG/M-CAG	7.6 cM	ScC3b2
rC6b6	AFLP	E-AGG/M-CAG	5.3 cM	ScC6b6
rGM-73a	SSR	-	73.3 cM	-

1.2. Bands confirmation

Eluted Bands were confirmed by PCR amplification with respective AFLP+3 primers that produced them; to establish the right cut of specific bands, they were visualized in 1.5% agarose gel and 6% acrylamide gel, if more that one band were cut or if the band size did not fit with the proper expected band size, band excision and elution were made again.

1.3. Bands ligation and cloning

Once Bands size were confirmed, they were ligated into *PGEM-T easy* ligation system, ligation reaction includes 5 µl of ligation buffer, 1 µl of *PGEM-T easy* vector, 1 µl of ligation enzyme, 2 µl of PCR product and 1 µl of PCR water; reaction was incubated for 1.5 hours at room temperature, afterwards was cleaned using sec-butanol. For cloning procedure 3 µl of clean ligation was mixed up with 20 µl of *DH5α E. coli* competent cells and electrophored at 406Ω; cells were recovered on LB medium during 1 hour at 37°C with constant agitation. 80 µl of recovered cells were plated on IPTG/X-gal/ampicillin /LB-solid medium, plates were incubated at 37°C overnight and selection of transformed cells was made by means of white/blue colony selection.

1.4. Plasmid extraction and bands re-amplification

Some white colonies were selected picked up and grown on LB medium with ampicillin at 37°C with constant agitation overnight, afterwards cells were centrifuged and plasmid-DNA was extracted using *Quiagen* mini-preps protocol. Plasmid DNA was amplified by PCR using T7/SP6 primers and 1/200 diluted plasmid DNA. Bands size was confirmed visualizing PCR amplification in 1.5 agarose gel (Figure 1).

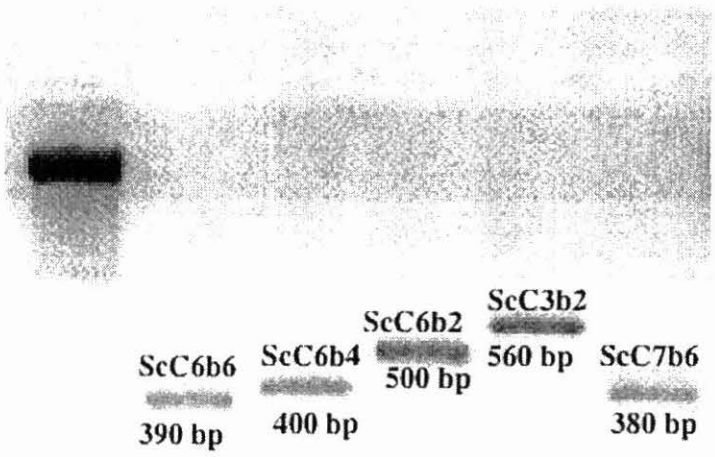

Figure 1. Bands re-amplification after plasmid extraction, where bands size corresponds with the expected one.

1.5. Bands sequencing, editing and primers design

Scars ScC6b6, ScC6b4 and ScC76 were sequenced from T7 side, while Scars ScC6b2 and ScC3b3 were sequenced from both sides T7 and SP6. Sequence reaction included 2 µl of big dye, 2 µl of sequencing buffer, 1 µl of primer, 1.5 µl of plasmid DNA and 3.5 µl of PCR water. After PCR amplification reaction was cleaned using ethanol/sodium acetate cleaning protocol. Produced sequences edition and contig establishment were made using *sequencher 4.5* program. Primer design was carried out following Abd-Elsalam recommendations (Abd-Elsalam, 2003) by means of *Vector NTI 8.0* program (Table 2).

Table 2. Designed primers for SCARS amplification

Primer Name	Product Length	Primer Length	TM Temperature	GC content
ScC7b6F	200 bp	20 bp	50.7 °C	50 %
ScC7b6R		25 bp	51.6 °C	40 %
ScC3b2F	430 bp	22 bp	55.6 °C	54 %
ScC3b2R		23 bp	53.2 °C	52%
ScC6b2F	220 bp	20 bp	50.7 °C	55 %
ScC6b2R		20 bp	51.6 °C	55 %
ScC6b6F	160 bp	24 bp	52 °C	42%
ScC6b6R		20 bp	53 °C	55%
ScC6b4F	100 bp	21 bp	53.7 °C	48 %
ScC6b4R		22 bp	53.9 °C	50%

1.6. SCARS mapping

SCAS were amplified by PCR (using previously designed primers) and those ones with appropriate band pattern (*B. decumbens*, band present and *B. ruziziensis* band absent) and properly band size were amplified on *B. decumbens* X *B. ruziziensis* population, data were taken as band present (1) or band absent (0); information was used for matrix construction and SCARS were mapped using LOD= 12, a recombination frequency r= 0.3 and Kosambi function by means of *MapMaker/EXPv3.0b* software (Figure 2).

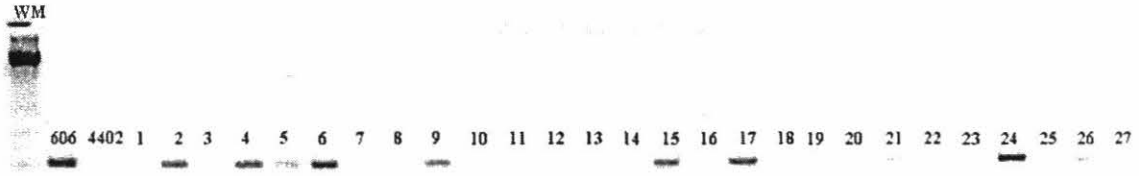

Figure 2. Some individuals of SCAR ScC6b2 that were read on *B. decumbens* X *B. ruziziensis* population generating a band present (1) or band absent (0) matrix.

Results and discussion

From bands sequencing, five new SCARS were generated (Figure 3). Two of them (ScC6b2 y ScC6b6), were mapped in population; ScC6b2 were mapped at 35cM of SCAR N-14 which is expected since SCAR ScC6b2 was produced from C6b2 AFLP band located at 31 cM of SCAR N-14 and ScC6b6 does not mapped within molecular map. This marker showed distortion on segregation analysis being statistically far from 1:1 segregation ratio, affecting its ligation to the map. SCAR ScC3b2 is being standardized to obtain a defined band that could be read in population. Scar ScC7b6 does not produce any amplification and SCAR ScC6b4 was amplified in *B. decumbens* and in *B. ruziziensis* also, this could suggest that the primers that were used to this last two SCARS amplification have some kind of problems, being necessary a new primers design from AFLP sequences. In the case of SCAR ScC6b4 the band presence on both parents could suggest that a band present on *B.decumbens* and *B.ruziziensis* was cloned instead of the desired one, this because of the difficulty to cut close bands even if they were run on acrylamide gels.

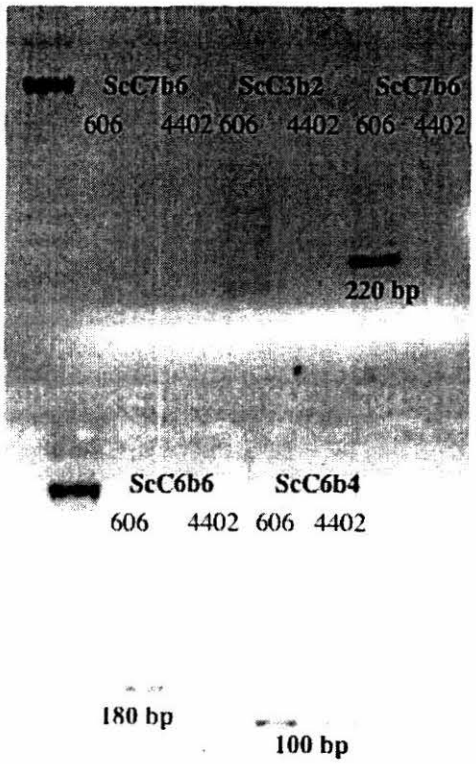

Figure 3. SCARS generated from AFLP bands close to SCAR N-14 sequencing. ScC7b6 does not produced any amplification, SCARS ScC3b2 and ScC6b6 generated the expected band pattern; ScC6b4 amplified on both parents and ScC3b2 is being standardized.

Ongoing work

Standardization of SCAR ScC3b2 and new primes design for SCARS ScC7b6 and ScC6b4 have been developed.

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1.2.20 QTLs Mapping of Aluminum Resistance in *Brachiaria* sp.

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Highlight

- Found acceptable associations between aluminum resistance variables and moleculars markers from the linkage map of *Brachiaria decumbens,* localizating the first QTLs for this complex characteristic in this specie.

Introduction

Aluminum toxicity (Al) constitutes one of the biggest limitations for agronomic productivity in acid soils affecting almost all tropical and subtropical countries, wich represent 40% of the arable land of the earth. When the soil's pH is low Al turn soluble (Al^{+3}) and micromolares concentrations inhibit root development, decrease nutrient acquisition and grow of the most vegetal species (Ma J, et al., 2001). In Tropical America, where 50% of land has this condition, some species from *Brachiaria* gender are forage pasture of great importance commercial for cattling, being *Brachiaria decumbens* the specie more spread in the zone (Rao I, *et al.*, 1998) and the most aluminum toxicicity resistant.

Since awhile the mechanism for the plants resist under this condition has been studied, however great variation shown into and inter species has made difficult the understanding of this complex character (Hoekenga O, *et al.*, 2003). Therefore genetical approaches have been driven with the purpose to provide a strategy sustainable to increase crop productivity with low cost and enviromental impact (Rao I, *et al.*, 1998). The molecular markers based in DNA have made possible to elucidated genetic factors that govern complex characteristic, using QTL´s mapping, finding zones inside the genome with genes that contribute the variation of one characteristic. Given that, the aluminum resistant response was broached from quantitative trait loci (QTL´s) view, applying genetic mapping strategies over F1 segregant population of *Brachiaria* with AFLPs, SSRs and Scar`s markers.

Materials y Methods

Plant Material and DNA extraction: An F1 hybrid population (263 plants) from the interspecific cross between *B. decumbens (*606, Al resistant) and *B. ruziziensis* (4402, susceptible), was used for this study.

Molecular Characterization: SSRs primers development from *B. decumbens* and AFLPs primers combinations (EcoRI/MseI), previously tested in the parents and determinate as polymorphics were evaluated in the population (for the PCR amplification conditions and methodology procedure information see SB-2 Annual Report, 2004). Additionally, SSRs primers from Rice were evaluated and few SCARs markers that were developed from AFLPs alleles were tested in this population.

Segregation Analysis and Linkage Map Construction: Polymorphics markers of each parental were subset, and a X^2 analysis was made (global α=0.05%) for each group for test the goodness

of fit to 1:1 and 3:1 or 5:1 ratios, for markers with single dosage (SDM) and double dosage (DDM). A linkage map was constructed (only with SDMs) with the Mac computer program MapMaker/EXP v3.0b (LOD min=12.0 and r= 0.3), calculating the distances (in cM) by Kosambi's mapping function.

Phenotypic evaluation of aluminum stress resistance Five quantitative variables (root length, root diameter, abundance of root tips, root length/shoot biomass and specific root length) indicative of Al resistance was employed for QTLs analysis. The Aluminum effect (eal) over each genotype of the population was calculated doing a LS mean over each data variable. Genotypes without replications were excluded of the posterior phase of the analysis, as well as the data with total length less than 90 cm, because its great response variability to Al stress conditions.

QTLs Mapping of Aluminium Resistance QTLs analysis was preformatted in WinQTLCartographer v2.5 program (9). Three kind of analysis was made: Single point analysis (SPA), Interval mapping (IM) and Compositive interval mapping (CIM). The threshold to report a QTLs (LOD) was calculated by permutations methodology with 1 000 permutations (α= 0.05).

Results and Discussion

Polymorphism type and SDMs detection: From 73 SSRs primers sets of *Brachiaria*, only 36 were tested in the population due to their polymorphism, these primers amplificated 66 alleles for parental 606 and 33 for 44-02. Because amplifications problems 27 SSRs were unable to evaluated. Five primers were monomorphics, and others five there are no standarizathed yet. Three SSRs primers from rice chromosome one, have been evaluated in the population producing five alleles for parental 606, also two Scar markers (ScC6b2 and ScC6b2) have been possible scored.

Twelve AFLP's primers combinations were evaluated for its high polymorphism, scoring 454 markers allelic loci due its complex banding pattern. Of these, 229 bands were shared, the rest were polymorphics, but from these ones almost 3/4 are bands from parental 606 (179). This amount of molecular information sharing shows a strong relationship between the genomes of 606 and 44-02.

A low percentage from AFLP's polymorphics alleles of each parental (14.5% for 606 and 15.2% to 4402) were not evaluated, because lack of segregation in the population, this last fact depends on the marker dosage, being one of the biggest inconvenient of polyploids species (Barcaccia G, *et al.*, 2003). The SSRs are more effective markers for SDMs detection, this separation is very important when its works with polyploids species because the others configurations use could be endanger the map precision, affecting the distance as well as the markers positions (Albertini E, *et al.*, 2003).

Linkage Map Construction: A linkage map with 19 linkage groups was constructed for the tetraploid specie *B. decumbens*, the map have 180 SDMs from which 114 are AFLPs, 64 SSRs and 2 SCAR markers, covering 1362.9 cM (Figure 1).

The 19 linkage groups obtained are closer to the haploid number of this specie (18 chromosomes). Perhaps, the small size of *B. decumbens* chromosomes (Do Valle C & Sadivan, 1998), explain the fact that with only 180 markers we achieve a linkage groups number closer to the search, and point out a considerable genome coverage avoid to get a good saturation level in the present linkage map.

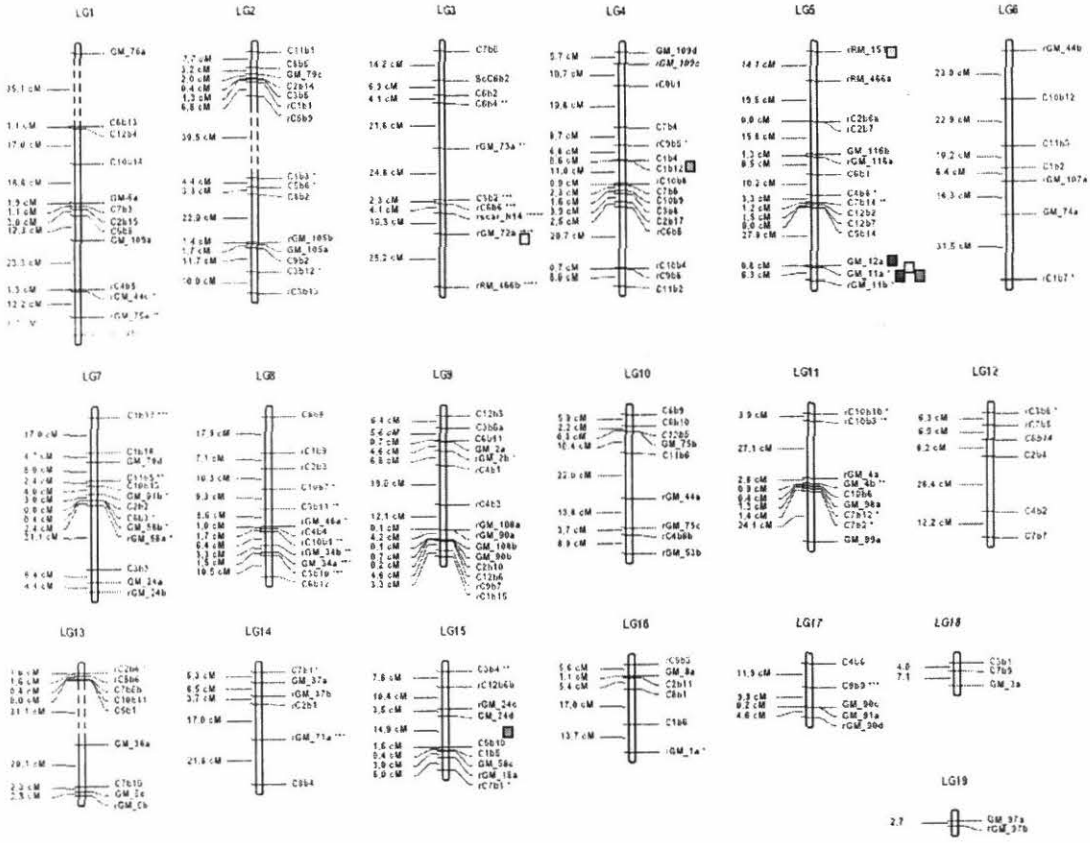

Figure 1. Genetic Linkage Map of *B. decumbens*, with Al resistance QTL's localization. SDMs of AFLPs, SSRs, and SCARs, in acople phase and repulsion (r) was used in this map, the distortion of the markers of the 1:1 proportion ratio are indicated by *, **, ***, ****. Distances between markers up 30 cM, are shown with discontinue line. The boxes show the most closer marker associated to the region with the characteristic of interest: eal_rl, eal_rd, al_rlsdw and al_srl.

Aluminum Resistance QTLs Mapping The variables useful to indicated the aluminum resistence (*eal_rl, eal_rd, eal_t, eal_rlsdw, eal_srl*) shows transgresivy, its could be due to the polyploid condition, contributing to heterosis o hybrid vigor.

With the three QTLs analysis methods (SPA, IM and CIM) was possible establishing putatives associations between moleculars markers, finding six putatives aluminium resistance QTLs. Three were localizated in LG5, the others in LG3, LG4 and LG15. The associations finding by the mapping analysis are compile in the Table 1 (Figure 1).

Table 1. Mapping analysis results. LG, position of the first marker, and the flanking markers associated with aluminum-resistance variable its indicated. In bold and underlying its shown the most closer marker with correlation.

Method	Variable	LG	Flanking Markers	QTL Position	Markers Position	LOD	Aditive	R2
IM	eal_rd	15	GM_24 - **C5b10**	36,68	21,7 - **36,6**	3,014	0,052	0,053
IM	eal_slr	3	**rGM_72a** - rRM_46b	112,16	**88,3** - 113,5	3,008	0,110	0,058
IM	eal_slr	5	**GM_11a** - rGM_11b	104,22	**104,1** - 110,4	2,297	-0,089	0,041
CIM	eal_lr	5	**GM_12a** - GM_11a	103,45	**103,3** - 104,1	3,260	-0,072	0,052
CIM	eal_lr	5	**GM_11a** - rGM_11b	108,22	**104,1** - 110,4	3,098	-0,072	0,053
CIM	eal_rd	5	**GM_11a** - rGM_11b	108,22	**104,1** - 110,4	2,993	0,051	0,050
CIM	eal_rd	15	GM_24 - **C5b10**	35,83	21,7 - **36,6**	3,317	0,052	0,054
CIM	eal_lrsdw	4	**C1b12** - rC10b8	52,34	**52,3** - 63,3	3,024	-0,081	0,050
CIM	eal_slr	3	**rGM_72a** - rRM_46b	112,16	**88,3** - 113,5	4,111	0,120	0,068
CIM	eal_slr	5	**rRM_151** - rRM_466a	0,01	**0,0** - 14,1	2,801	-0,091	0,043
CIM	eal_slr	5	**GM_11a** - rGM_11b	104,22	**104,1** - 110,4	3,010	-0,094	0,045

LG Linkage group, IM Interval mapping, CIM Compost interval mapping.

The small percentage of variation explained for each QTLs, could be due to complexity that involve the aluminium resistance characteristic, and maybe the six QTLs found, point out some genes of small efect that interacting produced the observated phenotype, however, more saturation level and/or fine mapping should be reached. Finally, its necessary confirm the QTLs here report, because only has made the phenotipic evaluation under one enviromental condition.

Further Works in Progress; (i) saturate the linkage map of *B. decumbens* (CIAT 606) with SSRs of species relates and some *Brachiairia* SSRs primers not standarizathed yet, (ii) conduct QTLs analysis for Al resistance with new data analyzed.

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1.2.21 Advances in the positional cloning of CMD2

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1. CIAT 2. Clemson University

Funding: CIAT

Important Outputs:

Construction of BAC contigs around the CMD resistance gene (*CMD2*)
Development of new BAC-end markers

Introduction

A TME-3 BAC library constructed last year at Clemson University Genomics Institute (CUGI) and was screened by a PCR-based method (Amplification of BAC pools) using 2 markers, NS158 and RME-1, tightly linked to the CMD resistance gene *CMD2* (CIAT 2004). Two and 14 positive clones were obtained for the markers NS158 and 14 RME-1 respectively. This year we initiated a chromosome walk to the *CMD2* gene, starting with contigs construction using the FPC program and then we applied two methodologies (allele specific primers and SSCP-SNP) to design the specific primers from the BAC ends to develop new molecular markers closer to *CMD2* gene region that permit another consecutives round of BAC library screening and development of marker closer to the *CMD2* gene.

Methodology

Positive BAC clones were digested with the *Hind III* enzyme and restricted fragments separated on a 2% agarose gel. The gel restriction profile was captured as a .tif file and analyzed with the Fingerprinting Contig (FPC) program (Marra et al 1997) using astringent parameters (tolerance=7 and cutoff= 1e12) to define clone overlapping. The clones located at the ends of each contig were identified and their BAC ends sent for sequencing at the Iowa State University sequencing facility (http://www.dna.iastate.edu). The sequences were edited from vector contamination and analyzed in the genome database with the Blastx algorithm (Altschul et al 1997); primers were designed from each sequence using the Primer 3.0 program to produce an amplification product of between 300-350 bp. PCR conditions were 2mM of $MgCl_2$, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase for a final volume of 25 ul, the amplification program consisted of a initial denaturation step at 95°C 2min and 30 cycles of 94°C 30 sec, 55°C 1 min, 72°C 1min and a final extension step of 72°C for 5min.

To develop allele specific primers, we amplified the resistant and susceptible parents, resistant bulk, susceptible bulk and two resistant and susceptible individuals of the fine mapping population with each primer pairs, PCR product was separated on a 1% agarose gel, and bands were eluted, cleaned using the Qiaquick PCR purification kit and cloned into the commercial vector pGemT-easy. The quality of cloned bands was evaluated by PCR with universal primers T7 and Sp6 and plasmid miniprep DNA were sent to sequencing at Iowa State University facility sequencing center. Sequences obtained with each primer were aligned using ClustalW (Thompson et al 1994) to find single nucleotide polymorphism regions and then allele specific primers were designed using the SNAPER 3 program.

To develop SSCP-SNP markers we denatured the PCR amplification products of the resistant and susceptible parental, resistant bulk and susceptible bulk for each pair of primers followed by separation by single-strand conformational polymorphism (SSCP) gels using a mutation detection enhancement (MDE) gel solution follow the conditions recommended for the author (Bertin et al 2005). The candidate markers were evaluated in each individual of each bulk and the recombinant susceptible individuals.

Results

Two contigs derived from positive clones obtained from BAC pool screening and one contig from NS158 positive clones were obtaining with the FPC program (Fig 1). The BAC clones # 9, 18,23, 33, 35 and 36 were identified as contigs ends. The BAC end sequences didn't show similarity with resistant gene sequences. The clustal W analysis for sequences obtained with the BAC #9 end primers permitted the identification of allele specific mutations that could distinguish resistant and susceptible individuals and eight pair of primers were designed on this region. The SNP primer PR2 showed differences between parentals and bulks but were not consistent in the opened bulk analysis (Fig 2). On the other hand, we obtained a clear difference between resistant and susceptible with the BAC 33b SSCP-SNP marker that was consistent in each individual of the bulk and just we found that one recombinant susceptible present the band (Fig 3)

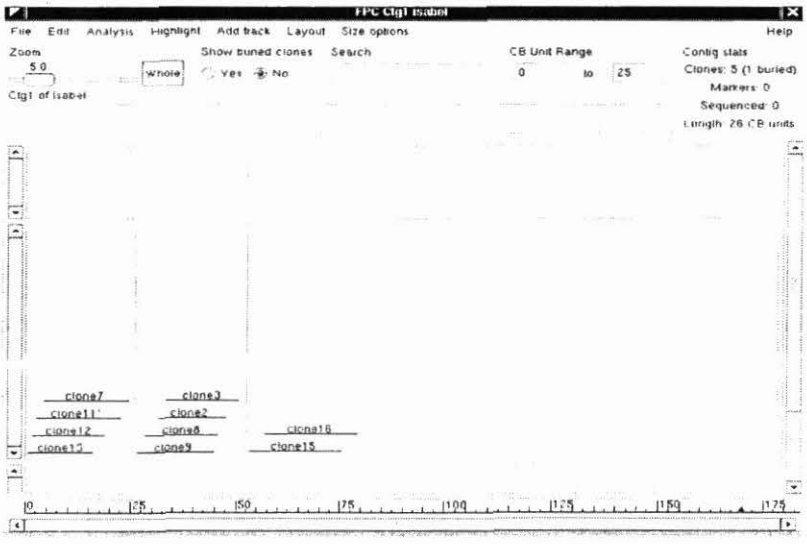

Figure 1. BAC Contigs obtained from the fingerprinting analysis using the FPC program

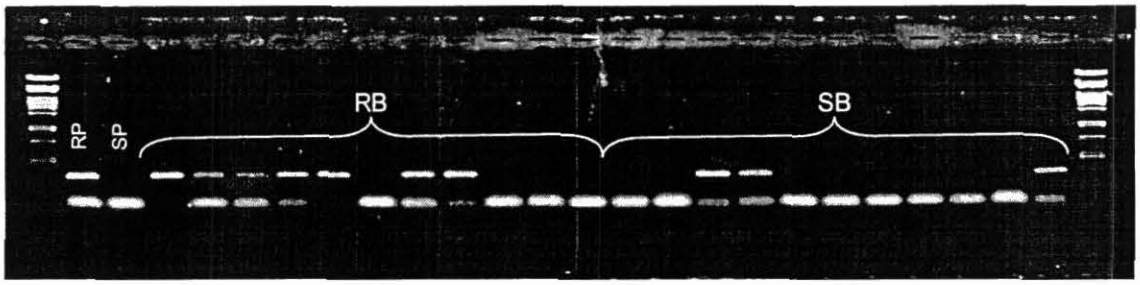

Figure 2. Segregant Bulk Analysis with PR-2 SNP primer. The sample order is: RP (Resistant parental), SP (Susceptible parental), RB (Resistant bulk) and SB (Susceptible bulk).

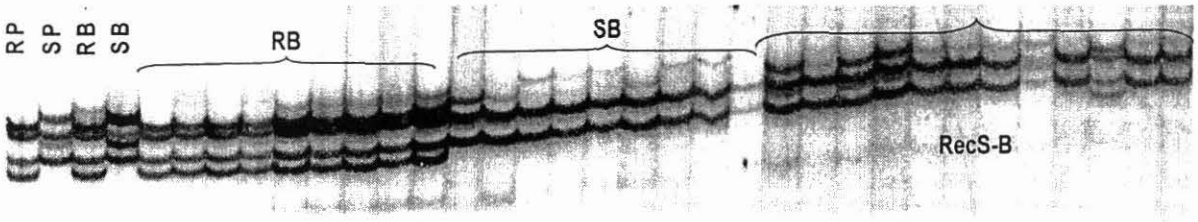

Figure 3. Segregant Bulk Analysis with the BAC33b SSCP-SNP primer. The sample order is: RP (Resistant parental), SP (Susceptible parental), RB (Resistant bulk), SB (Susceptible bulk) and RecS-B (Recombinant susceptible-Bulk).

Conclusion and perspectives:

Three BAC contigs were constructed from the NS158 and RME-1 positive clones around the *CMD2* gene. We developed a new SSCP-SNP markers from the BAC ends to initiate a new round of screening of the BAC library to continue the process of chromosome walking towards bridging the interval between the 2 markers closely linked to the *CMD2* resistant gene. The second library screen is ongoing and will be completed shortly

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1.2.22 Molecular characterization of the avocado (Persea americana Mill.) genetic resources held at Colombia: Genetic diversity and redundancy level of the Colombian collection (collaborative project with CORPOICA, funded by Ministerio de Agricultura y Desarrollo Rural, Colombia)

Contributors: C. H. Ocampo (GRU), G. Gallego (BRU), M. C. Duque (BRU), I. Sánchez (CORPOICA[1]), J. Tohme (BRU) and D. G. Debouck (GRU).
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Introduction

The cultivate avocado (*Persea americana* Mill.) is in the genus *Persea* (subgenus *Persea*) of the laurel family (*Lauraceae*). Is a sub-tropical fruit-tree, diploid with 2n = 24 chromosomes and a moderate genome size of 883 Mb (8.83 x 10^8 bp). Genome analysis and breeding of avocado is quite difficult mainly because of the size of the trees, a long juvenile phase, and a lack of satisfactory genetic knowledge (Fiedler et al., 1998).

This fruit the Aztecs called ahuacatl from the nahuatl language ahuacatl ("testicle"), referring to its shape. The avocado probably originated in a broad geographical area stretching from the eastern and central highlands of México through Guatemala to the pacific coast of Central America (Costa Rica). However, it was cultivated from the Rio Grande to central Peru before the arrival of Europeans (Bergh, B.O., 1992). Based on their presumed center of origin, the species *P. americana* has beeen commonly divided into three distinguishable ecotype or horticultural races, know as the Mexican, Guatemalan and West-Indian (Antillean) races and wich have been respectively designated as *P. americana var. drymifolia, P. americana var. guatemalensis and P. americana var. americana* (Bergh & Ellstrand, 1986). Recently, a new P. americana race (*P. americana var. costaricensis*) native from mountain region of Costa Rica, has been described (Ben-Ya'acov1 et al., 2003). *P. americana* presents a wide variety of genetic diversity, probably because avocados evolved in a part of North America, Central America, Colombia and the Caribbean region characterized itself by considerable diversity in climates, related to the varied topography of these regions. The diversity of avocado germplasm has tremendous impact on the development of the avocado industry worldwide (Bufler and Ben-Ya'acov, 1992). The molecular markers promise to help overcome of the obstacles of conventional breeding and genome analysis. In the last few years, several types of molecular markers have been used for the genome analysis of avocado, such as the isozymes, RFLPs ((cloroplast DNA and ribosomal DNA) DNA fingerprints (DFP), RAPDs, microsatellites and AFLPs (Clegg et al. (1999).

In this study we have proposed the following objectives: (1) to know the genetic diversity present in an avocado collection conserved ex—situ, using for it molecular markers (AFLPs) and (2) to know the redundancy level (possible genetic duplicates) present in the characterized collection.

Materials and Methods

Plant material consisted of 57 *Persea americana* Mill. accessions maintained by CORPOICA in Palmira (Colombia). These accessions represent a wide geographical and botanical diversity from

Colombia, Mexico, Guatemala and Trinidad y Tobago. In Addition, two wild species of *Persea* also were included as outgroup: *Persea caerulea* (young leaves collected of native trees from Valle del Cauca, Colombia) *and Persea rigens* (young leaves collected of native trees from Quindío, Colombia) (Table 1). The molecular marker selected was the AFLP technique because of the magnitude of genome coverage. A typical AFLP fingerprint contains between 50 and 100 amplified and analyzable fragments. The AFLP fingerprintin was performed basically as described manual protocol provided by Vos et al. 1995 as well as additions and changes made in our Laboratory (BRU) for avocado.

Table 1. Indentification of avocado varieties and wild species of *Persea* used in AFLP analysis with indication of their horticultural race.

Accession	Horticultural races/wild species	Accession	Horticultural races/wild species
Persea caerulea	Wild species	Nativo 2011	Antillean
Persea rigens	Wild species	Booth 5	Hybrid GuatemalanxAntillean
Trappica (C)	Antillean	Hulumana	Antillean
Lorena (C)	Antillean	1607	Mexican
Trappica (PF)	Antillean	Bacon	Mexican
Lorena (PF)	Antillean	135 27	Hybrid GuatemalanxMexican
Oriente	Antillean	Collin Red	Hybrid GuatemalanxAntillean
Hass	Hybrid GuatemalanxMexican	Booth 7	Hybrid GuatemalanxAntillean
Jim	Hybrid GuatemalanxMexican	Waldin	Antillean
HX 48	Hybrid GuatemalanxMexican	Semil 44	Hybrid GuatemalanxAntillean
135 - 15	Hybrid GuatemalanxMexican	Monroe	Hybrid GuatemalanxAntillean
Papelillo	Antillean	135-21	Hybrid GuatemalanxMexican
Simonds	Antillean	Trapp	Antillean
Peterson	Antillean	Pollock	Antillean
Itzamna	Guatemalan	Booth 1	Hybrid GuatemalanxAntillean
Nabilico	Guatemalan	Lula	Guatemalan
Kanola	Guatemalan	G 755	Guatemalan
Tumaco	Antillean	Puebla	Mexican
Winslowson	Hybrid GuatemalanxAntillean	Gottfried	Mexican
Hayes	Hybrid GuatemalanxMexican	La selva	Hybrid GuatemalanxMexican
Fuerte	Mexican	Booth 8	Hybrid GuatemalanxAntillean
135 -20	Hybrid GuatemalanxMexican	Dr. Sardi	Antillean
Costa Rica	Hybrid GuatemalanxMexican	Zutano	Mexican
143 - 77	Hybrid GuatemalanxMexican	Ibague	Antillean
Trinidad	Hybrid GuatemalanxAntillean	Los Silos	Antillean
Choquette	Hybrid GuatemalanxAntillean	Gwent	Hybrid GuatemalanxMexican
Gripiña	Hybrid GuatemalanxAntillean	Marcus	Antillean
Mayapan	Guatemalan	Ruelhe	Antillean
Duke 7	Hybrid GuatemalanxMexican	Ecuatoriano	Mexican
Marzala	Antillean	Fairchild	Hybrid GuatemalanxAntillean
Oculta 1	Antillean		

For the amplification a total of sixteen primer combinations EcoR1/Mse1 were selectively tested to identify at least four that can shows polymorphism among accessions: E-AG/M-CAC, E-AG/M-CAA, E-AG/M-CAG, E-AG/M-CAT, E-AG/M-CTA, E-AG/M-CTC, E-AG/M-CTG, E-AG/M-CTT, E-AC/M-CAA , E-AC/M-CAC, E-AC/M-CAG, E-AC/M-CAT, E-AC/M-CTA, E-AC/M-CTC, E-AC/M-CTG, E-AC/M-CTT. As a result, the four most-polymorphic primer combinations (E-AC/M-CAC, E-AC/M-CTC, E-AG/M-CTA, E-AG/M-CAT) producing clearly readable fragments and overall reproducibility of the AFLP amplification patterns was good. These four primer combinations were selected for the subsequent analysis with the avocado complete collection. AFLP markers were manually scored as binary data with presence as "1" and absence as "0". Cluster analysis was performed on the similarity matrix employing the "unweighted pair group method using arithmetic means" (UPGMA) algorithm provided in the computer program NTSYSpc, version 2.11S (Exeter Software Co., New York).

Results and Discussión

Genetic diversity analysis. With this work a genetic diversity analysis among cultivated avocado varieties in Colombia based on AFLP data was accomplished. The four primer pairs used in this study (E-AC/M-CAC, E-AC/M-CTC, E-AG/M-CTA, E-AG/M-CAT) resulted polymorphic. These combinations have in average 43 polymorphic and 62 monomorphic fragments across the cultivated avocado germplasm characterized (Fig. 1).

The UPGMA dendrogram obtained using the primer combination E-AC/M-CAC scored in all genotypes, fails only in separate two accessions, identifying the 97 % of studied genotypes. Corroborating the usefulness of this marker type to genotype identification (Fig. 2). Though no AFLP primer pair was capable identifying all the studied genotypes, combining data from at least two or more AFLP primer pair, each genotype could be characterize by specific band patterns and therefore certainly identified. This result agree with those obtained by Fiedler et al., (1998) using RAPD markers. In order to investigate the relationship among the different avocado accessions, dendrogram based on AFLP data shows that the accessions could be grouped instead according to three distinguishable avocado horticultural races. This way, presumable ecological groups were adequately represented in the dendrogram according to their putative origin, since Antillean plus hybrid GuatemalanxAntillean, Guatemalan plus hybrid GuatemalanxMexican and Mexican accessions were positioned in different groups. Although dendrogram based on AFLP data failed in grouping two accessions: one Mexican (Ecuatoriano) and one Antillean (Tumaco c) wich grouped independently. This dendrogram based on AFLP data supported the idea that Guatemalan and Antillean races are more closely related one another than either is to the Mexican race, although a more large sample of Mexican ecotypes are necessary to corroborate this hypothesis.These results are concordant with those obtained by Fiedler et al., (1998) and Ramirez et al., (2004).

Possible genetic duplicates. Among the 57 characterized accessions, two genotypes (Lorena and Trappica) were gruped together: the banding patterns were identical both in terms of number of bands and position. This is also confirmed by analyzing the Similarity Index and plotting a dendrogram showing the genetic distance (Fig. 2). This identical grouping was present in four primers combinations used in this study. They represent a possible genetic duplicate. The data from at least AFLP primer pair (E-AC/M-CAC), each genotype could be characterize by specific band patterns and therefore certainly identified, without including both duplicated genotypes previous (Lorena and Trappica).

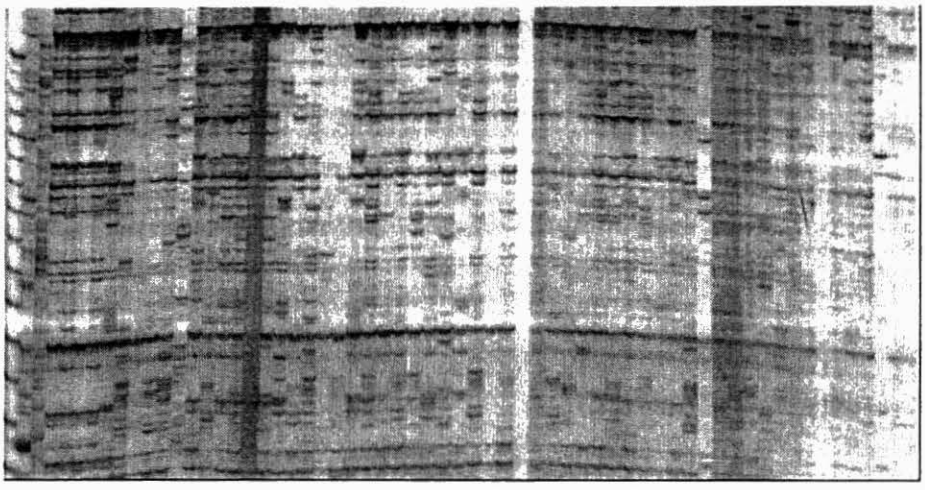

Figure. 1 Polymorphisms detected by AFLP with primer combination, E-AC/M-CAC between avocado varieties and also among two wild *Persea* species (*P.caerulea* and *P. rigens*).

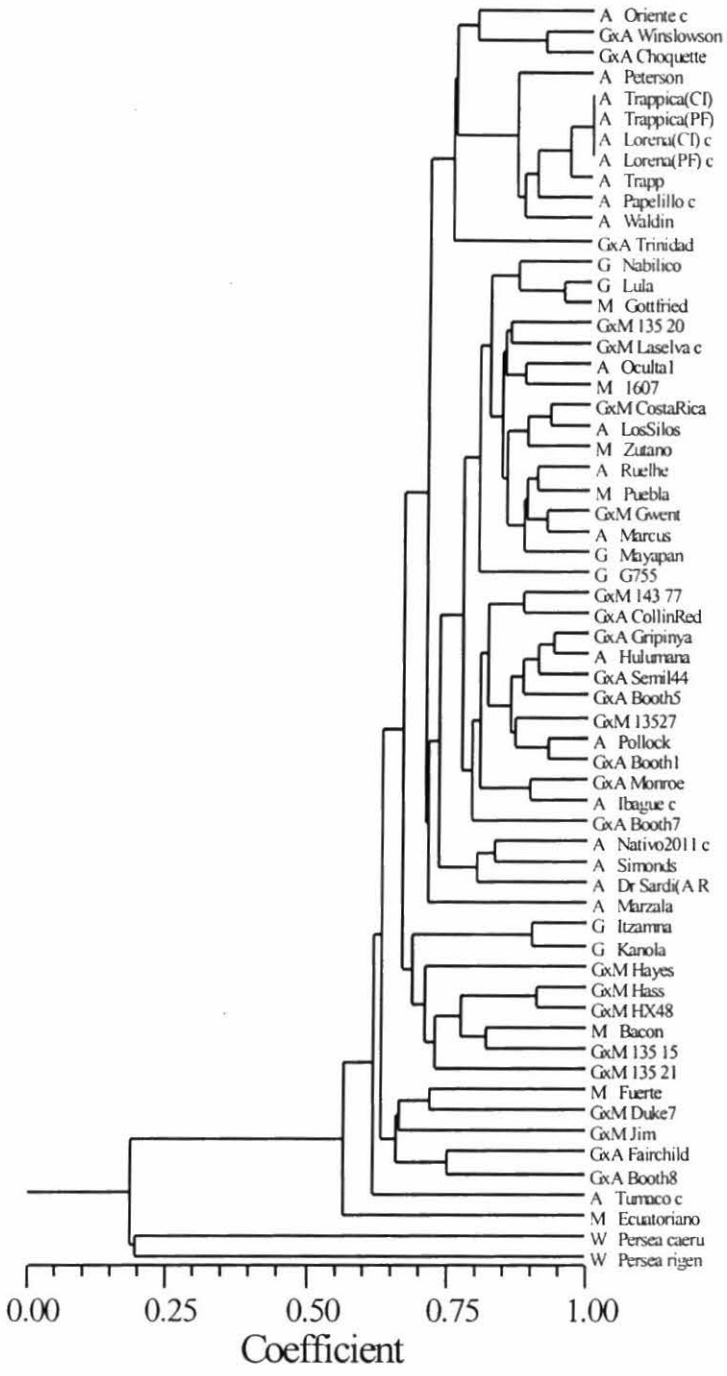

Figure 2. Dendrogram of 57 accessions of *Persea americana* Mill, and one *P. caerulea* and *P. rigens* generated by UPGMA clustering analysis based on AFLP data (primer combination E-AC/M-CAC). Indicates the race or the species (left) to which belongs each of the analyzed materials: w: wild species, A: Antillean race, G: Guatemalan race, M: Mexican, GxM: Hybrid GuatemalanxMexican and GxA: Hybrid GuatemalanxAntillean.

Several conclusions were derived from this study: (1) a high degree of variability can be detected in the Colombian avocado collection maintained at CORPOICA using AFLP markers, corroborating the usefulness of this marker type to genotype identification (2) the 97 % of studied accessions are genotypically unique (3) only there is a possible redundancy level of 3 % in the characterized collection (4) the cultivars clusters inside racial groups in a certain way that the genetic data confirms the ecological and/ or botanical designation.

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Activity 1.3 Identification and Mapping of useful genes and gene combinations

1.3.1 Identification of QTL for drought tolerance in common bean

S. Beebe, M. Blair and E. Tovar (SB-2); I. Rao, M.A. Grajales, C. Cajiao, and J.B. Cuasquer (IP-1). Carried out under a project funded by BMZ, Germany.

Introduction

Most beans are produced under low input, rainfed conditions. Drought is one of the primary risks that small bean producers face, especially in Central America, highland Mexico, northeast Brazil and eastern and southern Africa. CIAT has a long history of breeding for drought tolerance in common bean, and this effort has accelerated in the past five years. The present effort sought to identify QTL for drought tolerance. In the short run this will serve to elucidate inheritance and the relationship of QTL for yield with those for physiological traits. In the long run this could lead to Marker Assisted Selection. The objective of this study was to: identify QTL for drought tolerance; and to associate drought tolerance with physiological parameters, by finding genomic areas that contributed both to tolerance and to specific traits.

Methodology

Recombinant Inbred Lines (RILs) were developed from a simple cross of two common bean bred lines: MD 23-24, a drought susceptible line selected in the Escuela Agrícola Pan-americana (Zamorano); and SEA 5, a drought tolerant line developed in CIAT, Colombia (Terán and Singh, 2002). The population was advanced by single seed descent through the F_5 generation, when $F_{5:8}$ lines were created to establish the population designated MDxSEA. Phenotypic data were obtained from yield trials of 119 RILs and the two parents arranged in an 11 x 11 lattice design, and planted in three repetitions under rainfed and irrigated conditions in each of two years (2003 and 2004). Irrigation was applied in the drought trials to supply adequate moisture until shortly before flowering, at which time stress was expected to initiate. In 2003 the pattern of rainfall during the cropping cycle resulted in an intermittent drought, while in 2004, almost no rainfall occurred and therefore the drought was essentially terminal. The control treatments received irrigation water for the entire crop cycle. The field trials were sampled for physiological traits as well, to relate these to yield: seed weight; seed density; stomatal conductance; non-structural carbohydrates (NSC) in shoot and seed; ratio of pod wall to seed; pod harvest index (PHI) as proportion of pods in total biomass at late seed fill; seed P; seed N; leaf area index (LAI).

The parental genotypes were assayed for polymorphism among available SSR markers and combinations of AFLP primers, and polymorphic markers were evaluated on the 119 RILs. A genetic map was constructed applying the Mapmaker program (Lander et al, 1987) to 63 SSR and 62 AFLP markers. Most SSR markers had been mapped previously to the standard CIAT map and to the central bean map (Blair, et al, 2003), and therefore it was possible to associate the groups of the MDxSEA map with known linkage groups. Single point analysis (SPA) was

performed on all variables using the QGene program. The graphics of the QGene program serve to visualize readily the possible QTL for yield under drought and their possible relation to other traits, and thus to identify combinations of traits for subsequent study. Composite interval mapping (CIM) analysis was then applied to data for: yield under drought stress; irrigated yield; and for physiological traits that appeared to share genomic regions with drought yield QTL. Finally, joint CIM analysis was applied to the data for drought yield and for those same physiological traits. This last analysis was performed to seek evidence of a physiological relationship, under the expectation that the identification of a QTL through joint analysis may imply that the QTL so identified is associated with both traits.

Results

Ample and statistically significant differences in yields were obtained among genotypes, for moisture regimes, and for years (Table 1). All interaction terms were likewise highly significant. The interaction of genotype x moisture regime (drought or irrigation) implies differential reaction to drought and the presence of drought tolerance genes. However, the interaction of year x moisture regime suggests that the reaction of genotypes varied over the two seasons and patterns of drought.

All eleven linkage groups of the bean genome were recognized, with from 2 to 17 markers assigned to each linkage group. The map covered 495 cM of the bean genome, or about 40% of the total genome length, while 25 markers remained unlinked. The initial SPA suggested the existence of QTL for many traits, including drought yield, irrigated yield, and several physiological traits. CIM confirmed the existence of QTL for yield under drought and also in the irrigated control (Fig.1). Under drought three out of four putative QTL were derived from SEA 5, as expected, and these appeared on linkage groups b01, 05, and 08 (Table 2 and Figure 1.a). A fourth QTL on b08 that only expressed under terminal drought in 2004 derived from MD 23-24.

Some QTL were expressed under both moisture regimes. Under irrigated conditions two QTL were identified on b01 and b08 that derived from MD 23-24 (Table 2 and Figure 1.b), including the one expressed in terminal drought. Only one QTL in the irrigated treatment in 2004 derived from SEA 5, this being common to a drought QTL on b01 but with a smaller effect under irrigation. Thus, two out of four QTL (on b01 and b08) that expressed under drought were common to those that expressed under irrigated conditions, and the same allele increased yield under both conditions. Selection for these alleles would have a positive effect across environments. The other two drought QTL (on b05 and b08) had weak (statistically neutral) effects on irrigated yield, and selection for these alleles would not significantly reduce irrigated yield. The same could be said for the QTL for irrigated yield on b01, which could be selected without a significant negative effect on drought yield. Thus, positive alleles for both drought and irrigated yield could be combined in a single genotype.

In a joint analysis of drought and irrigated treatments involving four trials, still more QTL were identified on b03 and b10, and some previously identified effects were amplified. These additional QTL resulted from the negative relationship at some loci whereby an MD 23-24 allele gave a modest advantage under irrigation, and a SEA 5 allele gave a modest advantage under drought. The QTL effects within each moisture regime were not statistically significant, but the combined (negative) mathematical effect in the joint analysis led to significance in these cases. At such loci, it would not be possible to combine genes to optimize yield under both conditions, but in any case, these effects were not as important as those for QTL referred to above.

Both SPA and CIM analysis of the physiological traits suggested that drought tolerance genes might share genomic regions with PHI, seed weight, podwall-to-seed ratio, Non-Structural Carbohydrates (NSC) in seed, and LAI. Therefore, drought yield was analyzed jointly by CIM with these traits, to establish physiological relationships. Results are presented in Table 2. Drought yield appeared to share QTL with all these traits, suggesting that all may be associated with drought yield either causally or because they reflect the same yield processes. However, significance in the joint analysis may be driven by significance of the individual traits. The significant loci that are of most interest in the joint analysis are those that either did not express significance for the individual traits, or for which the level of significance increased dramatically in the joint analysis.

Seed weight, PHI, and NSC are all influenced by photosynthate transport to seed which we have speculated is a key drought tolerance trait. Among QTL identified in the joint analysis, those shared by drought yield and non-structural carbohydrate in seed displayed the most consistent positive relationship (i.e., positive values of NSC-seed were consistently associated with positive values of drought yield). There were no significant QTL identified for NSC in seed *per se*, but a QTL was revealed in the joint analysis of NSC-seed and drought yield on b11 that was not significant for either trait separately (although a peak falling short of statistical significance was observed here for drought yield). The positive effect was derived from SEA 5. Ironically, values for NSC in seed did not correlate with yield. If NSC in seed is controlled primarily by minor genes, it might be subject to greater environmental effect, resulting in a poor correlation with yield. Another QTL that appeared to relate Leaf Area Index to drought yield was also expressed on b11 with much greater significance than for LAI alone.

The relationship with drought yield was not consistent for other traits, based on the effects of their respective QTL. For example, under drought most seed size effects were positively associated with yield (i.e., greater seed size contributed to greater yield), but QTL on b10 expressed a negative relationship of seed size and yield, as has been observed in other physiological studies (Sexton et al, 1997). Thus, the seed size QTL are probably associated with different mechanisms controlling seed size, some of which contribute to drought tolerance and others not. Other traits (PHI, LAI) presented an even more erratic relationship. The relationship of drought yield to the podwall-to-seed ratio was especially unstable over the two seasons. In 2003 the relationship was negative for most loci, but in 2004 it was positive. In 2003 about 32 mm of rain fell at flowering time, and this might have generated more pod set than what was subsequently possible to fill with grain. This would have increased the relative podwall biomass in 2003, creating a more negative relationship. This was reflected in a more negative correlation of podwall-to-seed ratio and drought yield in 2003 than in 2004 (-0.51*** versus –0.29***). This illustrates the complexity of identifying selection criteria for drought tolerance under different drought regimes.

Conclusions

Independent QTL influence yield under drought and irrigated conditions. As many as seven QTL were identified for drought, five for irrigated conditions, and another two were revealed in a comparison of drought and irrigated trials.
Several traits associated with photosynthate transport such as Pod Harvest Index and seed weight are associated with drought tolerance but the relationship of individual QTL was inconsistent.

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Table 1: Analysis of variance for yield of recombinant inbred lines (RILs) of the MDxSEA population under two moisture regimes (drought and irrigated) in two years.

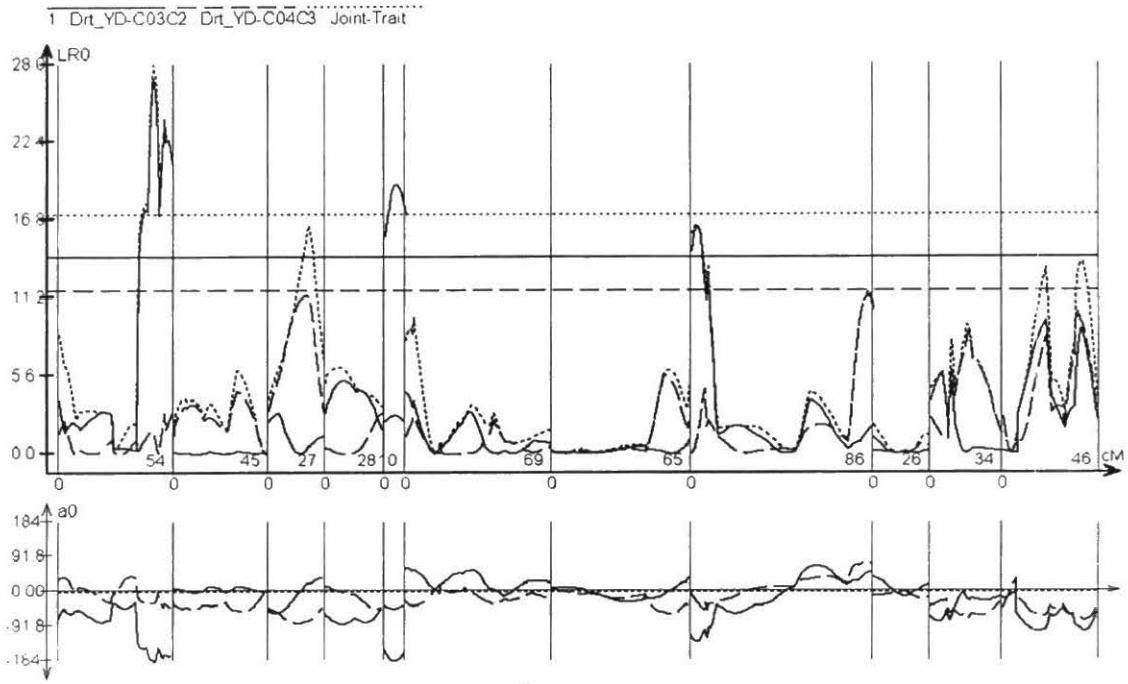
	df	F	P
Year (Yr)	1	168.89	<0.0001
Moisture regime (M)	1	1654.91	<0.0001
Year x M	1	368.28	<0.0001
Rep (Yr x M)	8	14.46	<0.0001
Genotype (G)	120	5.72	<0.0001
Yr x G	120	1.75	<0.0001
M x G	120	3.07	<0.0001
Yr x M x G	120	1.96	<0.0001

Table 2: QTLs identified by CIM –Cartographer of individual and multiple traits, and the relationship (positive or negative) with drought yield in a joint analysis. Two signs (+ and -) signify a reversal of this relationship in 2003 and 2004.

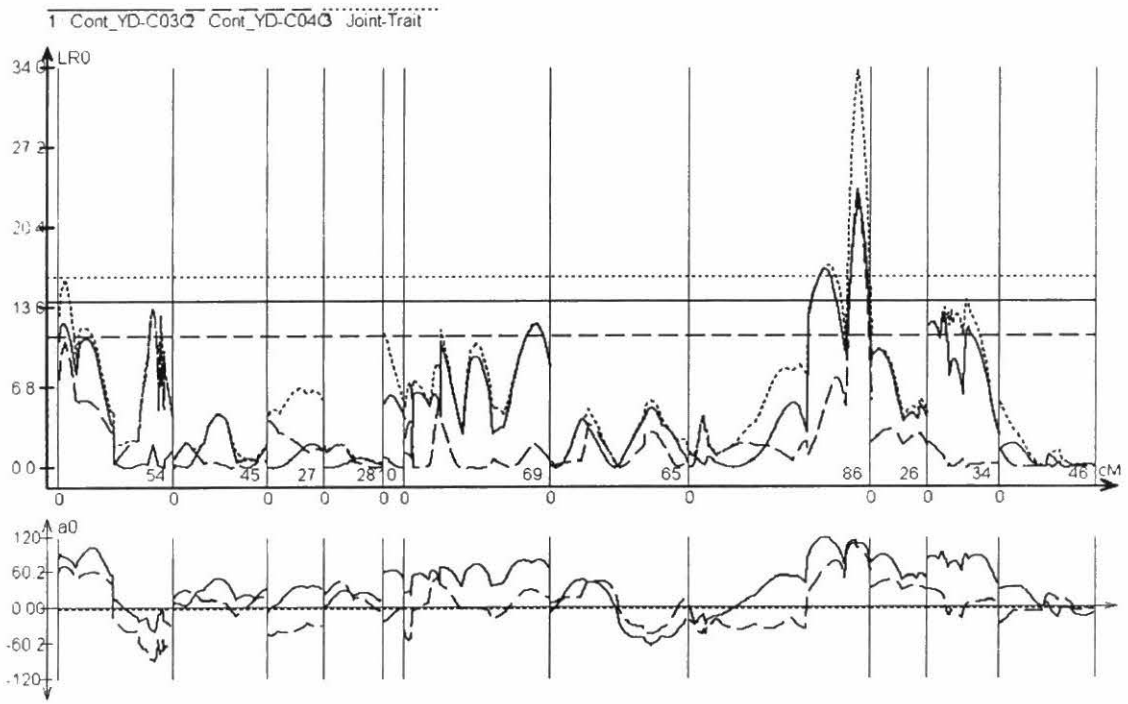
LG	Drt 03	Drt 04	Drt 03-04	Cont	PHI	100 sw	TNC s	TNC PA	IAF	Pod/s	D+ Con	D+ PHI	D+ 100sw	D+ TNCs	D+ LAI	D+ Pod/s
1				*	*						* -/+					
	*		*								* +	* +	* +	* +	* -	* -/+
2																* /+
3											* -/+					
																* -/+
4																
5			*			*					* -	* +	* +	* +		* -/+
6						*				*						* -/+
										*						
7																
8	*		*								* +	* +			* +	
		*	*	*						*	* +					* -
9						*							* +			
10						*		*			* -		* -			
											* -					
11								*					* +	* +		
									*						* +	

Figures 1a and 1b: QTL for yield in a population of RILs of MDxSEA in two seasons, and in joint analysis, under drought (1a) and irrigated (1b) conditions, as detected by Composite Interval Mapping

1.a.



1.b.



1.3.2 Development of SSCP-SNP based COS markers and other Allele Specific Markers as a tool for Cassava Improvement

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CIAT

Funding: Generation Challenge Program

Important outputs

Design of allelic specific primers to identify SNPs by differential PCR amplification in wild allelic variants of the gene GBBSI.
Implementation of SSCP-SNPs as a tool for genetic mapping of COS markers and ESTs.
Genetic mapping of 2 COS markers, DHRF and DREB-I, on the existing cassava genetic map.

Introduction

Genetic variation at the DNA sequence level is the basis for crop diversity and for genetic analysis and the principal tools for genome mapping and gene tagging. This year efforts were made to develop a new platform of molecular markers that better exploit single nucleotide polymorphisms (SNPs), by far the most abundant genetic variation at the DNA level. Thanks to the growing genomic information on cassava and other crops (EST database, gene sequences), SNPs can now be searched in coding and non-coding sequences of genes for the development of highly informative molecular markers for genetic studies. Our focus has been on the development of allelic specific, Conserved Ortholog Set (COS) and Single Strand Conformation Polymorphism (SSCP-SNP) markers. Allelic specific markers have been used to identify allelic variation on the gene GBSSI in wild relatives of cassava (Castelblanco and Fregene. 2005a). SSCP-SNPs has been validated as a system to identify SNPs or small insertions-deletions (INDELs) in non-coding regions of genes and finally COS markers takes advantage of comparative genomics to a specific gene controlling a trait of interest identified from functional genomics studies in other taxa by a computational approach that eliminates confounding effects of gene families or paralogs.

Methodology

Allelic specific primers
The allelic specific marker involves designing a primer that ends on a SNP and the addition of an extra mismatch at the end 3' to increase specificity of amplification of the mutant allele. Allelic specific primers were designed with the help of the program SNAPER (http://ausubellab.mgh.harvard.edu), several primer combinations were designed and evaluated by PCR.

Primer design for COS markers

Cassava homologs for three drought related sequences coming from *Musa sp* namely chalcone synthase (CHS), dihydroflavonol-4-reductase (DHRF), and drought responsive element binding

factor 1 (DREB-I) were searched in Genbank and the EST database of cassava (http://syrah.univ-perp.fr/~piegu/meEST/blast/blast) using tBLASTX to identify ESTs producing significant alignments. ESTs that showed the best E-value were selected as the homologous candidate genes in cassava. An EST was selected for each gene and its sequence was used as template in primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to design pairs primers. To identify if the ESTs selected for cassava are conserved genes, termed as conserved ortholog set (COS) markers, a computational comparison of the *Arabidopsis* genomic sequence with the ESTs was done. Cassava ESTs were scanned against the *Arabidopsis* genome (TAIR, http://www.arabidopsis.org/) using tBLASTX. An EST was defined as a conserved ortholog sequence (COS) if it matched a single Arabidopsis BAC at an expected value of E-15, and the next best *Arabidopsis* match was of lower significance (i.e., there was a difference of $\geq$ 10 between expect scores) as described by Fulton et al. (2002).

Primers designed for CHS, DHRF and DREB-I were tested for PCR amplification and polymorphism on SSCP gels and by sequencing DNA from TMS30572 and CM2177-2, parents of the F_1 genetic map progeny, (Fregene et al. 1997) followed by search for SNPs. The PCR products were denaturated and separated by SSCP gels using a mutation detection enhancement (MDE) solution. The gel mix was made in a 70-ml total volume containing a final concentration of 0.5x gel solution (Rockland , Me.) and 0.6x TBE buffer and polymerized by the addition of 0.28 ml of 10% ammonium persulphate and 28 µl of tetramethyllenediamine (TEMED). About 4 µl of PCR product was add to 10 µl of loading dye, followed by heating at 95 °C for 5 min and quenching on ice. Fragments were electrophoresed for 16 h at a constant power of 8 W at room temperature and then silver-stained as described by Bassam et al. (1991). Separately the PCR products of the parents were extracted from the agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen). They were cloned in the p-GEM-T easy vector (Promega), electroporated into DHα5 E. coli strain, cultured overnight and plasmid DNA miniprep carried out; five clones of each genotype for each gene were selected and sequenced. Sequences were analyzed by Clustal-W (http://www.ebi.ac.uk/clustalw/) .

Genetic mapping of the COS markers

COS markers showing polymorphism between parents were evaluated in 150 individuals of the genetic map population, segregation ratios of present, absent classes for each marker were tested for goodness of fit to the expectation 1:1 to identify single-dose markers by chi-square analysis. Data generated was added onto the existing data sets for the female- and male-derived maps. The segregating marker was placed onto the existing linkage groups of the cassava genetic map using the "group" command, with a LOD threshold score of 4.0, a recombination fraction of 0.30, and the "try" command of Mapmaker 2.0 running on a G3 Macintosh. Map units (in centimorgans, cM) were derived using the Kosambi function

Results

Allelic specific markers

Allelic specific primers were designed to amplify mutant GBSSI alleles found in *Manihot crassisepala* and *Manihot chlorostricta* based upon SNPs previously identified in the glycosyl transferase region of the gene (Fig 1). These markers are now being used to introgress these mutant alleles into cassava

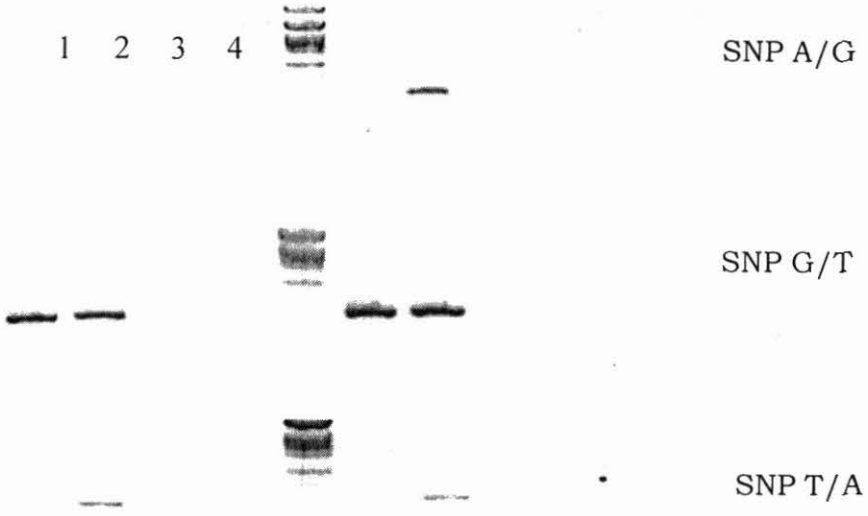

Figure 1. PCR amplification of mutant GBSS alleles in two wild *Manihot* species. Order of samples is: *M. Crassisepala (1)*, *Manihot chlorostricta* (2), MCOL1508 (3) and MCOL2269 (4)

Identification of COS markers

The blastx procedure permitted the identification of the genes CHS, DHRF and DREB-I in cassava, the most significantly alignments were ESTs mb_dsc.03.E3.u (CHS), rni.03.L23.5 (DHRF) and c.07.P13.5 (DREB-I). The CHS and DHRF ESTs can be defined as COS markers because sequences with significant homology with *Arabidopsis* were found. The EST c.07.P13.5 (DREB-I) showed similar E-values to different AP2 domains suggesting that it is not a conservative gene. A set of three pair primers were designed from the cassava ESTs to produce PCR amplification products ranging from 300 bp to 1000bp.

Sequence analysis and search for polymorphism by the SSCP-SNP approach

SNPs and INDELs variation were obtained after sequence alignment of sequences of the DREB and DHRF genes from different clones of parents of the cassava map population. A deletion (GACCAC) in a tandem motif and several SNPs were found for the DREB-I gene, SNPs were also identified for the DHRF gene, but no polymorphism was observed for CHS. The marker technique SSCP-SNPs, that looks for polymorphism in non-coding regions of the genes, permitted the identification of polymorphism between the parents of the mapping population for the DREB-1 and DHRF gene without the need for sequencing (Fig 2). The SSCP-SNPs marker technique is now been routinely used for mapping genes

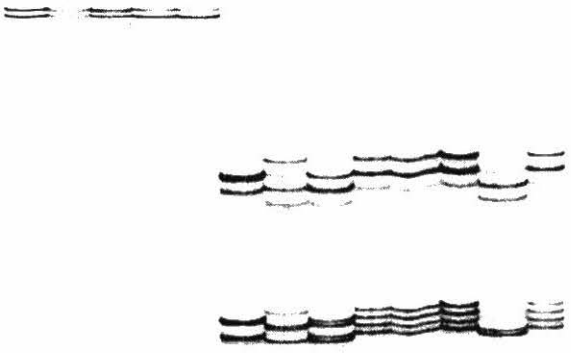

Figure 2. SSCP-SNPs gel of PCR amplification of the DHRF and DREB-I genes in the parents of the cassava map population. Polymorphic bands can be observed in the female parent (TME30572) for DHRF and the male parent (CM2177-2) for DREB-I. Sample order is TME30572 (lane 1), CM2177-2 (lane 2), K1, K2, K3, K4, K5 AND K6 correspond to a small subset of the progeny (lanes 1-6).

Genetic mapping of COS markers

DHRF and DREB-I showed SSCP-SNP polymorphism between parents that segregated in the F1 progeny and could therefore be mapped. The expected segregation ratio of 1:1 was observed for both genes and they were subsequently mapped on to the existing cassava genetic map. DHRF was mapped on the linkage group R of the female map between the SSR markers NS656 and NS1099 at 9.5 and 8.6 cM respectively. DREB-I was mapped at an end of the male linkage group K near to the marker J3C at a distance of 22.1 cM

Conclusions and perspectives

Thanks to a new SSCP-SNP molecular markers platform, we have been able to map two COS markers in cassava (Castelblanco and Fregene. 2005b), a computational protocol to identify COS markers and development of allelic specific markers have also been implemented. These new marker tools should accelerate the development of marker tools for accelerating breeding in cassava.

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Castelblanco, w and fregene m. (2005b) sscp-snps based conserved ortholog set (cos) markers for comparative genomics in cassava (manihot esculenta crantz) (*m. esculenta* crantz). submitted to plant science(in review)

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1.3.3 Genetic mapping of 36 new EST-SSR markers in cassava

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CIAT

Important Output:

Design of primers for 175 EST-SSRs markers
Genetic mapping of 35 EST-SSRs markers on to the existing cassava genetic map

Introduction

EST-SSRs are ESTs having simple sequence repeat motifs within them and are useful molecular markers because they often represent known genes. Recent development of cassava ESTs have led to the identification of over 500 new EST-SSRs, we describe here the design of primers for 175 EST-SSRs, parental survey using 100 of these markers and genetic mapping of 35 EST-SSRs onto the existing genetic map of cassava. The new EST-SSR markers have now increased the total number of SSR markers available for cassava to over 800 and also have led to an increased saturation of markers on the cassava map.

Methodology

Identification and primer design for EST-SSR markers
The EST resource of cassava was evaluated for SSR motifs using the SSRIT tool (http://143.48.220.116/db/searcher/ssrtool/) to identify motifs with a minimum number of repeats of 5 and a pentamer as the maximum motif-length group. Of 532 ESTs searched, 175 were selected and primers were designed from flanking sequnces to produce PCR amplification product of between 200-300 bp. PCR amplification conditions for EST-SSR markers were: 2mM of $MgCl_2$, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase, and a final volume of 25 ul; the thermal amplification profile was an initial denaturation step of 95°C 2min and 30 cycles of 94°C 30 sec, 45°C or 55°C 1 min, 72°C 1min and a final extension step of 72°C for 5min.

Genetic mapping of the EST-SSR markers

EST-SSR markers showing polymorphism between parents were tested in 150 individuals of the genetic map population, segregation ratios of present, absent classes for each marker were tested for goodness of fit to the expectation 1:1 to identify single-dose markers by chi-square analysis. Data generated was added onto the existing data sets for the female- and male-derived maps. The segregating marker was placed onto the existing linkage groups of the cassava genetic map using the "group" command, with a LOD threshold score of 4.0, a recombination fraction of 0.30, and the "try" command of Mapmaker 2.0 running on a G3 Macintosh. Map units (in centimorgans, cM) were derived using the Kosambi function

Results

Of the 175 EST-SSR markers for which primers were designed only 100 markers have been tested to date in the parents of the genetic map population. Of this number 35 markers showed polymorphism in either or both parents and could be mapped (Fig 1) Placing of the 35 EST-SSR markers onto the existing cassava genetic map is ongoing.

Conclusion and perspectives

A new set of ESTs-SSR markers have been developed for genetic mapping in cassava, 35 such markers are being added to the genetic map of cassava. These markers represent a way of increasing markers that are known genes on the genetic map of cassava.

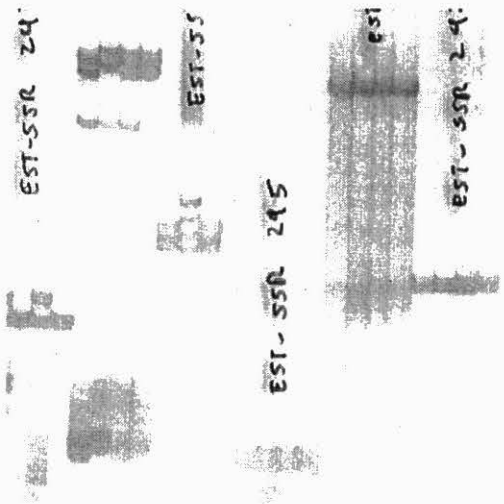

Figure 1. Silver-stained PAGE electrophoresis of PCR amplification of the parents of the cassava map population and 2 F1 progenies using EST-SSR markers. Sample order is TMS30572, CM2177-2, K1 and K2. The first 3 markers from the left are polymorphic in the parents and segregate in the progeny.

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

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

Introduction

Manihot esculenta is a tropical shrub highly tolerant to low soil fertility and drought, and one of the most efficient carbohydrate-producing crops. It develops succulent roots used as energy storage, which are currently the fourth most important source of calories in the tropics after rice, sugar cane and corn. According to FAO, more than 600 million people depend on the cassava roots in Africa, Asia and Latin America. Cassava is grown by poor farmers, many of them women, often on marginal land. For these people, it is a subsistence crop vital for both food security and income generation. It is mostly produced on small-scale family farms for village

markets, but it is also an important industrial and cash crop that can promote rural development. However its short post-harvest storage life is a major constraint towards developing its economic potential which would be particularly beneficial for the poor. 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. In the long term, the purpose of this work is to design a genetic construct with some of these genes, to modulate the PPD on a genetically transformed cassava variety.

In the present report, we show the analysis of the available data obtained during wet-lab work since 2003.

Methodology

The high throughput cDNA microarray technology was used to perform a genome wide expression analysis of the PPD process in cassava roots. As described earlier PPD-related microarrays were constructed (Cortés, 2003) and hybridized (Bernal, 2004) with samples of cassava roots and leaves in order to perform two gene expression experiments. The first one is a time-course experiment, which enabled us to acquire a root six-time-point gene expression profile during the PPD, at 0, 12, 24, 48, 72 and 96 hours after harvest, and identify genes regulated during this process. The second one is a leave vs. root gene expression comparison to identify root specific genes, and genes that are expressed during the healing of wounded leaves and are not expressed during the PPD process.

Gene expression analysis: Microarrays of the three biological replicas of both experiments were scanned using the VersArray Chipreader scanner from BioRad. Expression data was extracted with VersArray Analyzer (BioRad) from the TIFF (Tagged Image File Formats) files. Background was calculated with the local corners function available on VersArray Analyzer. Spots which had a net intensity below 2 times the standard deviation (SD) of background were eliminated. The following procedures were done using MIDAS (Saeed, 2003). Spots that were not present in at least two of the three biological replicas were eliminated. Between channel normalization was performed using the Intensity dependant Lowess method as proposed by Yang et al. (2002) on a local basis using the subgrids as units of normalization. Between-grid and between-slide SD filter was applied to regularize dispersion of the log2(Rt/R0) among subgrids, and among slides. In-slide replica function was used to calculate the log2(Rt/R0) arithmetic average of the four technical replicas within each slide. This left us with a value of the log2(Rt/R0) for each clone of the PPD library, for each biological replica.

For the PPD time-course experiment, using excel, we calculated the log2(Rt/R0) value for each clone of the PPD library as the arithmetic average of the respective values for each biological replica. These values were arranged along the log2(Rt*R0), and the SD of the log2(Rt/R0) was calculated on static 95-point windows. Those points which had a log2(Rt/R0) outside 1.96 SD of their local window were considered as differentially expressed on each time comparison. The same analysis was performed at the University of Bath (UK) with the following differences: Expression data was extracted with the program ArrayVision, between channel normalization was performed on a global basis using the whole microarray as the unit of normalization. Finally, in both analysis, only those points which were up regulated in at least two time-point

comparisons, or were down regulated in at least 4 time-point comparisons were sent to the University of Bath and sequenced.

A clustering analysis only for the regulated clones during PPD was performed on MeV (Saeed, 2003), using the normalized signal-ratio of each time-point after harvest to 0 hours. First, the signal-ratios of different comparisons were normalized dividing the signal-ratio of each gene by the SD of signal-ratios across the 5 time-point comparisons. Then a principal component analyis was run (it takes the first three principal components found and plots the data on that 3D space). Next, a figure of merit (FOM) analysis was run; it evaluates the predictive power of a clustering algorithm. The implementation of it on MeV gives an estiamtion of how well the data are clustered depending on the number of clusters in which the data is divided. Finally a supported K-means clustering (SKC) analysis was run with 150 K-mean runs, 7 clusters, using the Euclidean distance, and a 60% threshold of co-occurrence to accept that 2 genes cluster together.

For the leave vs. root gene expression comparison, using excel, we used the one-sample design of the significance analysis of microarrays (SAM) method (Chu, 2002), choosing 5000 permutations and a false discovery rate of 0.1% or lower. We decided to use SAM, in stead of the local SD criteria, because since all the clones we were evaluating with the microarray came from roots, we found that the majority of the clones are more expressed in the root samples than in the leaves samples (as was expected), so if we assume no tendency to over-expression on roots of the group of clones evaluated, as needed with the SD criteria, we were not able to find the clones which we knew were found only on leaves (spiked clones). SAM analysis is based on permutations of the data, so it takes into account this expression tendency of the whole microarray.

Results and discussion

490 genes were identified as regulated during PPD in cassava roots. 39% of these clones came from the analysis performed at the University of Bath, 28% came from the analysis performed at CIAT, and 32% came from both analysis. From the leaves vs. roots comparisons, 76 clones seem to be expressed only in roots and not in leaves. These clones are interesting because the promoter of a root specific gene is needed to localize the expression of any genetic construct to the roots of cassava. 570 clones are expressed in roots 24 hours after harvest and are not expressed in leaves after 24 hours of detachment from the plant; 149 clones of these are regulated during PPD. These are also interesting clones because they might be involved with the unstopped wound/defense response observed in roots due to the lack of any healing mechanisms, which ends up in their deterioration, unlike leaves, which heal after a wound.

The principal component analysis of the 490 clones regulated during PPD, allowed us to distinguish only two clusters, which are the up-regulted (colored red on figure 1), and down-regulated clones (colored green in figure 2) according to the SD criteria.
The Figures of Merit analysis is shown in figure 2. The lower the adjusted FOM value is, the higher the predictive power of the algorithm, so as it can be seen in figure 2, as the number of clusters increases, the score goes down; actually it goes down very steeply until around 8 clusters, and then it keeps on decreasing but more slowly. Therefore, the SKC was run with 8 clusters, but there was always a group of clones which couldn't be assigned to any cluster, that is why the algorithm was finally run with 7 clusters, and the parameters mentioned above (see methodology). Also, hierarchical trees were constructed to organize the genes of each cluster using the Euclidean distance metric and a complete linkage to organize genes.

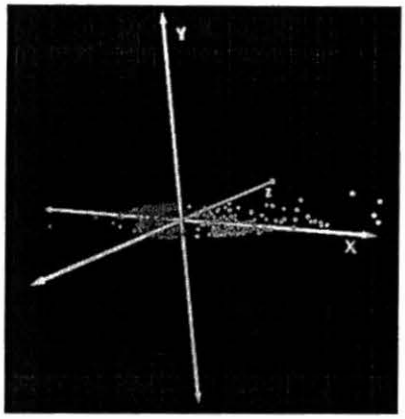

Figure 1. Principal component analysis. Regulated genes during PPD graphed on a 3D space, where each axis is one of the first three principal components found.

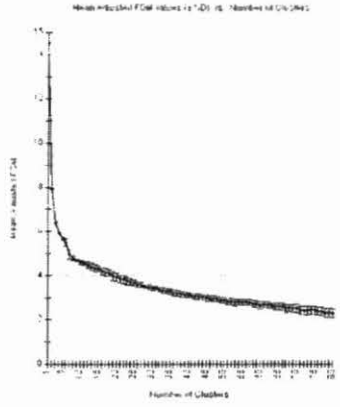

Figure 2. Figure of Merit. It shows that ~8 clusters are enough to reflect in an acceptable manner the inherent division of the genes according to their expression profile.

Figure 3 shows the expression patterns of each one of the seven clusters found, and the hierarchical clustering within each one.

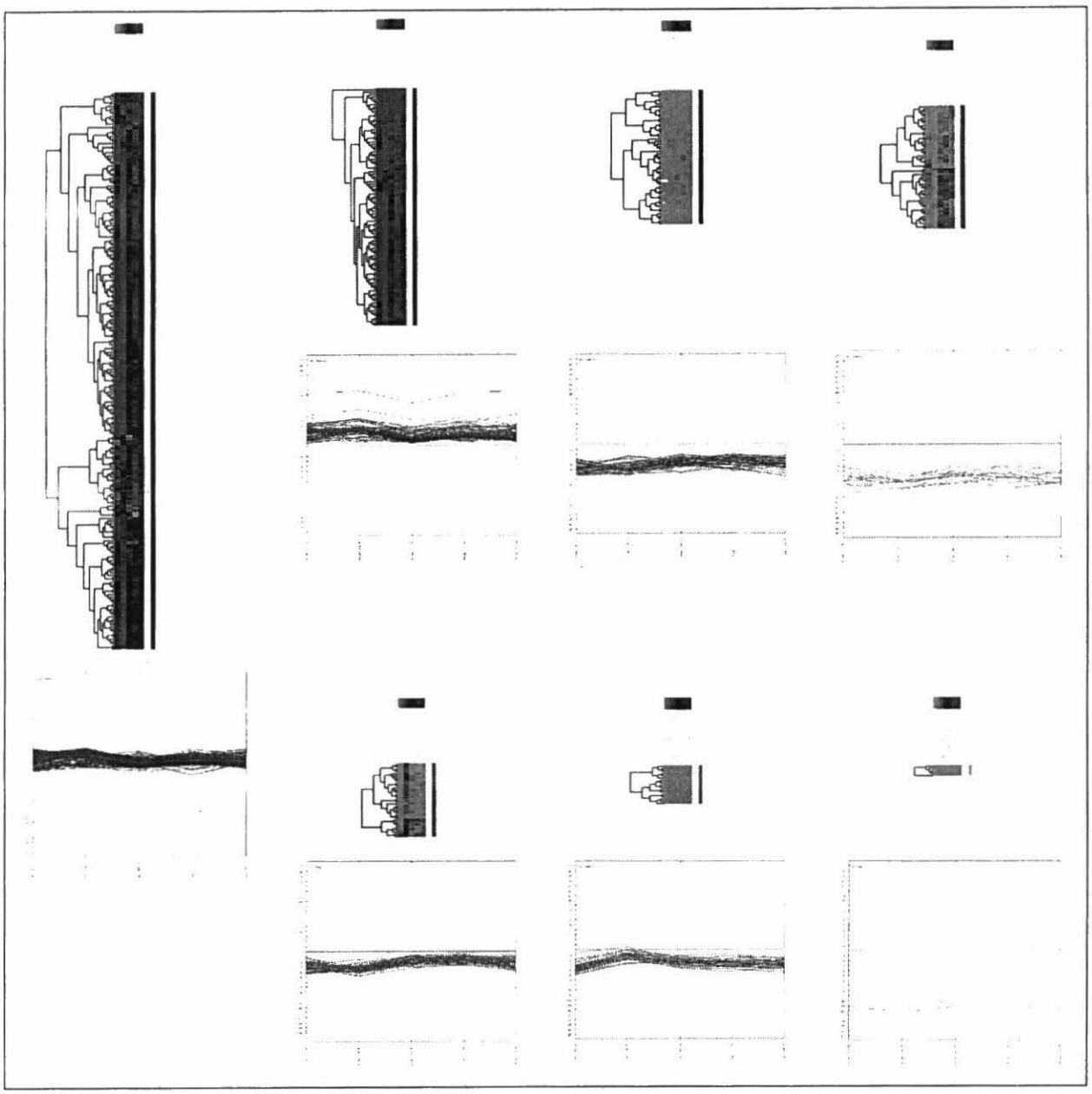

Figure 3. Expression profile of regulated genes during PPD on cassava roots, and hierarchical clustering of the genes in each cluster.

Finally, according to the expression pattern during PPD, the comparison between leaves and roots, and the putative function of the sequenced clones, the expression pattern of eight clones with the following putative functions: cysteine protease, germin-like protein, peroxidase, auxin induced protein, ethylene response factor, ascorbate peroxidase, and catalase, was confirmed at the University of Bath by Kim Reilly, who used standard northern techniques.

Future work and recommendations

A scientific article is being written to publish the results obtained so far.
The PPD experiment was done on the cassava Unigene set (Lopez, 2003), amd is currently being analyzed.

The genes corresponding to the regulated clones during PPD after performing the analysis over three biological replica should be mapped via CAPs (Cleaved amplified polymorphisms) in the cassava molecular genetic map in order to see if there is any association with QTLs.
A computer driven annotation of the regulated clones should be done associating a functional GO (Gene Ontology) category to each sequence, to determine which metabolic process are involved during PPD besides the well recognized oxidative stress response.

The position within the respective metabolic pathway of sequences which are enzymes should determined, to identify those genes which are at the beginning of the multiple metabolic pathways activated during PPD in cassava roots, because they might be the key activators of them, and therefore are adequate for modulating PPD.

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1.3.5 Identification of Genomic Regions Responsible for Conferring Resistance to Whitefly and Isolation of Expressed Sequences During the Defense Response of MEcu-72 to Whitefly Attack in Cassava

A. Bohorquez, J. Vargas, A. Bellotti, B. Arias, M.C. Duque and J. Tohme

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 Tropical Whitefly IPM Project, with the financing of the New Zealand Agency for International Development identified Resistance to the White fly *Aleurotrachelus socialis* (Bondar) in cassava clones from Ecuador (genotype MEcu-72). Due to the importance whitefly as pest, it is necessary to know about the nature of genes that confer resistance to whitefly in genotypes like MEcu-72. For this purpose we are using F1 segregation and the genetic expression of cross MEcu-72 (resistant genotype) x and 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 we hypothesized that these resistant genes may also be effective against other whitefly species, especially *Bemisia tabaci,* the species that is a vector of CMD, 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 (Progress report 2003).

An additional step toward a better understanding of attack response of white fly to cassava, it is characterize genes that are involved in the downstream signaling cascades in plant defense responses. One tool that permits the unraveling of the complexities of gene expression is the establishment of a cDNA library, which has been developed with a highly effective method known as Subtractive Hybridization. Using this approach, two mRNA populations, extracted from both resistant and susceptible genotypes, were examined to elucidate the differential gene expression between them.

Functional genomics tools such as the Microarray give a first comprehensive overview of the molecular basis of the cassava defense response to the whitefly attack and will help to understanding the defense mechanisms to other important pests and diseases. Microarray-expression profiling will be used to identify putative early response regulatory and/or signaling genes. 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. Lopez et al, (2004) constructed a cassava Unigenes Microarray (5700 sequences), which is an unvalued resource to study global gene expression profiles.

The objective of this work is the contribution towards a better understanding of the plant-insect interaction and this information should help in the development of strategies for managing whitefly attack in cassava.

Methodology

Mapping

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) 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), we designed 105 pairs of SSRs primers from ESTs sequences (Bohorquez et al, 2004) which 25 were evaluated. We are using AFLPs (Vos, et al, 1995) and 15 random primers RAPDs to find markers associated to resistance for mapping and ultimately cloning the resistant genes.

Functional Genomics

Greenhouse trials

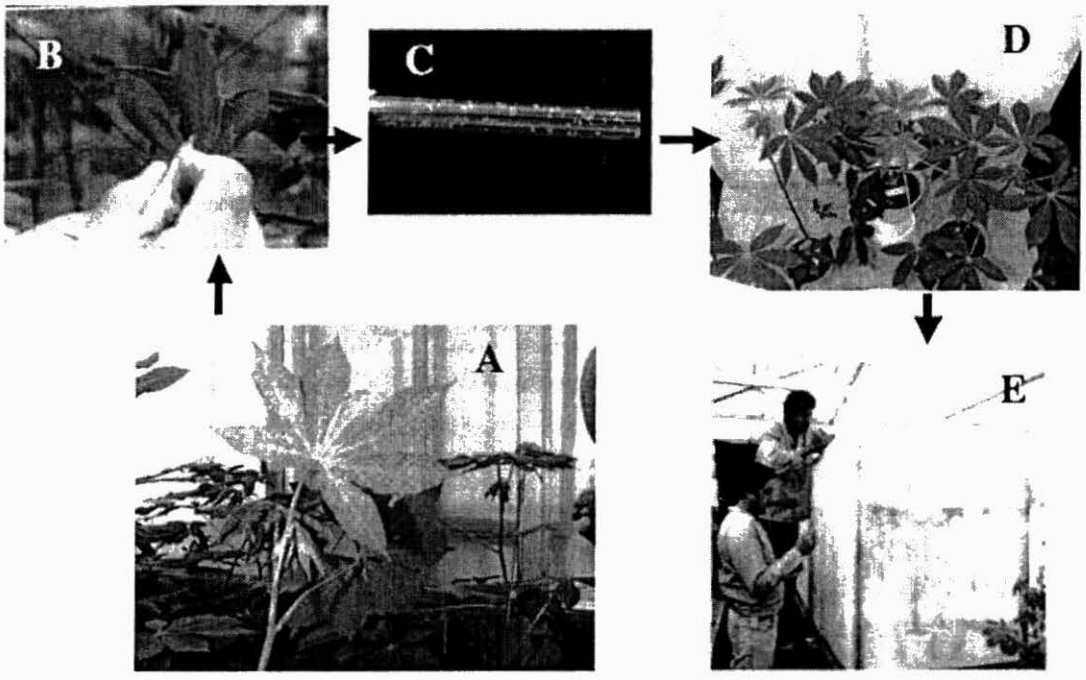


Figure 1: Scheme of the greenhouse trials. A: CIAT colony of A. *socialis* developed on CMC-40. B: Two thousand adult male and female of A. *socialis* were removed from the CIAT colony with the aid of a bucal aspirator. C: tube containing the adults. D,E: These were placed in the cages, as previously described, and attached to the undersides of MEcu-72 and MCol-2246.

For the isolation of expressed sequences, we are using 28 plants of 40 days planted in pots, 7 of each genotype, (MEcu-72 resistance and MCol 2246 susceptible) infested and 7 of each genotype without infest. These plants were carry out to greenhouse. Then were infested with 300

whitefly adults per plant. A. *socialis* adults are obtained from the CIAT colony that is maintained in the greenhouse (27 ± °C temp. and 60 – 70% RH), on the cassava cultivar CMC-40. Finally were 2100 whiteflies per cage (Fig. 1). The 2nd day the adults are removed, allow the to develop of the eggs (oviposited for females). We collected leaves in six different times for the RNA extraction, according to the whitefly life cycle. First time before the infestation, 2nd time to one day after the infestation, third time to four days, 4th time to six days, 5th time to ten days and the 6th time to 14 days after the infestation.

Differential Subtraction

For the isolation of expressed sequences we using the follow strategy: the genotype MEcu-72 infested was using as tester and the genotype MCol- 2246 infested was using as driver. The objective is to obtain constitutive Resistant genes. The representational difference analysis of cDNA is divided in several phases, like the generation of a PCR amplicon which is representative of the original mRNA from MEcu-72 and MCol-2246, then the subtractive hybridization of these amplicons MEcu-72 (Tester) and MCol-2246 (Driver), during which amplified portions of differentially expressed genes are enriched and common sequences are depleted, and ultimately the cloning and screening of the resulting products. In this moment we are performance the Differential Subtraction Chain (DSC) technology according to Luo et al, (1999).

The RNA was isolate from young leaves collected in the greenhouse. For the isolate total RNA we are using the *Rneasy Plant Mini Kit™ QIAGEN.* Genomic DNA was removed prior to isolation of poly(A)$^+$ RNA with DNAse I. We are using *SV™ Total Isolation System of Promega.* The first-strand cDNA synthesis and the cDNA amplification were done using *SMART™ PCR cDNA Synthesis kit BD Biosciences.* The PCR products were purified using *QIAquick™ PCR Purification kit QIAGEN.* Once cDNAs amplified were purified, was done the Digestion-Ligation, which the cDNA is digested with DpnII and then an adapters (BamI and BamII) are ligated. We standardized the follow procedure of Digestion-Ligation process:

Procedure:

REAGENT	Volume (µl)
One-Phor-all Buffer 10X (*Amersham*)	2,5
DpnII Enzyme (10 U/µl)	1.0
cDNA (500 ng)	M=7,7 E= 12,5
Distillated Water	Complete to 25, M= 13,8 E= 9

2-Incubate to 37°C for 2 hours.
3-Inactivate the enzyme to 65°C during 20 min.
4-Add directly to the reaction (Final Volume 25 µl):

REAGENT	Volume (µl)
Distillated Water	12,5
One-Phor-all Buffer 10X (*Amersham*)	2,5
ATP (5 mM)	2,0

Adapters (*BamI, BamII*)* (10 µM)	6,0
T4 DNA Ligase (1U/µl)	4,0

**BamI* for MEcu-72 and *BamII* for MCol-2246

5-Incubate to 20°C for 2 hours.
6-Dilute with 10 µl of TE, pH: 8,0 (Dil. 1:1)

Then the amplicons are generated:

REAGENT	Volume (µl)
Distillated Water	37.3
Advantage Buffer 2™ 10X	5.0
dNTPs 20 mM (5mM cada uno)	0.5
Primer BamI, BamII 10 µM*	2.0
Advantage 2 Polymerase Mix™ (*BD Biosciences*)	0.2
Diluted Ligation Products	5.0

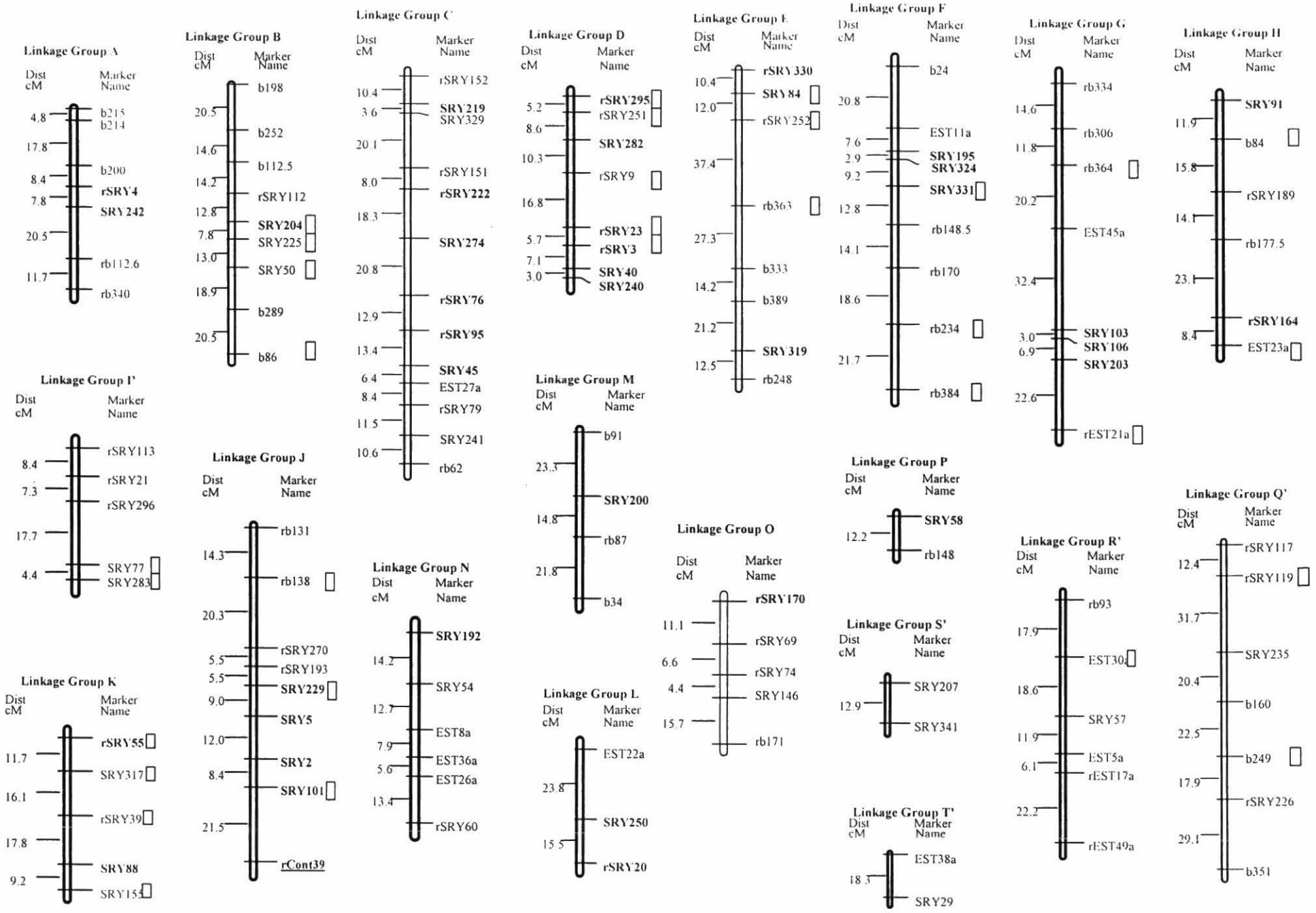
**BamI* for MEcu-72 and *BamII* for MCol-2246

Results

Mapping

We evaluated the OPERON™ set random primers in parents, which 43 were selected for evaluated in the cross. An AFLP analysis was made of 128 combinations of primers with both parents (MEcu 72 and MCol 2246) and both bulks of 10 whitefly-resistant DNA and 10 susceptible DNA. We obtained 53 polymorphic combinations, in which there were 425 polymorphic bands between the resistant and the susceptible. All combinations were amplified in the F1. Approximately 155 of the SSRs evaluated were polymorphic in the parents and were evaluated in the F1 (286 individuals). For the construction of linkage map 103 SSRs, 1 RGA, 15 RAPDs and 57 AFLPs were analyzed of which 129 were anchored. A genetic linkage map of cassava was constructed with 129 markers segregating from the heterozygous female parent (MEcu-72) of an intraspecific cross (see Figure 2). The map consists of 20 linkage groups, which represent approximately the haploid genome of cassava. These linkage groups span is 550,2 cM and the average marker density is 1 per 7,9 cM. The position of 129 markers, are 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 Mapmaker 2.0. So far, 41 SSRs markers were mapped (bold) on the cassava framework map (Fregene et al, 1997), the other 88 markers are new. The molecular data are being analyzed using QTL packages (QTL cartographer) to determine linkages between the SSR, RGA, RAPDs and AFLPs markers and the phenotypic characterization.

Figura 4: Preliminary Cassava framework Map of MEcu-72 for Resistance to White Fly, consisting of SSRs, AFLPs and a RGA (Contig39) (Lod = 25 and theta = 25)


Linkage Group A
Dist cM
Marker Name
4.8 b215 b214
17.8
8.4 b200
7.8 rSRY4 SRY242
20.5
11.7 rb112.6 rb340
Linkage Group B
Dist cM
Marker Name
b198
20.5
b252
14.6
b112.5
14.2
rSRY112
12.8
SRY204
7.8
SRY225
13.0
SRY50
18.9
b289
20.5
b86
Linkage Group C
Dist cM
Marker Name
rSRY152
10.4
3.6 SRY219 SRY329
20.1
rSRY151
8.0
rSRY222
18.3
SRY274
20.8
rSRY76
12.9
rSRY95
13.4
SRY45
6.4
8.4 EST27a
rSRY79
11.5
SRY241
10.6
rb62
Linkage Group D
Dist cM
Marker Name
5.2 rSRY295 rSRY251
8.6
SRY282
10.3
rSRY9
16.8
5.7 rSRY23 rSRY3
7.1
3.0 SRY40 SRY240
Linkage Group E
Dist cM
Marker Name
10.4 rSRY330
12.0 SRY84 rSRY252
37.4
rb363
27.3
b333
14.2
b389
21.2
SRY319
12.5
rb248
Linkage Group F
Dist cM
Marker Name
b24
20.8
EST11a
7.6
2.9 SRY195 SRY324
9.2
SRY331
12.8
rb148.5
14.1
rb170
18.6
rb234
21.7
rb384
Linkage Group G
Dist cM
Marker Name
rb334
14.6
rb306
11.8
rb364
20.2
EST45a
32.4
3.0 SRY103
6.9 SRY106
SRY203
22.6
rEST21a
Linkage Group H
Dist cM
Marker Name
SRY91
11.9
b84
15.8
rSRY189
14.1
rb177.5
23.1
rSRY164
8.4
EST23a
Linkage Group I'
Dist cM
Marker Name
rSRY113
8.4
rSRY21
7.3
rSRY296
17.7
SRY77
4.4
SRY283
Linkage Group J
Dist cM
Marker Name
rb131
14.3
rb138
20.3
rSRY270
5.5
rSRY193
5.5
SRY229
9.0
SRY5
12.0
SRY2
8.4
SRY101
21.5
rCont39
Linkage Group K
Dist cM
Marker Name
11.7 rSRY55
SRY317
16.1
rSRY39
17.8
SRY88
9.2
SRY155
Linkage Group L
Dist cM
Marker Name
EST22a
23.8
SRY250
15.5
rSRY20
Linkage Group M
Dist cM
Marker Name
b91
23.3
SRY200
14.8
rb87
21.8
b34
Linkage Group N
Dist cM
Marker Name
SRY192
14.2
SRY54
12.7
EST8a
7.9
5.6 EST36a EST26a
13.4
rSRY60
Linkage Group O
Dist cM
Marker Name
rSRY170
11.1
rSRY69
6.6
rSRY74
4.4
SRY146
15.7
rb171
Linkage Group P
Dist cM
Marker Name
12.2 SRY58 rb148
Linkage Group S'
Dist cM
Marker Name
SRY207
12.9
SRY341
Linkage Group T'
Dist cM
Marker Name
EST38a
18.3
SRY29
Linkage Group R'
Dist cM
Marker Name
rb93
17.9
EST30a
18.6
SRY57
11.9
6.1 EST5a rEST17a
22.2
rEST49a
Linkage Group Q'
Dist cM
Marker Name
rSRY117
12.4
rSRY119
31.7
SRY235
20.4
b160
22.5
b249
17.9
rSRY226
29.1
b351

Association between Molecular Markers and Resistance

Preliminary analysis (X^2 and Simple Linear Regression at the 5% level) was done using SAS. Subroutine associations were found between 32 markers SSRs, RGA and AFLPs, shown by blue squares in the Figure 2 and the field phenotypic characterization (score 1.0 to 2.0 of the levels of damage and populations). We observed that all markers anchored in the linkage group B, D, E, F. J and K are associated with the resistance. The molecular data are being analyzed using QTL packages (QTL cartographer) to determine linkage between the markers and the phenotypic characterization.

Functional Genomics: Differential Subtraction

RNA extraction

We are isolate RNA of MEcu-72 and MCol-2246 with high quality (Fig. 3).

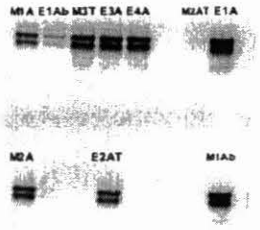


Figure 3: RNAs isolates with *Rnesy Plant Mini Kit QIAGEN* E=MECU-72 M= MCOL-2246 T=Witness plants

Poly A+ mRNA isolation, cDNA amplification y purification.

The Poly A+ mRNA was isolate from total RNA and was using like substrate for the generation of cDNA. The first-strand cDNA synthesis and the cDNA amplification were done (Fig. 4). Then of the amplification of cDNA, these PCR products were purified

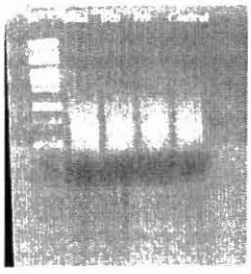


Figure 4: M: λ DNA digested with Pst I. cDNAs amplified with kit *SMART™*

Once cDNAs amplified were purified, was done the Digestion-Ligation and the amplicons generation.

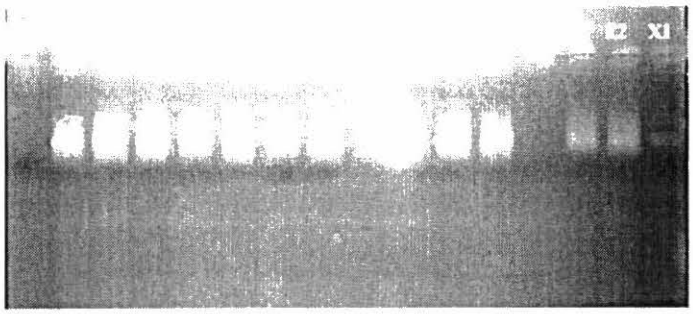


Figure 5: M: λDNA digested with Pst I. Amplicons of E=MECU-72 M= MCOL-2246 X=negative controls

Ongoing activities

-QTL analysis for whitefly resistance.

-Subtractive hybridization of the amplicon MEcu 72 (tester) and MCol 2246 (driver), the DSC technology according to Luo et al, (1999).

-Estrategy 2: the genotype MEcu-72 infested as tester and the same genotype without infest as driver, for to obtain induced defense genes.

-Cloning, sequencing 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.

-Hybridization with Cassava Unigenes Microarray.

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1.3.6 Identification of genes related to resistance to *Xanthomonas oryzae* pv. *Oryzae* using suppression subtractive hybridization and microarray analyisis

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Introduction

Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most important disease of rice in irrigated environment causing 20-80% yield reduction in severe epidemics (Gnanamanickam, S.S. *et al.* 1999; Datta, K. *et al.* 2002) The identification of genes associated with defense responses is one of the most critical steps leading to the elucidation of disease resistance mechanisms (Datta, K. *et al.* 2002). The objective was to identify genes associated with rice defense responses to *Xoo* combining suppression subtractive hybridization, microarray analysis. International mutant collections are also being used to validate some of the candidate genes.

Methodologies

Subtractive library construction

The incompatible interaction *Nipponbare-Xoo* (strain *PXO339*) was studied for differential gene expression over time after inoculation. Leaf tissues were collected at 2, 4, 8, 12, 24 hours post-inoculation (pi) and 3, 5 and 7 days pi. For subtraction we followed the SSH method performing forward and reverse subtractions.

Microarray analysis

SSH libraries were spotted on glass arrays and used along with a rice defense microarray provided by IRRI for gene expression analysis. Two hybridizations were performed: one comparing a cDNA pool from inoculated plants vs. a cDNA pool from healthy plants and one comparing three different time points (0-12 hpi; 0-24 hpi; 0-48 hpi). Two different biological and technical replicates with a dye-swap were performed. A rice oligo array (Génoplante, 60mers, indica genotype 93.1, 60K) was also used for gene expression analysis at 24hai.

Sequence analysis

Differentially expressed genes (from the SSH array analysis) were sequenced and compared to the GenBank databases. We compared the specific or common sequences among the different analysis.

Validation by quantitative RT PCR
A set of genes that were differentially expressed at different time points during infection were selected for confirmation by Q RT-PCR.

Results and Discussion

Identification of differential expressed genes

Using the SSH microarray we showed a clear expression of up-regulated genes 24 hours after inoculation (hai) (Fig.1). Differentially expressed genes were sequenced resulting in 22 singletons and 21 contigs. A functional classification showed a high percentage of genes related to secretory pathway and defense response (Fig. 2). Using the rice defense microarray (IRRI), 190 genes were differentially expressed. Cluster analysis showed five clusters with a clear expression of genes at 12 and 24hai. Sequence data were provided by IRRI for 33 of the differentially expressed genes. These genes are related to signal transduction and regulatory pathways.

Given that after pathogen recognition rice produces rapid defense responses, we selected one time (24hai) to identify differential expressed genes using a whole genome oligomicroarray (Génoplante). 146 genes were up and 41 down regulated 24hai. Using the TIGR annotation we found a high percentage of genes that are related to regulation, defense responses but also of unknown function.

In order to identify common genes involved in the defense response to PXO339, we selected 278 annotated sequences resulting from the different array analysis. They were grouped into functional categories using GO classification scheme (Fig. 3).
We observed that no sequence were common among the 3 array analysis. 4 sequences were common to the SSH and oligo array analysis (Fig. 4).

Validation by Q RT-PCR was confirmed for 15% of the selected genes that were upregulated at 24hai.

Search for insertion lines Using the OryGenes Database (CIRAD) 136 knockout mutants (Ac/Ds, Tos17, T-DNA) were identified in international mutant collections. 42 T-DNA mutants were identified from the Génoplante collection.

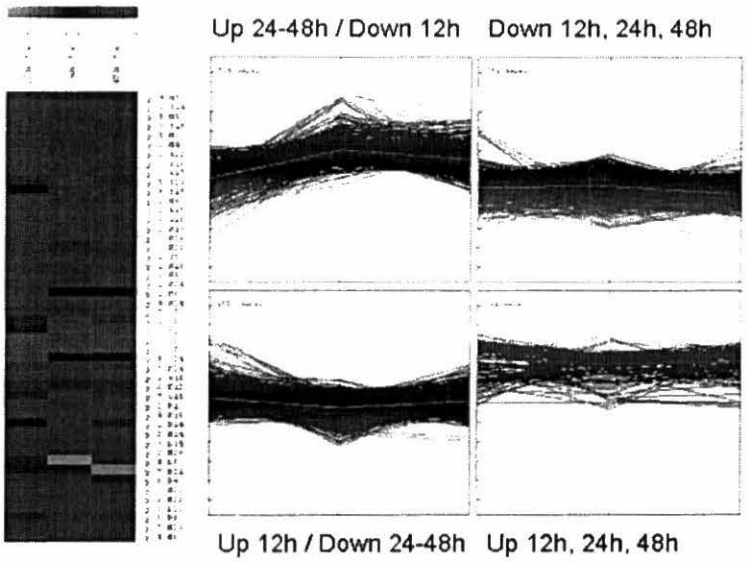

Fig 1. Cluster analysis showing four clusters with the SSH array. These graphics show a clear expression of up-regulated at 24 hours pi.

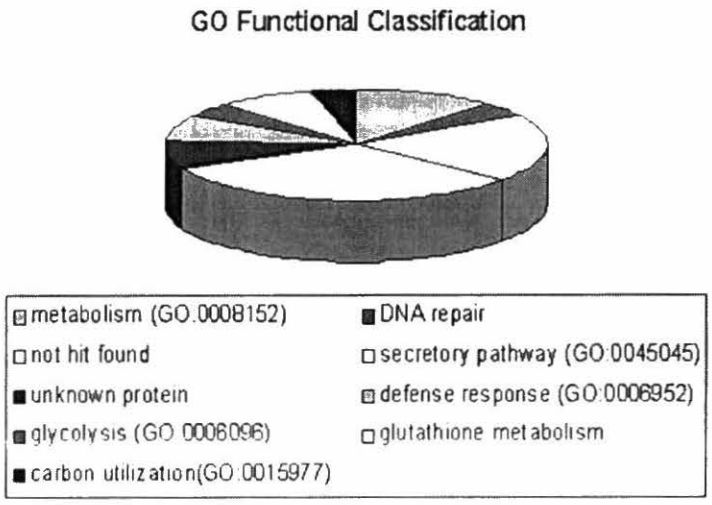

Fig 2. Go functional classification

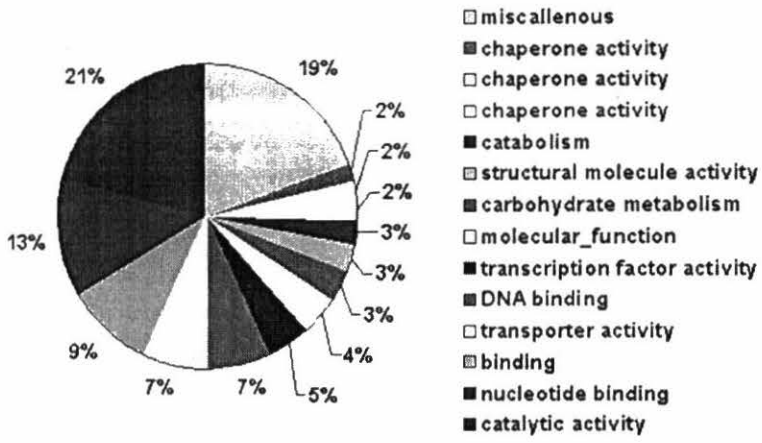

Fig 3 GO representation of differential expressed annotated genes

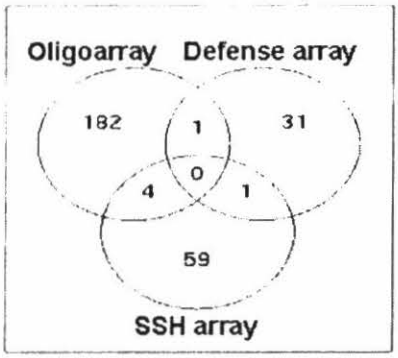

Fig 4. Venn diagram showing the distribution of the sequences present in each of the array analysis

On going activities

Functional validation of selected genes
Mapping the differentially expressed genes on the rice pseudo molecule
Screen the mutant T-DNA lines (Génoplante collection) for validation

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1.3.7 Identification and Molecular Characterization of Two Wild Rice Accessions Collected in Colombia

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SB-2 Project

Abstract

The un-known samples collected in Salahondita and Santa Rosa, Villavicencio, Meta were shown to be a tetraploid species (CCDD) belonging to *O.latifolia*. After several backcrosses to O.sativa, it was possible to recover fertile plants having introgressed traits from the wild progenitor; some plants presented additional chromosomes. These hybrid plants represent a very valuable genetic resource for genetic and breeding purposes. The presence of bivalents at diakinesis in F1 plants could be indications of recombination between genomes of different species. Chromosome behavior was abnormal in F1, BC2 and BC3 progenies, which causes high plant sterility. Polymorphic markers were identified which could be used to assess introgressions from the wild progenitor. Preliminary results showed that *O.latifolia* is resistant to rice blast, rice hoja blanca virus and *Tagosodes oryzicola*.

Introduction

There are two cultivated species (*O.sativa* and *O.glaberrima*) and about 20 related species in the genus Oryza. Some of them are diploid or tetraploids and 10 genome groups have been identified, mainly AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ, and HHKK (Ge et al., 1999).These species are widely distributed in the tropics and subtropical regions and represent great genetic variability. Four wild rice species have been reported in Latin America and the Caribbean, mainly *O.glumaepatula*(diploid, AA genome)and *O.alta*, *O.latifolia* and *O.grandiglumis* (tetraploids, CCDD genome). So far, Brazil and Cost Rica have reported the largest number of accessions; however, it is known that other regions in Latin America are rich in wild rice accessions. The purpose of this paper is to report on the characterization and agronomic value of two wild rice collections made in Colombia.

Materials and Mèthods

Two un-known samples were collected in contrasting environments in Colombia; one of them was collected along the banks of the Patia River in the Pacific Coast near a place called Salahondita(2° latitude north and 78° longitude best) in Nariño. The other collection was made in Santa Rosa Experiment Station, Villavicencio, Meta. These two sites differ in terms of soil and climatic conditions and disease pressure. Keys developed by Vaughan (1994) and Zamora et al, 2003, were used for the morphologic classification. Standard methodologies used by the Rice Project were used to determine the reaction of the two samples to rice hoja blanca virus, Tagosodes oryzicola and rice blast. The Salahondita collection was crossed to elite rice varieties and the resulting progenies were evaluated for hybrid sterility and cytogenetic behavior.
The wild accesión was used as the male parent in crosses to Fedearroz 50(female).Embryo rescue was used 10 days after pollination to recover F1 plants; MS ½ media (Murashige & Skoog, 1962) was used to grow the immature embryos, and 10 cm tall-seedlings were transferred to the screenhouse. Highly sterile F1 plants were backcrossed to BCF1720, BCF1658, and Fedearroz

50; a pollen mixture from BCF1720, BCF1658, Fanny, Pi9 and Fedearroz 50 was used to get BC2 and BC3 FI. Embryo rescue was also used for BC2 and BC3.
Somatic cells taken from 2-days old root tips were used for chromosome counts. Mitotic cells in metaphase were fixed with Farmer's solution and observed under a light microspcope. Immature panicles collected from F1, BC1 and BC2 plants were also fixed with Farmer's solution and used to determine meiotic behavior of chromosomes. Pollen viability was conducted as an assessment of plant sterility. Finally, 250 SSRs markers selected from the Gramenea database (www.gramene.org) were run on parental lines to find polymorphism in the resulting progenies.

Results

Several stands of wild rice were observed along the banks of the Patia River near Salahondita. This ecosystem is classified as humid tropical forest with about 9,000 mm of rainfall/year and low light intensity (3.5-4.5 hours/day). Based on morphological traits and chromosome counts (Fig.1and 2) the sample was classified as tetraploid species (CCDD), probably *O.latifolia*. This was further confirmed by using CAPs markers Bao and Ge (2001) based on the amplification of an ITS sequence of a ribosomal gene using specific primers for the region ITS 1 (5'-AGAAGTCGTAACAAGGTTTCCGTAGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). One accession of *O. alta* (N°. 100161), two accessions from *O. grandiglumis* (N°.105664 and 105669) and one from *O. latifolia* (N°. 100167)were used as control for the banding pattern characteristic of species belonging to the CCDD genome. Results confirmed that the two un-known samples collected in Colombia are *O.latifolia*.

F1 plants were almost completely sterile and seed set was lower that 1%; these plants were triploid (genome ACD) with 36 chromosomes: 24 chromosomes from Salahondita and 12 from the improved cultivar. At diakinesis presence of 2 and 8 bivalents (Figure 2) was observed, suggesting recombination between genomes of the different species. However, bivalents could also be the result of recombination between chromosomes of the two genomes carry by the wild progenitor. Chromosome incompatibility resulted in abnormal meiotic behavior in all phases causing plant sterility; evidence of chromosome elimination is presented in Fig.3. Seed set was also very low in BCF1 and BC3F1. However, embryo rescue allowed the recovery of 28 plants (11 BC2 and 17 BC3) with different morphology and plant characteristics, suggesting introgressions from the wild parent. The meiotic behavior of six plants was carried out (Table 1). Data shows that plants 2 and 4(BC2) and plants 13, 14, and 22 (BC3) are diploid, have a normal meiotic behavior, and highly fertile. In addition, some plants have chromosome additions with different degree of fertility. These plants represent a valuable genetic resource for genetic and breeding purposes.

Out of 250 SRRs markers evaluated, 77 were found to amplify in *O.latifolia* but only 74 were polymorphic (Fig.4), and relatively well distributed on the 12 chromosomes (Fig.5).
Furthermore, evaluations done under greenhouse conditions showed that the two new accessions of *O.latifolia* are resistant to rice blast, rice hoja blanca virus and *Tagosodes oryzicola*. Further work is underway to confirm preliminary results, which will have important implications for our breeding work and the rice community at large.

Conclusions

The un-known samples collected in Salahondita and Santa Rosa were shown to be a tetraploid species (CCDD) belonging to *O.latifolia*. After several backcrosses to O.sativa, it was possible to recover fertile plants having introgressed traits from the wild progenitor; some plants presented additional chromosomes. These hybrid plants represent a very valuable genetic resource for genetic and breeding purposes. The presence of bivalents at diakinesis in F1 plants could be indications of recombination between genomes of different species. Chromosome behavior was abnormal in F1, BC2 and BC3 progenies, which causes high plant sterility. Polymorphic markers were identified which could be used to assess introgressions from the wild progenitor.

Future Plans

Use molecular markers to characterize introgressions in BC2 and BC3 progenies.
Continue agronomic evaluation and selection of BC2 and BC3 progenies.
Continue crossing program to transfer traits of agronomic importance from *O.latifolia* to *O.sativa*.

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Figure 1. Chromosomes from *O. latifolia* in mitotic metaphase 2n=48.

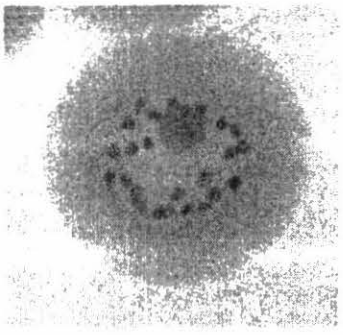

Figure2. Pollen mother cell from *O. latifolia* at diakinesis showing 24 bivalents.

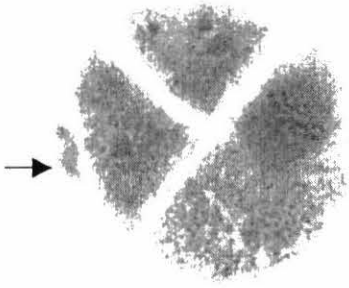

Figure 3. Cell from an F1 plant *O. latifolia* x *O.sativa* showing abnormalities at meiosis. Anaphase II (right) and telophase II (left). Arrow indicates chromosome elimination

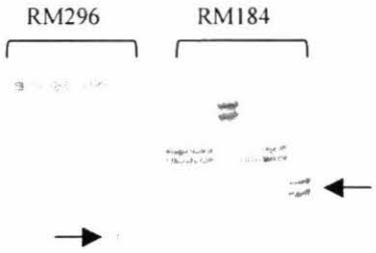

Figure4. Polimorphic SRRs for *O. latifolia* RM296 y RM184. Upper bands correspond to *O. sativa* (Fed 50, Pi9, Fanny, BCF1720 y BCF1658) and the lower band to *O.latifolia* (arrow)

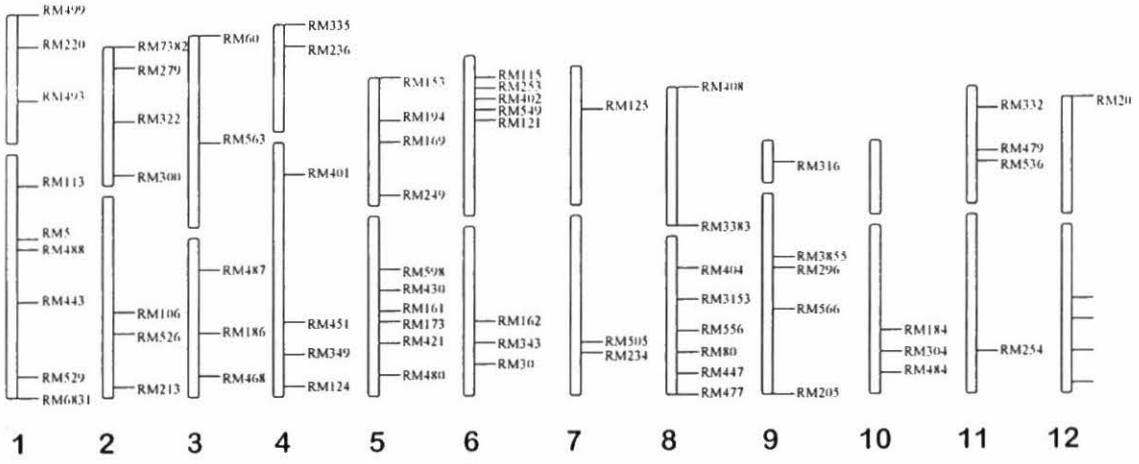

Figure 5. Distribution of SRRs markers that showed polimorphism between *O. sativa* and *O.latifolia.*

TABLE 1. Meiotic chromosome behavior and pollen viability in F1, BC2 and BC3 plants from crosses between *O.sativa* x *O. latifolia*. N (normal), A (abnormal), ND (not determined)

	Profase I (diacinesis)	Metafase I	Anafase I	Telofase I	Profase II	Metafase II	Anafase II	Telofase II	%pollen viable
O. sativa	12 II	N	N	N	N	N	N	N	91%
O. latifolia	24 II	N	N	N	N	N	N	N	87%
F1	2II, 32 I - 8 II, 20 I	A	A	A	A	A	A	A	0.01%
plant 2 (BC2)	12 II	N	N	N	N	N	N	N	86%
plant 4 (BC2)	12 II	N	N	N	N	N	N	N	86%
plant 5 (BC2)	12 II 2I	N	A	N	A	N	A	N	88%
plant 8 (BC2)	12 II 2I	N	A	N	A	N	A	N	ND
plant 11 (BC3)	12II 2I	N	A	N	A	N	A	N	ND
Plant 12 (BC3)	12 II 1I	N	N	N	N	N	N	N	52%
Plant 13 (BC3)	12 II	N	N	N	N	N	N	N	94%
Plant 14 (BC3)	12 II	N	N	N	N	N	N	N	92%
Plant 16(BC3)	12 II 1I	N	A	N	A	N	A	N	65%
Plant 17 (BC3)	12 II 1I	N	A	N	A	N	A	N	ND
Plant18 (BC3)	12 II 2I	N	A	N	A	N	A	N	ND
Plant 19 (BC3)	12 II 1I	N	A	N	A	N	A	N	44%
Plant 20 (BC3)	12 II 2I	N	A	N	A	N	A	N	37%
Plant 21 (BC3)	12 II 1I	N	A	N	A	N	A	N	19%
Plant 22 (BC3)	12 II	N	A	N	A	N	A	N	ND
Plant 25 (BC3)	12 II 2I	N	A	N	A	N	A	N	ND
Plant 26 (BC3)	12 II 1I	N	A	N	A	N	A	N	20%
Plant 27 (BC2)	12 II 2I	N	A	N	A	N	A	N	ND
Plant 28 (BC2)	12 II 2I	N	A	N	A	N	A	N	67%

1.3.8 Development of interspecific CSSLs and associated marker systems in rice

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Partners: IRD-UMR5096, Cornell University, Embrapa-CNPAF, Fedearroz, WARDA
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

O. sativa x *O. glaberrima* populations
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, Cali, Colombia. This population was derived from the cross Caiapo (tropical *japonica*) x IRGC103544 (*O. glaberrima*) and was made of more than 600 lines.

A BC2F5 & BC3F4 population produced by M. Lorieux and A. Ghesquière at IRD, Montpellier, France. The parents of this population are IR64 (tropical *indica*) and TOG5681 (*O. glaberrima*).
O. sativa x wild AA genome rice species populations

Choice of recurrent parents: A scale from 1 to 5 was used during the launching meeting to assess the merits of candidates presented by participants from CIAT and CNPAF. The following cultivars were selected: Liderança, CNA 8557, Bonança, Linea 30 and CNA 9025. Some NERICA lines were also suggested by WARDA. Five accessions from each of the following wild rice species have been used as donor parents: *O. glumaepatula*, *O. meridionalis*, *O. rufipogon*, *O. nivara* and *O. barthii*. CNPAF is responsible for developing populations with *O. glumaepatula* whilst CIAT, Fedearroz and WARDA are taking care of the crosses with the remaining wild species.

A flow chart for the development of these populations was also established and shared with participants.

Seed of recurrent parents was exchanged among participants following quarantine regulations from each country. Dr. Susan McCouch for Cornell University provided data and seeds of about 100 wild rice accessions to facilitate the identification of the best accessions for the development of the populations.
CIAT, Fedearroz and CNPAF got started in their crossing program. Due to difficulties in seed exchanges, the WARDA F1 crosses were done at CIAT.

Marker analyses

To help at the genotyping of the all six populations, we designed and started to develop a *universal rice core genetic* map. 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. (See the 2005 Generation Challenge Program Annual Report for details.) Only

microsatellite markers showing a unique locaiton in the genome were selected (according to the TIGR v. 2 data obtained from the Gramene database, http://www.gramene.org/).

The 312 lines of the Caiapo x IRGC103544 population were screened for additional SSR markers, in order to fill the gaps in the genetic map. Standard conditions for PCR and gel migration (PAGE/silver staining) were used.

The two sub-populations from the cross IR64 x TOG5681 were evaluated using 90 primer pairs selected after a screening for polymorphism within a set of 144 SSRs well distributed along the genome.

The PCR products were separated either high-throughput agarose gels and or denaturing polyacrylamide gels, depending on the polymorphism size. The PAGE technique was used when the polymorphism was lower than 8 bp. As a result, 60 markers could be visualized in agarose gels and 30 were visualized in polyacrylamide gels.

Phenotypic survey

Each line of the Caiapo x IRGC103544 population was phenotypically evaluated for yield traits and yield components traits traits (panicle length, plant height, yield, sterility, 1000-seed weight, and tillering) at CIAT HQs during the 2003 dry season.
The IR64 x TOG5681 lines were grown at CIAT HQs during the second semester of 2004 and were measured for a series of standard agronomic traits.

Data analysis

All the marker data produced from the two *O. sativa* x *O≥glaberrima* populations were analyzed with the CSSL Finder program (M. Lorieux, 2005, http://genetics.software.free.fr). The program uses a heuristic to determine the subset of lines that maximizes the representation of the donor genome and minimizes the undesired genetic background. It also displays graphical genotypes of the lines. Several parameters values (targeted segment size) were tried in order to search for the best subset of lines suitable for the CSSL population construction. Graphical genotyping of the final set was used to verify the final quality of the subset (total genome coverage).
QTL analysis was carried out using the programs MapDisto (M. Lorieux, 2005, http://genetics.software.free.fr) and CSSL Finder. CSSL Finder allows to display the graphical genotypes of the lines that show extreme values for a given trait, along with the F-test computed for each marker as criterion. It also computes permutation tests to determine local and overall thresholds for each marker/trait combination.

Results

Completion of two *O. sativa* x *O. glaberrima* populations
IR64 x TOG 5681 population
The data were first analyzed with the full set of 90 markers and, from the 353 lines evaluated, 64 were selected to be part of the introgression lines population. As the number of lines selected by the heuristic may depend on the number of markers selected for the analysis, we analyzed again the data with a subset of 75 markers, pulling out the ones that were redundant in terms of genome location. This way, a subset of 56 lines could be selected. The size of the introgressions

was measured based on the physical positions of the SSR markers on the rice genome (TIGR release v. 2).

The *O. glaberrima* genome was almost entirely conserved in the two sub-populations, but it appears that some small segments of the genome of *O. glaberrima*, especially on chromosomes 3, 4, 6, 10 and 11 were lost. Anticipating this result, we developed new BC2F1 lines from the same cross that we will check for the lost segments. The analysis also revealed that for 42 lines from the 46 selected, the bulks must be analyzed individually in order to find the plants that are in homozygous state for the target fragments. The remaining lines were already fixed for the target fragments.

We could identify QTLs for tillering, panicle size, and plant height. The QTLs were confirmed by comparison of the genotypes at the QTL locations of the lines showing extreme phenotypes. For *tillering*, three QTLs were identified, one on the chromosome 1 associated to the SSR RM104, another on chromosome 3 related to RM232 and another on chromosome 4 associated with RM348. For *panicle size*, two QTLs were found, one in the chromosome 3 associated to RM232 and one on chromosome 4 with RM348. Two QTLs were found for *plant height*, the first one on chromosome 5 associated with RM13 and the second one on chromosome 6 with RM136.

Caiapo x IRGC103544 population

Seventy-six SSRs markers were added to the previous data set, leading to a total of 174 mapped markers for the 312 lines. Using the program CSSL Finder, a subset of 72 lines was selected according (1) to the presence of contiguous chromosome segments that maximized the genome coverage of *O. glaberrima*, and (2) to the percentage of recurrent genetic background. As a result, in each line, one or few different chromosomal segments of *O. glaberrima*, was substituted in the genetic background of the cultivar Caiapo. The substituted chromosome segments in the 72 introgression lines represented the complete genome of *O. glaberrima*, except for small regions of chromosomes 2, 4. Moreover, for some introgressions it was unclear if they formed overlaps with other segments, due to the small size of the introgressed segments. These areas are understood among chromosome 2 (RM71-RM300), chromosome 4 (RM261-RM241), chromosome 8 (RM308-RM284), chromosome 9 (RM316-RM296) and chromosome 10 (RM474-RM239). It will be necessary to screen these regions with additional markers.
The QTL analysis by means of the programs MapDisto v.1.5 (Lorieux 2005), CSSL Finder (Lorieux 2005) and QGene v.3.07 (Nelson 1997) allowed to detect 12 QTLs for 5 quantitative traits in chromosomes 1, 3, 4, 5, 6 and 11, and are being compared to QTLs found in the literature and to those obtained with the IR64 x TOG5681 population.

Generation of *O. sativa* x wild AA genome rice species populations

At CIAT, we are currently collecting F1 seeds and doing backcrosses from single crosses between recurrent parents and *O. barthii, O. nivara, O. rufipogon, O. meridionalis, and O. glumaepatula*. F1 seeds from 17 different wild accessions are being planted to produce BC1F1 (six from *O. nivara*, four from *O. rufipogon*, two from *O. barthii*, three from *O. meridionalis* and two from *O. glumaepatula*). Another 10 F1 seeds from different wild types accessions are ready to be planted, by the end of this year.

At CNPAF, the F1 seeds of 20 interspecific crosses are being sowed, to obtain the BC1 seeds. The F1 crosses among Linea 30 and five wild *O. glumaepatula* accessions are being obtained at the moment, due to the delay in seed exchanges for the recurrent genitor.

Tissue samples for those F1 plants that have germinated are being collected to verify their identity with molecular analysis, as well for the wild types accessions that are used at CIAT.
Also, during the period of time from July 11th to October 25th, pictures were taken for fifty-one wild types of the genus *Oryza* at CIAT HQs. These types are distributed in eight types of *O. nivara*, seven types of *O. rufipogon*, six types of *Oryza barthii*, twenty-four types of *O. meridionalis* and six types of *O. glumaepatula*. This activity was due to describe some phenotypic characteristics (qualitative) of the wild parental that will be used in the interspecific crosses, in order to keep a data record, and compare them during the production of the F1 crosses and the backcrosses.

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).
Also, the IR64 x TOG5681 lines already permitted 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.

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1.3.9 Molecular analysis of a BC3F2 population from the cross Lemont x *O. barthii*

Giraldo Olga Ximena and Martinez, C.
SB-2 Project

Abstract

Further molecular analysis was carried out in this population this year.Linkage map was saturated with 10 additional microsatellite markers for a total of 123 microtatellite markers; additional SSRs were distributed on chromosomes 1, 5 and 10. A total of 12 agronomic traits, including yield and yield components, were evaluated. The association between phenotype and marker genotype was done using single-point analysis (SPA), interval mapping (IM) and composite interval mapping (CIM) using QTL Cartographer Vers. 1.17d software. Transgressive segregants were observed for all traits examined. For those traits for which two or more QTLs were detected, increases in the traits were conditioned by *O. barthii* alleles at some QTLs and Lemont alleles at others. Chromosome 1 carries QTLs associated whit grain yield (RM5, and RM9). 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. Markers RM 184 and RM 304 on chromosome 10 are associated with days to heading. Advanced breeding lines with different flowering dates have been selected out of this cross.

Introduction

Rice (*Oryza sativa*) is one of the main food crops; 50% of the world population use rice as main base of their alimentary needs and approximately 85% of world rice production is for human consumption (He et al. 1999; Shen et al. 2001, before IRRI 1997). The 21 – 23 wild species and 2 cultivated species (*Oryza sativa* and *Oryza glaberrima*) represent wide genetic variability for rice breeding programs. Several studies have suggested that wild species are valued as a unique source of genetic variation, but they have rarely been used for the genetic improvement of quantitative traits. To identify trait-improving quantitative trait loci (QTL) alleles from exotic species, a BC_3F_2 population derived from cross between an accession of *Oryza barthii,* (donor parent) and Lemont (recurrent parent), were used. The results of applying AB-QTL analysis to 327 individuals characterized with 123 microsatellite markers are presented. Marker data were used to identify QTLs associated with yield and yield components.

Materials and methods

Lemont, the recurrent parental, is a elite *japonica* cultivar, developed in Texas, US, that has been reported as a high yielding variety with excellent grain quality (http://www.cropscience.org.au/icsc2004/poster/1/4/789_katoyv.htm). *Oryza barthii* (Accession #104119), used in this study as a donor parental, is an annual rice wild specie originated in Africa and it is the ancestor of *O. glaberrima*. There was very high sterility so three backcrosses to Lemont were done. BC_3F_2 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 leave tissue was isolated according to Dellaporta DNA extraction protocol (McCouch et al. 1988), modified for PCR probes in the laboratory of the Biotechnology Research Unit of CIAT. DNA quantification was performed with DNA standard of λ phage, and DNA dilutions of 4 ng/µl were storage at -20°C and used for the molecular analysis.

The hybrid population was evaluated with 123 polymorphic microsatellite markers to parentals Lemont / *O. barthii,* and 12 quantitative traits including 1000-grain weight (*gw*), number of panicles per plant (*mtn*), number of tillers per plant (*tpl*), number of grains per panicle (*gpp*), total grains per plant (*tg*), total plot weight (*pw*), plant height (*h*), panicle length (*pl*), percent sterility (*ps*), days to harvesting (*dhv*), yield (*yld*) and grain weight (*gw*) were evaluated.

Results

Segregation ratio expected in BC_3F_2 population would be 90,625% homozygotes (Lemont/Lemont): 6,250% heterocygotes (Lemont / *O. barthii*): 3.125% homozygotes (*O. barthii / O. barthii*). Segregation distortion at $P < 0.01$ was detected for 108 marker loci located in 12 chromosomes. In map construction, the heterozygotes of individual loci were treated as missing data. The order of 123 microsatellite markers 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). Mapmaker analysis at LOD 3.0 resolved the distances in centimorgans between the markers. Our linkage map has a total length 1.150,86 cM. The number of the markers mapped on their respective chromosomes ranged from 5 (Chr.6) to 20 (Chr. 2). Transgressive segregants were observed for all traits examined (fig. 1). The association between phenotype and marker genotype was investigated using single point análisis (SPA), interval mapping (IM) and composite interval mapping (CIM) using QTL Cartogrpher Vers. 1.17d software. Moreover, permutations allow the estimation of experimentwise significance. For SPA, the threshold was established by doing 10.000 permutations, while threshold, for IM and CIM were established by carrying out 1000 permutations. Composite interval mapping at LOD threshold 2.843 identified a total of 17 QTLs for the 12 traits (table1). In chromosomes 2, 4, 5, 6, 7 and 9, neither marker was associated with neither of the traits; while, there were several genetic regions associated with more than one trait in chromosomes 1, 3, 8, 10, 11 and 12 (Table1). 11 of the QTLs were detected before and after adding the ten new microsatellite markers, and the remaining 6 QTLs were detected only after. For yield two QTLs were detected this year, located on chromosome 1, the RM 5 was simultaneously detected in both years. Three markers RM81B, RM42 and RM21 located in the chromosomes 3, 8 and 11 respectively, were associated with number of panicles per plant (*mtn*) and number of tillers per plant (*tpl*), in which the marker RM42 are associated with a region derived from *O. barthii.* Markers RM184 and RM304 on chromosome 10 are associated with days to harvesting (*dhv*); 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.

Table 1. QTLs identified as significant by all three analytical procedures.

Variable	Cro.	SSR	Position	SPA	Interval mapping				Composite interval mapping			
					LR	LOD	R^2	A	LR	LOD	R^2	A
number of panicles per plant (*mtn*)	3	RM81B	0.4006	36.413	36.144	7.851	0.106	1.142	25.882	5.622	0.063	0.907
	8	RM42	0.0947	25.306	25.053	5.442	0.074	-1.679	14.951	3.247	0.035	-1.183
	11	RM21	0.4429	29.270	29.251	6.353	0.086	1.221	18.384	3.993	0.043	0.888
number of tillers per plant (*tpl*)	3	RM81B	0.4006	29.852	29.769	6.466	0.089	1.219	14.406	3.129	0.036	0.802
	8	RM42	0.0947	34.928	34.758	7.549	0.102	-2.295	17.693	3.843	0.043	-1.570
	10	RM222	0.0001	16.967	16.606	3.607	0.051	-3.007	20.256	4.400	0.048	-2.921
	11	RM21	0.4429	29.275	29.280	6.360	0.086	1.427	16.695	3.626	0.041	1.010
number of grains per panicle (*gpp*)	12	RM19	0.0589	28.846	29.675	6.445	0.093	29.747	24.152	5.246	0.069	26.447
	12	RM247	0.0614	22.697	22.659	4.922	0.067	30.984	18.193	3.951	0.048	25.446
1000-grain weight (*gw*)	3	RM114	1.8525	17.534	17.479	3.796	0.053	0.542	18.831	4.090	0.054	0.555
days to harvest (*dhv*)	10	RM184	0.0443	90.136	96.511	20.962	0.280	15.941	85.448	18.559	0.185	13.404
	10	RM304	0.0455	38.407	88.231	19.164	0.280	15.925	77.436	16.819	0.187	13.241
panicle length (*pl*)	3	RM114	1.8525	49.763	51.270	11.136	0.153	-1.047	70.140	15.234	0.157	-1.065
	8	RM210	0.5339	33.257	33.616	7.302	0.101	0.869	14.999	3.258	0.030	0.508
	8	RM256	0.7735	19.682	20.177	4.382	0.067	1.025	13.088	2.843	0.035	0.807
Yield (*yld*)	1	RM9	0.9505	18.555	31.182	6.773	0.099	570.720	23.892	5.189	0.066	471.093
	1	RM5	0.9735	29.986	29.882	6.490	0.088	528.687	23.455	5.094	0.060	439.943

Future plans

Evaluate advanced breeding lines selected based on phenotype, with microsatellite markers associated with the different traits evaluated to confirm presence of QTLs reported in this study.

Develop Chromosome Segment Substitution Lines (CSSLs) targeting alleles from *O. barthii* on a Lemont genetic background to make them available as a genetic resource for the rice communities.

1.3.10 Study of gene expression during pistil development in apomictic and sexual *Brachiaria* spp

D. Bernal, J. Tohme

Introduction

Apomixis is a plant reproductive mode through seeds, which contain an embryo produced without the fusion of the egg and sperm cells. It has been identified as an enormously advantageous characteristic for plant breeding programs since it could allow breeders to fix highly heterozygous genotypes through seeds. Since map-based positional cloning has not been successful at cloning the gene(s) responsible for apomixes, another approach such as the characterization of differences in the transcriptome of pistils from sexual and apomictic plants could give us insight into the mechanisms involved in the regulation of plant reproductive mode. Previously, pistil EST's associated to late stages of the apomictic reproductive development in *Brachiaria* were identified using subtractive hybridization coupled to microarray gene expression

analysis as described earlier (Bernal, 2003). Currently we are retaking this project and we will use the former strategy with the purpose of extending the transcriptome characterization to the first stages of the reproductive development by acquiring a three-point gene expression profile of apomictic and sexual *Brachiaria* during this process. Also the gene expression analysis was revised.

Methodology

Revised gene expression analysis

The microarray data of the comparisons between different apomictic and sexual mRNA samples was normalized using MIDAS (Saeed, 2003) by applying the intensity-dependent normalization method LOWESS by sub-grids, followed by a standard deviation regularization between subgrids and between slides. Differentially expressed genes were identified using the one-class design of the significance analysis of microarrays method (Chu, 2002), choosing 500 permutations and a false discovery rate of 0.1% or lower. Cluster analysis was performed on MeV (Saeed, 2003) on all the genes called differentially expressed at any comparison by the SAM analysis. First, principal component analysis (PCA) was used to find any clusters among the genes. Then the Figures of Merit (FOM) analysis was run to determine the advisable number of clusters to group the genes. Next, a supported K-means cluster (SKC) analysis was run to identify groups of genes with an interesting gene expression profile considering all the comparisons between different apomictic and sexual genotypes.

Microarray construction.

The microarray will contain both full-length cDNA and subtractive hybridization clones. The libraries containing these clones will be constructed as described earlier (Bernal, 2003), but the plant material used to extract mRNA is from the first stages of reproductive development. Pistils are classified into I and II stages. Pistils I are less than 0.2 mm, and pistils II are between 0.21-0.4 mm. Pistils are stored in RNA later solution at room temperature during dissection (maximum during 12 hours), at –20C for a month, and at –80C for longer storage. Microarray construction, hybridization, scanning and normalization will also be performed as described earlier (Bernal, 2003).

Gene expression analysis.

Gene expression analysis during reproductive development of apomictic and sexuall plants will be made using *B. decumbens* (606, apomicitic) and *B. rusisiensis* (44-2, sexual), at the first stages of reproductive development because:

- There is a molecular map available for *B. decumbens*
- Higher fertility in crosses between *B. rusisiensis* and *B. decumbens*, than between *B. rusisiensis* and *B. brizantha* (Lutts et al, 1994)
- Regarding reproductive behavior, *B. decumbens* is more uniform than *B. brizantha* when comparing greenhouse and natural conditions

Results

In the previous analysis, 47 clones were identified as up-regulated in the apomictic mRNA samples, and none as down-regulated. In the new analysis we could not obtain an ideal number of clusters to group the genes since there was not any trend on the PCA graphic or any noticeable slope change on the FOM graph, as seen in figure 1 and figure 2 respectively.

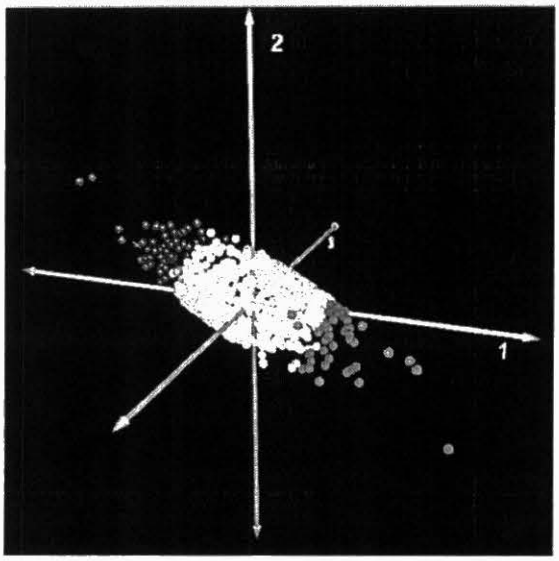

Figure 1. Principal component analysis graph on a 3D space where the axis are the first three principal components of the variability found in the gene expression differences.

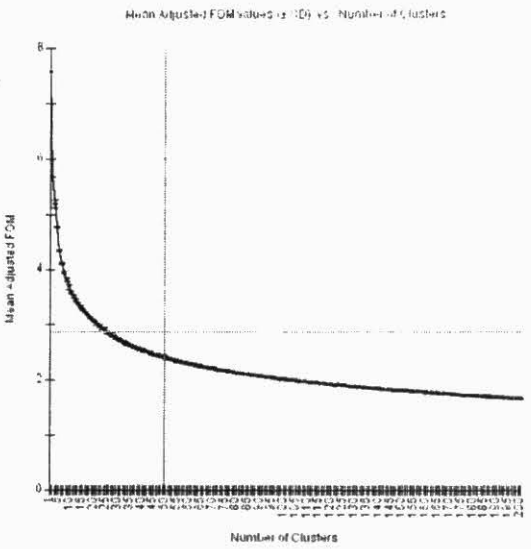

Figure 2. Figure of Merit graph showing a smooth curve as one increases the number of clusters (up to 200) to group the genes according to their relative gene expression between different apomicitc and sexual samples.

In spite of this, as shown in figure 3, the SKC analysis allowed us to identify a group of clones that were up-regulated (figure 3 b, 76 clones) and a group of clones that were down-regulated (figure 3 c, 45 clones) in all the comparisons performed; 45 of the 76 up-regulated clones had been identified in the previous analysis and sequenced. Also, other two groups of genes can be made according to their similar pattern of expression in the comparisons made. However they don't show a differential expression in all the comparisons, so they are most likely due to differences not involved with the apomictic reproductive mode, since all the comparisons are made between an apomictic and a sexual mRNA sample. Finally there are many clones that could not be assigned to a cluster (figure 3 a)

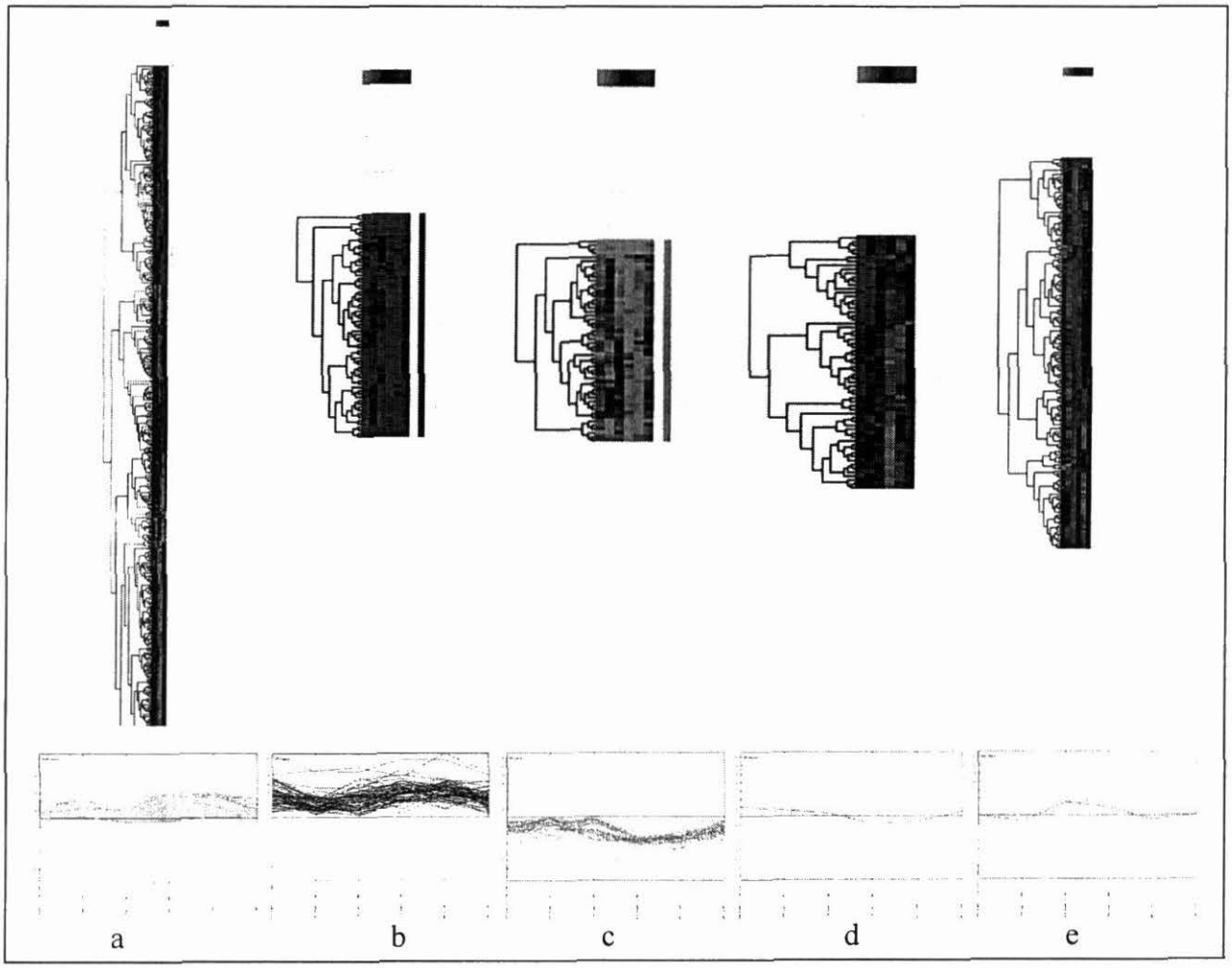

Figure 3. SKC analysis showing 4 clusters identified (b, c, d and e) and a group of unassigned clones (a).

Primers were designed for the previously sequenced clones in order to isolate the complete transcript of those which show a more differential expression in all the comparisons and have an interesting putative function. Consequently, we will gain more information to be able to comprehend their possible role in the reproductive development.

Microarray construction

Pistils were dissected from *Brachiaria* inflorescences to extract RNA. Table 1 shows the number of pistils dissected from each genotype at stage I and II. RNA has been successfully extracted from all samples and full-length cDNA has been synthesized.

Identification	Stage I (0-20 u)	Stage II (21-40 u)
MX 1319	253	223
MX 1388	169	121
MX 1423	74	94
MX 1614	166	146
MX 2090	52	95
MX 3213	118	74
SX 4346	58	21
SX 4357	102	98
SX 2987	144	117
SX 2071	79	73
SX 2068	178	136
SX 2226	61	99
SX 0770	245	168

Table 1. Number of pistils dissected from each genotype at stage I and II. MX means apomictic and SX means sexual.

Full length cDNA is ready to be used in the construction a full-length cDNA library and perform a subtractive hybridization to construct a subtractive library enriched in apomicitic and sexual related clones.

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OUTPUT 2. Genes and gene combinations use to broaden the genetic base

Activity 2.1 Transfer of gene and gene combinations using cellular and molecular techniques

2.1.1 Overcoming crossing incompatibility between selected genotypes of tepary & common bean through double congruity backcrossing

A. Mejía-Jiménez, L. Galindo, H. Jaimes, A. Criollo, C. Cardona and J. Tohme
[1]Agrobiodiverstity-Biotechnology Project SB2; [2]Project IP1

Introduction

The tepary bean (*Phaseolus acutifolius*) possesses multiple traits that are important for common bean breeding (for a review see Singh, 2001). Furthermore, this species has been the only one *Phaseolus* species, in which *in vitro* tissue culture methods could be reproducibly applied to produce transgenic plants through *Agrobacterium tumefaciens* (*Agro*-transformation. Dillen *et al.*, 1997; Mejía-Jiménez, 2000; Clercq *et al.* 2002; Zambre *et al.* 2005). Due to the difficulties encountered in the *Agro*-transformation of common bean cultivars, the tepary bean has been proposed as a bridging species for the introgression of transgenes to this species through sexual crosses (Dillen *et al.*, 1997).

Although difficult to cross, morphological and biochemical markers, as well as agronomic important traits, have been introgressed from tepary into common bean (Waines et al, 1988). Through recurrent and congruity backcrosses performed at CIAT (Mejía-Jiménez *et al.* 1994), the lines of common bean with the highest levels of tolerance to bacterial blight (*Xanthomonas campestris pv. phaseoli*) available have been produced (Singh and Muñoz, 1999). These lines are being used around the world to introgress the resistance to other local commercial cultivars (see Bean Improvement Cooperative reports at: www.css.msu.edu/bic/). Nevertheless the introgression of other agronomic important traits of tepary bean such as the resistance to drought, leafhopper (*Empoasca kraemeri*) or the bean weevil (*Acanthoscelides obtectus*), has not been possible applying the same backcross strategies as for bacterial blight. Incompatibility between most tepary bean accessions and common bean cultivars if crossed directly, self sterility of the hybrids, linkage between desirable and undesirable traits (Waines *et al.* 1988) and/or low levels of trait introgression from tepary bean (Muñoz *et al.* 2004) may be the constraints preventing this. During 2005 we continued to apply a novel backcross strategy called Double Congruity Backcrosses (DCBC) which may help to overcome some of the above mentioned constraints.

This backcross strategy was initiated in 2000 to introgress into common bean the traits responsible for *Agro*-transformation competence, as well as resistance to *A. obtectus*. During 2005 a large number of fertile hybrids showing the presence and segregation of traits of both species were produced.

Methodology

Our DCBC backcross program is described in http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf. The parentals used during 2005 were DCBC hybrids chosen using the following criteria: advanced DCBC hybrids showing high fertility and vigor, stem and flower pigmentation (male parents) or no pigmentation (female parents), good response to *in vitro* culture methodologies or high transient expression of the GUS intron transgene after *Agro*-transformation, resistance to the bruchid *Acanthoscelides obtectus*, a large seed size and/or fertility. All embryos resulting from interspecific and from most of the intraspecific DCBCs, were rescued and cultured *in vitro* (Mejia Jimenez *et al.* 1994). All plants resulting from a backcross on fertile hybrids (interspecific DCBCs), were verified as such by the presence of morphological markers derived from the male parent (hypocotyl and flower color or primary leaf petiole size).

Results

Production of hybrid lines using parental accessions with response to *in vitro* culture, plant regeneration and *Agrobacterium* mediated genetic transformation.

Different accessions of tepary bean such as the wild genotype NI576 (Dillen *et al.* 1997; Clercq *et al.* 2002), the cultivated ones TB-1 and PI- (Zambre *et al.* 2005) and the intraspecific hybrids G40065 x NI576 and G40022 x NI576 (Mejia *et al.* 2000 and 2002) have been *Agro*-transformed. Methodologies used to transform these genotypes have been applied to common bean wild and cultivated accessions without success in the production transgenic regenerable tissues or plants (see bean transformation report and unpublished results).

As in other crops such as rice and maize have been demonstrated, the response to *in vitro* tissue culture methodologies for plant regeneration or genetic transformation is influenced by genetic traits. Traits for competence to *Agrobacterium* mediated transformation seem to be present in selected accessions of tepary bean that could be transformed using this vector, and absent in common bean.

In order to transfer these traits to common bean, a crossing program was started in 2000, in which a tepary bean accession, competent to *Agro*-transformation, and other tepary and common bean accessions selected for their response to *in vitro* culture were included.

The direct crossing of the selected common and tepary bean accessions using recurrent or congruity backcrosses yielded no fertile or cross-fertile plant. It was necessary to develop the DCBC backcross strategy in order to generate fertile progeny involving the selected accessions as parentals.

For accumulating alleles which may play an important role in competence to *Agro*-transformation, we transformed 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.

During 2005, 3 interspecific and 10 intraspecific DCBC hybrid generations with the common bean cytoplasm and 3 interspecific and 9 intraspecific DCBC hybrid generations with the tepary bean cytoplasm succeeded, and more than 500 fertile hybrid lines were produced. The number of crosses performed and the embryos rescued have not been yet evaluated to calculate and compare hybridization efficiencies among the different DCBC generations.

Several of the DCBC hybrids produced during 2005 involve in their pedigree, lines selected for improved competence for *Agro*-transformation (Tables 1 and 2). These lines will be tested for competence to *Agro*-transformation during 2006.

Development of hybrid lines using parentals resistant to *A. obtectus*

During the past five years interspecific hybrid lines produced in our DCBC program were provided to CIAT's bean entomology for testing for resistance to the bruchid *A. obtectus*. At least 10 different lines have been identified as resistant (IPM report 2004). One of the resistant lines has the cytoplasm of common bean (code T7K-2E), the other (codes GNVAV, GVV, GKVGAG, NNIQLAC, and BWG) the cytoplasm of tepary bean.

The most stable resistance to *A. obtectus* has been found in DCBC hybrids with tepary bean cytoplasm. In order to pyramid different resistance genes in common bean lines we are attempting to cross the different lines that showed resistance, including the resistant progeny of the line T7K-2E with the most advanced V-DCBC lines. During 2005 some of this crosses succeeded in the production of fertile or cross fertile hybrids (Table 1 and 2). These lines are being multiplied or have been already provided to CIAT's bean entomology for resistance testing.

Optimization of the screening methodologies for identifying lines resistant to *A. obtectus*

One of the bottlenecks of the screening methodologies for identifying resistant hybrids to *A. obtectus* are the long incubation periods under high temperature and relative humidity. During this time many of the resistant seeds loss their viability. Therefore, no progeny is obtained to confirm or stabilize the resistance . At the end of 2005 a M.Sc. thesis was started (Hugo Jaimes) at the National University of Palmira (Valle del Cauca, Colombia) with the main objective of developing faster and non-destructive methodologies for screening segregating progenies of interspecific hybrids for resistance to the insect. The methodology proposed in this research intends to screen individual halves of seeds, without embryo axes, while the other halves are to be used to grow the plant and produce the next progeny.

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-DCBC x A-DCBC). Underlined parentals correspond to hybrids selected for resistance to *A. obtectus*. Bold underlined parentals correspond to hybrids selected for its good *in vitro* response (for the complete pedigree of the hybrids see the table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf).

Cross abbreviation/ hybrid code	Female parent/ code	Male parent/ code	Characteristics of the parent lines	Verification of hybrids	Nr. of fertile or cross-fertile hybrids
V-DCBC$_{11B}$ STRB	V-DCBC$_{10D}$ STR	A-DCBC$_{14C}$ *BB-1*	BB-1 includes in its pedigree an A-DCBC line resistant to *A. obtectus*	Red hypocotyl and flower color from the male parent	2
V-DCBC$_{12D}$ STRBR	V-DCBC$_{11B}$ STRB	V-DCBC$_{12B}$ RGRR		Female parent self-sterile	3
V-DCBC$_{12E}$ G24R	G10024	V-DCBC$_{12C}$ RGRTS, RGRRR	G10024 wild common bean cultivar from Argentina	Scarlet flower color from the male parent originaly from P. coccineus	8
V-DCBC$_{12F}$ G24RR, G24RS	V-DCBC$_{12E}$ G24R	V-DCBC$_{10E, -12C}$ STR, RGRTCR		Red hypocotyl and flower color from the male parent	5
V-DCBC$_{13A}$ RGRTC	V-DCBC$_{12B}$ RGRT	A-DCBC$_{16B}$ **CWB**	CWB includes in its pedigree hybrids selected for good *in vitro* response	Red hypocotyl and flower color from the male parent	2
V-DCBC$_{14A}$, RGRTCR, RGRTCS	V-DCBC$_{13A}$ RGRTC	V-DCBC$_{10E, -12A, -12B, -12C}$ STR, RGR, RGRR, RGRT, RGRTS,		Female parent self-sterile	20
V-DCBC$_{14B}$ DV	V-DCBC$_{14A, 12D, 10F}$ RGRTCS, RGRTCR, STZS, STRBR	V-DCBC$_{10B, -10E, -10G, 12B, -12C, -12D, -14A}$ RR, STR, RGRT, STRR, STRBR, RGRRR, RGRTS, RGRTCS	STZS includes in its pedigree the tepary bean accession G40199, which is resistant to *A. obtectus*	Red hypocotyl and scarlet or red flower color from the male parent	92
V-DCBC$_{14C}$ DVG, DVB, DVD	V-DCBC$_{14B}$ DV	V-DCBC$_{5E, -12F, 14B}$ BKIM, G24RS, DV	BKIM includes in its pedigree the accession Bayo Madero, a common bean cultivar showing good *in vitro* response	Red hypocotyl and scarlet or red flower color from the male parent	84
V-DCBC$_{14D}$ DVDD	V-DCBC$_{14C}$ DVD	V-DCBC$_{14B}$ DV		Red hypocotyl and scarlet or red flower color from the male parent	12
V-DCBC$_{15A}$ ET	V-DCBC$_{14A}$ RGRTCR	A-DCBC$_{16D}$ CWBBG	CWBBG includes in its pedigree a resistant *A. obtectus* line	Red hypocotyl and flower color from the male parent	1
V-DCBC$_{16A}$ ETD	V-DCBC$_{15}$ ET	V-DCBC$_{14B}$ DV		Female parent self-sterile	1
V-DCBC$_{16C}$ ETDR	V-DCBC$_{16A}$ ETD	V-DCBC$_{14A}$ RGRTCR		Female parent self-sterile	6

Cross abbreviation/ hybrid code	Female parent/ code	Male parent/ code	Characteristics of the parent lines	Verification of hybrids	Nr. of fertile or cross-fertile hybrids
V-DCBC$_{16D}$ ETDRD, EDTRT	V-DCBC$_{16C}$ ETDR	V-DCBC$_{6,-4C}$ DVG, T7K-2E	T7K-2 is an *A. obtectus* resistant V-DCBC line	Red hypocotyl and scarlet or red flower color from the male parent	83
TOTAL					319

Table 2. New DCBC (Double congruity backcross) hybrid progenies with the cytoplasm of tepary bean (A-DCBC) developed during 2005. V-DCBC=DCBC with the cytoplasm of common bean. The shaded boxes correspond to interspecific DCBC (A-DCBC x V-DCBC). Underlined parentals correspond to hybrids selected for resistance to *A. obtectus* (for the complete pedigree of the hybrids see the table DCBC hybrids: http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf).

Cross abbreviation/ hybrid code	Female parent/ code	Male parent/ code	Characteristics of the parent lines	Verification of hybrids	Nr. of fertile or cross-fertile hybrids
A-DCBC$_{16C}$ CWBB	A-DCBC$_{16B}$ CWB	A-DCBC$_{12B,-14B,-14C}$ BWG, BWTZBB, CWB	BWG is A-DCBC line resistant to A. obtectus	Red hypocotyl and flower color from the male parent	15
A-DCBC$_{16D}$ CWBBB, CWBBG, GBC, BBC	A-DCBC$_{16C}$ CWB, GB, BB, CWBB	A-DCBC$_{12B,-16B,-16C}$ BB, GB, CWB, CWBB	All male parents include in their pedigree A-DCBC lines resistant to *A. obtectus*	Red hypocotyl and flower color from the male parent	29
A-DCBC$_{16E}$ G01C	G40001	A-DCBC$_{16B}$ CWB	G40001 *is a facilitator genotype for common x tepary bean crosses*	Red hypocotyl and flower color from the male parent	1
A-DCBC$_{16F}$ G01CC	A-DCBC$_{16E}$ G01C	A-DCBC$_{16B}$ +CWB+32	CWB+32 carries a GUS transgene	Red hypocotyl and flower color from the male parent	1
A-DCBC$_{17}$ CWBR, GBCR	A-DCBC$_{16B}$ CWB, GBC	V-DCBC$_{12C,-14A}$ RGRRR, RGRTCR		Red hypocotyl and red or scarlet flower color from the male parent, long petiole of the primary leaves	2
A-DCBC$_{18A}$ CWBRG, GBCRG, GBCRB	A-DCBC$_{17}$ CWBR, GBCR	A-DCBC$_{16B,-16D}$ CWB, GBC, BBC	All male parents include in their pedigree A-DCBC lines resistant to *A. obtectus*	Red hypocotyl and flower color from the male parent	8
A-DCBC$_{18B}$ CWBRGG	A-DCBC$_{18A}$ CWBRG	A-DCBC$_{16D}$ GBC	CWBRG carries the scarlet flower color of *P. coccineus*	Female parent self-sterile	2
A-DCBC$_{19}$ G01CCD	A-DCBC$_{16F}$ G01CC	V-DCBC$_{14B}$ DV		Long petiole of the primary leaves	2
A-DCBC$_{20A}$ G01CCDC, G01CCDG	A-DCBC$_{19}$ G01CCD	A-DCBC$_{16B,-16D}$ CWB, GBC		Female parent self-sterile	5

Cross abbreviation/ hybrid code	Female parent/ code	Male parent/ code	Characteristics of the parent lines	Verification of hybrids	Nr. of fertile or cross-fertile hybrids
A-DCBC$_{20B}$ YZ	A-DCBC$_{18A,-20A}$ G01CCDC, CWBRGG, GBCRC	A-DCBC$_{-4C,-8G,-12B,-16B}$ GVV, NNIQLAC, GKVGAG, BWG, CWB	Underlined codes are A-DCBC lines resistant to *A. obtectus*	Female parent self-sterile or red hypocotyl and red or scarlet flower color from the male parent	70
A-DCBC$_{20C}$ TBY, TBG	TB-1	A-DCBC$_{18A,-20B}$ YZ, G01CCDC	TB-1 is a tepary bean accession competent to Agro-transformation	Red or scarlet or lila flower color from the male parents	100
A-DCBC$_{21}$ WD	A-DCBC$_{18A}$ GBCRC	V-DCBC$_{16D}$ ETDRD		Red hypocotyl and red flower color from the male parent, long petiole of the primary leaves	2
Total					237

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). The fertile hybrid populations produced may be a source for other desirable traits of tepary bean.

Future plans

* To continue with the DCBC strategy to produce more advanced DCBC hybrid generations
* 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 or other molecular techniques.

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2.1.2 Modification of flowering in cassava by genetic transformation Background

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Important Outputs

Several independent transgenic lines were obtained using the model transformation cassava genotype 60444 and ethanol-inducible or steroid- inducible constructs containing the flowering genes Constans and Apetala

Introduction

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 gene expression system). The AlcR gene-expression system functions in many plants and works in controlled and breeding environments (Sweetman et al.2002).A similar approach is being attempted for cassava to increase flower number and specifically target the timing of flowering to provide cassava breeder greater flexibility in the development of breeding populations. The principal object of this project is to assess the effect of the expression of a set of floral developmental genes that are known to modify flowering time in cassava. The immediate benefits of this work include opening up 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 gateway cloning technique was used to clone several flower meristem identity genes of *Arabidopsis thaliana*, generously donated by the Prof Coupland's lab of the Max Planck Institute of Plant Breeding Research (MPIZ), into two inducible vectors: an ethanol- inducible promoter and an estrogen receptor-inducible promoter. These constructs were introduced into cassava by agrobacterium mediated transfer as described by Schopke et al 1996 but with some modifications made at CIAT that has been used to successfully produce several independent transgenic events.(CIAT 2002).

Two binary vectors containing the ethanol inducible systems and the flowering genes Constans (ABI-pNew CO) or Apetala (ABI-pNew API) and a vector with the steroid inducible gene and

the Constans genes (AGL1-pER8 CO) were used for transformation. Friable Embryogenic Callus (FEC) used for this transformation were obtained from the cassava variety TMS 60444.

Agrobacterial cultures having the constructs ABI-pNew-CO, ABI-pNew-API and AGL1-pER8-CO were grown overnight with shaking (250rpm) in LB medium containing 19.6µl of acetosyringone (100mg/ml) with the selective antibiotics Kanamycin 50mg/ml, Chloramphenicol 30mg/ml and Carbenicillin 100mg/ml for ABI constructs and Carbenicillin 100mg/ml, Spectinomycin-Streptomycin 50mg/ml for AGL1 constructs at 28°C until the Optical Density, O.D 560 was between 0.5-1.0 Acetosyringone is a phenolic inducer of the virulence (vir)genes of the agrobacterium tumefaciens which functions in the processing and transfer of the T-DNA from the Ti plasmid into susceptible plant cells, with subsequent integration into the host genome.

Bacteria were pelleted and resuspended in LB media, more acetosyringone was added and 10µl of the bacterial culture used to inoculate FECs that were then re-distributed in clusters of 5mm width and about 0.082g weight, about 20 clusters per dish of solid media GD2-50Pi + Acetosyringone. The plates were placed in a vacuum apparatus to remove air trapped within the FEC to ensure direct and total contact of the agrobacterial culture to all the FEC units. The cocultivation was carried out in darkness for 48hours at 21°C. Ten plates of TMS 60444 FEC were inoculated per construct. After cocultivation, transformed FEC were collected with a sterile spatula and washed 4 times with GD2-50Pi liquid media supplemented with Cefotaxin (0.5mg/ml), the washing procedures were repeated each day for one week and for an additional week under appropriate selection pressure. Phosphinothricin -ppt (1mg/l) for pNew constructs and Hygromycin 5mg/ml for the pER8 constructs.

Individual cell lines of transformed FECs were allowed to proliferate in the GD2-50Pi solid media for 4weeks after which the medium was changed twice after 2weeks each under the appropriate selection pressure. The transgenic lines were selected on MS2-1µM NAA α-naphthalene acetic acid for more than 5weeks to allow the development of somatic embryos. Somatic embryo development is however asynchronic, because some transgenic lines are still in this medium. The green somatic embryo from the transgenic FEC lines were transferred from the maturation medium to MS2-0.5% Activated Charcoal for cleaning, the procedure took over a month (some transgenic lines are still at this stage). Young matured green transgenic lines were transferred to elongation media MS2-0.2µM Gibberillin for elongation which takes about 3weeks or more. After elongation, they are transferred in 17N for the development of roots for 4weeks and maintained in-vitro after which they will be hardened in the screen house and later used for preliminary molecular and physiological analysis.

Results

Agrobacterium mediated transformation of the 60444 variety using 2 floral genes is at different stages (Fig 1, Fig 2, and Table 1). Several independent transgenic lines have been obtained for the ABI-pNew-CO construct and are presently being elongated (Fig 1) after which they which they will be transferred to 17N rooting media. There are several 70 API lines and 60 CO lines in the proliferation phase in activated charcoal (Fig 2).

Figure 1. These are the independent ethanol inducible transgenic constans (CO) lines.

Conclusions

Significant advances have been made in the flowering genes transformation work but considerable ground remains to be covered. Several independent transgenic lines for the constans gene construct are expected soon and molecular and physiological analysis to confirm the presence expression of the inserted gene will be carried out. Ethanol induction of the inserted gene under screen house conditions on flowering will also be conducted before the eventual field trials.

Figure 2. The independent transgenic cassava lines for both ethanol inducible Constans and Apetala 1 in activated charcoal.

Table 1. Summary of the transformation procedure with the 4 different flowering gene constructs.

Constructs	ABI-pNewCO	ABI-pNewAPI	AGL1-pER8CO	ABI-pER8CO
Start date	23rd May	23rd May	23rd May	15th July
Number of plates	30	30	30	30
First stage of growth	3rd June	3rd June	3rd June	28th July
First isolation of transformed clusters	5th –8th July	5th –8th July	11th July	No transformed cluster
number of plates	34	55	8	-
Selection media	GD2-50Pi +Cefotaxin+1mg/L ppt	GD2-50Pi +Cefotaxin+1mg/Lppt	GD2-50Pi +Cefotaxin+5mg/LHygromycin	-
2nd Isolation	1st –10th August	1st –10th August	1st –10th August	
number of plates	51	89	very low transformation efficiency	
Selection media	GD2-50Pi + 1mg/L ppt	GD2-50Pi + 1mg/L ppt		
Approx. total number of clusters	~ 650	~ 900		
Maturation Media MS2-BAP +1mg/L ppt	29th August 15plates, min.of 9clusters per plate	29th August 16plates, min.of 9clusters per plate	22nd September 3 clusters	
MS2-1uM ANA	30th Aug –6 Sept	30th Aug-12Sept		
number of plates	64plates, min.of 9clusters per plate	99plates, min.of 9clusters per plate		
MS3-0.5%CA Somatic Embryos	6th October 10lines 7th Nov, 60 independent lines	6th October 1 line 7th Nov, 70 independent lines		
MS2-0.2GA3	4 transgenic lines			

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2.1.3 Development of additional waxy cassava transgenic lines

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CIAT
Funding: Ministerio de Agricultura y Desarrollo Rural (MADR) Colombia, CIAT.

Important outputs

Production of 137 sense and 86 anti-sense GBSS transgenic of the variety 60444
2) Considerable improvement of the transformation process of cassava, positive *gus* assay of transformed FECs show a 90% increase in efficiency of transformation.
3) Application for permission to transfer transgenic plants to the field has been requested to the CIAT biosafety committee for biochemical evaluation of the waxy phenotype in the field.

Introduction

With funds from the Colombia Ministry of Agriculture and Rural Development, a project was initiated en 2002 to genetically engineer industrial cassava varieties to produce waxy starch via the sense and anti-sense down-regulation of the *GBSSI* gene. Two transgenic lines were obtained but work to obtain many more transgenic lines continued. We describe here the development of over 200 new independent transgenic lines which are now being tested for gene insertion and then transferred to the screen house for hardening and field establishment for biochemical evaluation of amylose free starch.

Methodology

Development of constructs with the GBSSI gene in the sense and anti-sense orientation has been described earlier (CIAT 2003). FEC were massively produced by culturing nodal cuttings from *in vitro* plantlets in 4E media at a density of about 25 cuttings per Erlenmeyer glass flask. Explants produced were used to produce somatic embryos according to standard procedures (Schopke 1996). Axilliary buds of the explants were finely shredded and placed in MS4 media in a glass jar, 10 pieces per jar, for 3 to 4 weeks. Somatic embryos formed were excised from the rest of the tissue and placed in GD2-50Pi media solid for the induction of friable embryogenic callus (FECs; maximum of 9 clusters per dish). After 30 days, FECs that have developed in the clusters were sub-cultured in fresh GD2-50Pi media solid to increase amount of FECs. FECs obtained above were cultured again in GD2-50Pi media solid for one month.

To transform the FECs, acetosyringone [200μM] was added to each petri-dish and the FECs collected and re-distributed in clusters of 5mm width and about 0.082g weight, about 20 clusters

per dish. Following, 10µl of *agrobacterium* already transformed with the construct GBSSI in orientation sense or anti-sense was added to each cluster and left for 2-3 days at 21°C. The transformed FECs was collected with a sterile spatula and washed with GD2-50Pi liquid media supplemented with Cefotaxima or Claforan [0.5 mg/ml] for one week and an additional week under appropriate selection pressure (Higromicina 5mg/ml). Individual cell-lines of transformed FECs were allowed to proliferate in the GD2-50Pi solid media for 5 weeks after which they were transferred to MS2-1µMANA media for 2 to 3 weeks to allow the development of cotyledenous embryos and to continue with the process of regeneration plants.

Result

A total of 123 independent transgenic lines with the GBSS in sense orientation and 86 lines with the GBSS anti-sense were obtained using the model transformation genotype 60444 using *Agrobacterium tumefaciens* as demonstrated by *gus* assay (Fig 1). This is a 90% increase in efficiency over previous transformation experiments with the GBSS construct. We hope to produce approximately 61 plants transgenic in orientation sense and 43 in orientation anti-sense to be hardened in the screen house and tested in the field. At the same time, an application for permission to transfer transgenic plants to the field has been requested to the CIAT biosafety committee for biochemical evaluation of the waxy phenotype in the field.

Conclusion and perspective

We have successfully transformed full-length sense and anti-sense constructs of the *GBSSI* gene into the model cassava transformation variety 60444 and obtained total of 209 independent transgenic lines at the FEC stage. The transformed callus are being regenerated and plantlets obtained will be transferred to the screen house and later to the field for biochemical evaluation of the waxy phenotype.

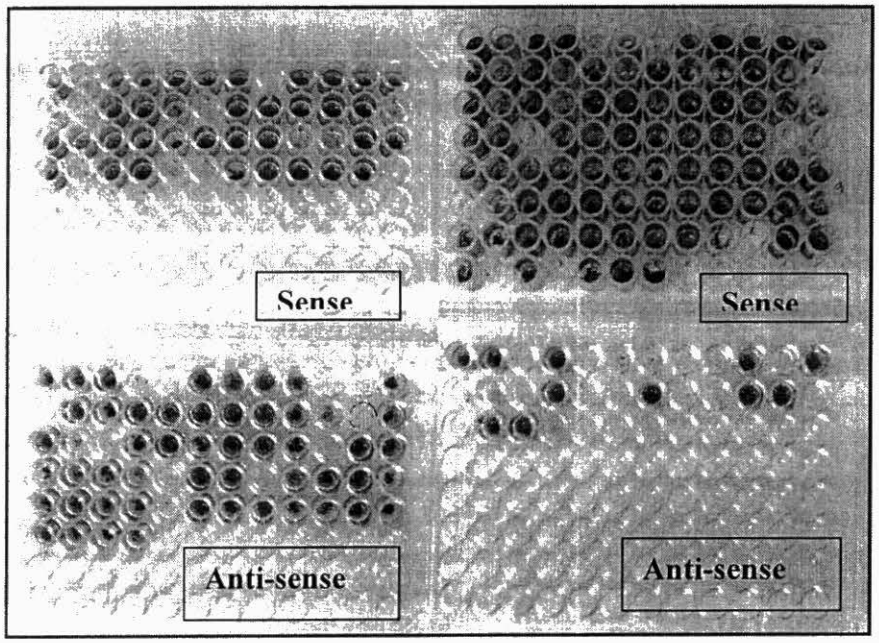

Figure 1. Positive *gus* assays of FEC variety 60444 using two different transformation constructs, each one of these samples are independent lines.

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2.1.4 Foreign genes as novel sources for increased drought tolerance in rice

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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 mechanisms 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. Last year we reported the regeneration of about 400 independent transgenic events generated via *Agrobacterium tumefaciens* transformation (E. Tabares *et al.*, 2004, Foreign genes as novel sources for increased efficiency of water use in rice SB2 Annual Report 2004) carrying either *Arabidopsis* or rice

DREB genes driven by the *Lip9* stress inducible promoter. This year we report the analyses of selected plants including molecular patterns of gene insertions and preliminary experiments to standardize the analysis of *Lip9* and DREB gene expression.

Materials and Methods

Plant material. A total of 432 independent transgenic events from 726 regenerated plants represented by 118 events of the *Lip9::AtDREB1A* and 228 independent events of the *Lip9::OsDREB1B* gene constructs were used (Figure 1). Transgenic plants were generated using three different rice genotypes adapted to Latin American agro ecosystems, the irrigated varieties Cica 8 and Palmar (*indica* type), and the upland genotype CT6241-1-15-1 (tropical *japonica* type). 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 *Lip9* stress inducible promoter and spliced into the plasmid *pBIG*, carrying the hygromycin resistance gene (JIRCAS). *Lip9* corresponds to a rice promoter whose expression is not affected by ABA. The DREB gene constructs were kindly provided by JIRCAS under material transfer agreement. Rice *Lip9::OsDREB1B* sequence was previously confirmed at JIRCAS. *Lip9::AtDREB1A* was confirmed using 7 primers specifically designed for *Lip9* (sequence provided by JIRCAS) and *DREB1A* (Gene bank accession AB007787). T_0 plants were grown in the greenhouse to maturity, evaluated for agronomic traits and T_1 seeds harvested from each plant.

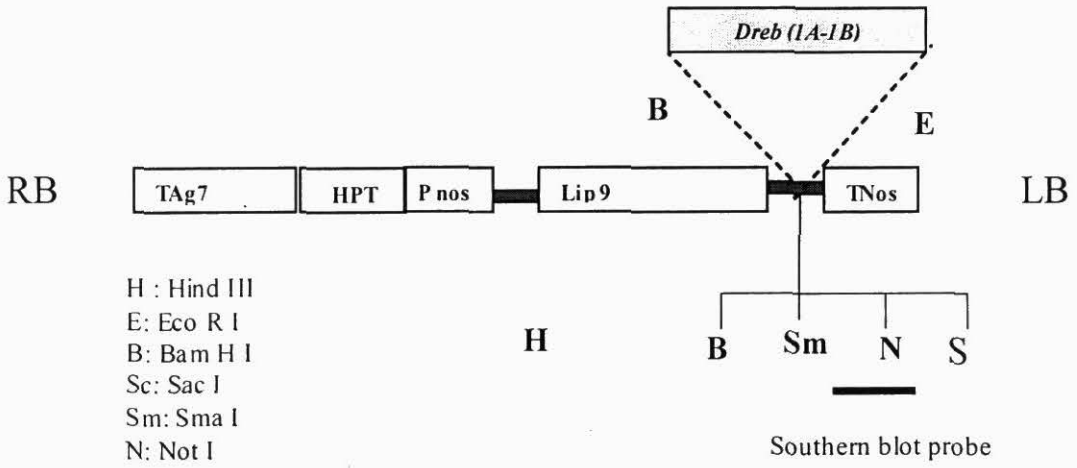

Figure 1. Gene cassette construct map, some restriction sites are showed. *HPT* hygromycin resistance gene, *Pnos* promoter, *Tag7* terminator, Lip9 promoter, *TNos* terminator, *RB* right border, *LB* left border.

Southern blot analysis of transgenic plants. Genomic DNA was extracted from 1g of rice leaves according to a modified Dellaporta (1983) protocol. Genomic DNA of each T_0 plant was digested with *EcoRI* or *EcoRI/HindIII* (Figure 1) to determine the number and patterns of gene insertions. The digested DNA was fractioned in a 1.0% (w/v) agarose gel. Gels were denatured and neutralized by standard procedures, and DNA transferred onto Amersham N^+ Hybond membrane according to the manufacturer's instructions. Membranes were hybridized using a labeled TNos specific probe (Figure 1) at 60°C (Sambrook *et al.*, 1989). Probe DNA was radio labeled following the Megaprime™ DNA Labeling Systems Amersham Biosciences protocol.

Endogenous gene expression analysis of lip9 and OsDREB1B

To examine endogenous expression of *lip9* and *OsDREB1B*, non-transformed Palmar was subjected to drought shock treatment. 3-week old plants were grown in pots, then removed from the soil, rinsed with tap water, and placed on the lab bench for 0, 1, 2, 5 and 10 hours. Total RNA was extracted from leaves using TRIzol reagent (Invitrogen Inc. USA) by following the manufacture's instructions with some modifications. Residual DNA was eliminated using Deoxyribonuclease I (Invitrogen Inc. USA). Total RNA was reverse transcribed to produce first strand cDNAs using the Superscript III reverse transcriptase (Invitrogen Inc. USA). Real Time PCR analysis was performed in Opticon II (Bio-Rad Inc. USA) using the Dynamo SYBR qPCR kit (Finzymes Inc. USA) with the following PCR conditions: 94°C for 2 min, 45 cycles at 94°C for 30 s, 58°C for 30 s, 74°C for 1 min. The 18s rRNA gene was used for normalization. Relative quantification analysis was conducted as described in Livak et al (2001).

Results and Discussion

Production of DREB transgenic rice plants

A total of 726 rice transgenic plants were regenerated via *Agrobacterium tumefaciens* from which 432 were independent events. These events were evaluated to maturity in the greenhouse. In general, independently of the DREB gene and genotype, transgenic plants were shorter, produced fewer tillers and panicles, and showed delayed flowering and higher sterility respect to the non-transgenic controls (mean values, Table 1). Interestingly, the most affected genotype was the upland line CT6241-1-15-1 drought tolerant, and the least the drought susceptible irrigated variety Palmar. Spite off this problem, some DREB plants performing similarly to the corresponding control plants were identified (see upper range values, Table 1) and selected for future analyses. It is important to point out that in contrast to DREB plants, transgenic plants with high fertility and normal agronomic traits similar to the non-transgenic controls had been routinely generated in our laboratory using a similar protocol and the same genotypes as herein but carrying other transgenes encoding for fungal resistance (PAPy123, Tabares *et al*, 2002 and Fory *et al.*, 2003, Foreign genes as novel sources of resistance for fungal resistance, SB2 Annual Report 2002 and 2003) or virus resistance (Lentini et al., 2003). These results suggest a potential detrimental physiological effect on plant development due to the DREB transgenes, effect that requires a more detailed analysis in the future. Based on the agronomic traits of the DREB plants, a total of 150 T_0 independent events were selected to include plants showing contrasting agronomic performance per genotype and gene construct, but ensuring having enough T1 seeds for future analyses.

Table 1. Agronomic performance in the greenhouse of T_0 transgenic DREB rice plants of the irrigated varieties Cica 8 and Palmar (*indica* type), and the upland genotype CT6241-1-15-1 (tropical *japonica* type).

Genotype	Transgene construct	Events	Height (cm)	Tillers	Panicles	Days to 50% Flowering	Sterility (%)
CT6241-1-15-1 upland	*AtDREB1A*	35	72 (30-95)	17 (2-40)	17 (2-40)	84 (69-113)	36 (5-99)
	OsDREB1B	31	61 (25-90)	17 (3-42)	17 (3-42)	90 (69-159)	45 (10-99)
	Events (A+B)	66					
	None (control)	7	89 (85-100)	23 (13-40)	25 (15-42)	81 (73-89)	38 (5-99)
Cica 8 irrigated	*AtDREB1A*	54	67 (35-90)	20 (6-49)	20 (7-49)	99 (75-117)	42 (5-97)
	OsDREB1B	23	81 (60-100)	19 (7-49)	18 (7-49)	94 (70-136)	35 (5-95)
	Events (A+B)	77					
	None (control)	8	90 (85-90)	24 (10-38)	26 (17-38)	101 (89-108)	25 (2-40)
Palmar irrigated	*AtDREB1A*	128	75 (55-100)	20 (2-48)	20 (2-48)	100 (80-140)	35 (3-100)
	OsDREB1B	161	65 (35-90)	16 (1-45)	16 (1-43)	103 (77-189)	33 (3-98)
	Events (A+B)	289					
	None (control)	8	87 (75-100)	17 (12-30)	17 (12-30)	98 (89-108)	20 (10-30)
	All events	432					

Mean values are followed by minimum and maximum values in brackets.

A total of 150 independent events were selected from the 432 generated based on their agronomic performance in the greenhouse (Table 1). Of the 150 independent events selected, herein we showed the Southern blot analyses of a first set of 43 transgenic events analyzed so far. The analyses of the rest 107 events are in progress. Southern blot analyses were carried out to determine the integration patterns of the DREB transgenes. The interpretation of the results is not simply due to the presence of the *Lip9* promoter and the *OsDREB1B* gene in the rice genome since these sequences were isolated from this crop (Dubouzet et al., 2003). For this reason a NOS terminator probe was used. This region is common in both constructs *Lip9::AtDREB1A* and *Lip9::OsDREB1B* (Figure 1). DNA digestion with *EcoRI* cuts the transgene constructs once outside the DREB coding regions (used herein to determine the number of transgene insertions in the rice genome), and double digestion with *HindIII/ EcoRI* excised the complete *Lip9::AtDREB1A* and *Lip9::OsDREB1B* cassettes (used herein to determine the presence of DREB gene rearrangements) (Figure 1).

Gene copy analysis of the DREB transgenic lines

Southern blot analyses indicated that of the 43 transgenic T_0 events analyzed so far, 28 of them (65%) showed simple transgene insertions (1-2 insertions) and no rearrangements (Table 2), which make these plants ideal candidates for future phenotypic and molecular characterization of

the transgenic trait. It is important to note that a significant higher number of plants (about twice as many) showed simple insertions (1-2 insertions) when using the *Lip9::OsDREB1B* constructs (90% of the plants) than when using the *Lip9::AtDREB1A* construct (41%). However, in most cases few rearrangements (about 12% in average) were noted spite of the number of transgene insertions (Table 2, Figure 2). Likewise, the most complex hybridizations patterns were observed in some plants (all genotypes) when transformed with the *Lip9::AtDREB1A* construct (Figure 2). Three to four apparent hybridization bands were noted in 32 % of plants, and five to seven bands were observed in 27 % of plants. These results indicate that there might be some construct defects or sequence related features that make the *Lip9::AtDREB1A* more prone to complex insertions in the rice genome Similar results had been reported by other groups when the gene encoding C-repeat/dehydration-responsive element binding factor 1 (CBF1/DREB1b) of *Arabidopsis* was introduced into rice. Southern blot analyses showed the integration of one to several copies of the transgene into the genome (Sang-Choon et al., 2004). However is important to note that in our case plants showing simple hybridization patterns of *Lip9::AtDREB1A* were also identified and selected for future analyses (Table 2). Having into account that from 43 T_0 plants analyzed so far 65 % showed a low number of transgene insertions (1 or 2) and without rearrangements, there is a high probability of finding enough number of independent events from the different genotype/construct combinations to elucidate the molecular mechanisms and phenotype underlining by these DREB transgenes.

Table 2. Comparative Southern blot analyses of 43 events of the total 150 events selected on their agronomic performance.

Genotype	Gene	N	1-2 Insertions	>3-4 Insertions	>5 insertions	Events without rearrangements (%)
Cica 8	*AtDREB1A*	**8**	**4**	**2**	**2**	**88**
CT6241		**6**	**2**	**2**	**2**	**67**
Palmar		**8**	**3**	**3**	**2**	**100**
Total events		**22**	**9 (41 %)**	**7 (32 %)**	**6 (27%)**	**85**
Cica-8	***OsDREB1B***	**7**	**6**	**1**	**0**	**86**
CT6241		**6**	**6**	**0**	**0**	**100**
Palmar		**8**	**7**	**0**	**1**	**88**
Total events		**21**	**19 (90 %)**	**1 (5%)**	**1 (5%)**	**91**
	Total (A+B)	**43**	**28 (65 %)**	**8 (19 %)**	**7 (16 %)**	**88**

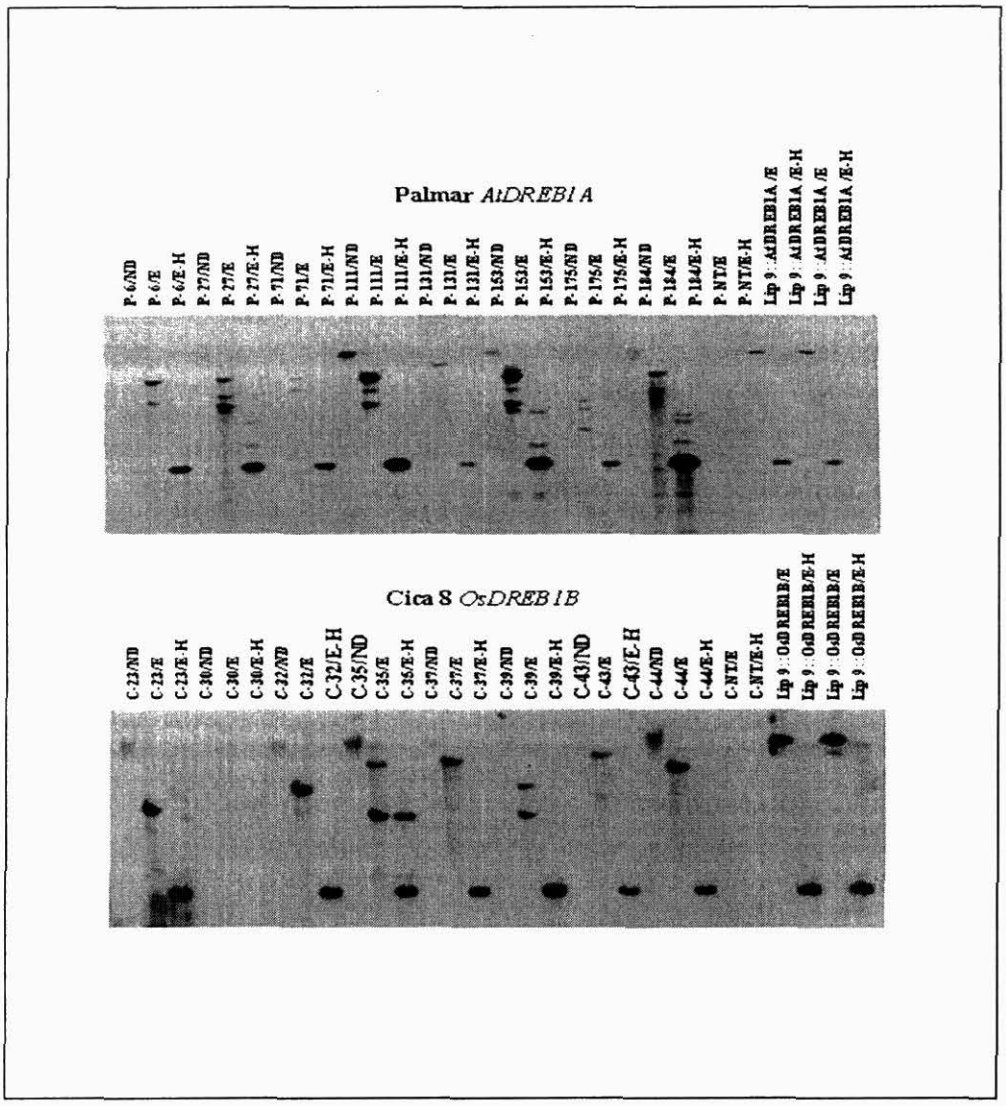

Figure 2. Southern blot analyses of some of the selected Palmar *Lip9::AtDREB1A* (upper) and Cica 8 *Lip9::OsDREB1B* (lower) transgenic events. Genomic DNA was not digested (ND, control) or digested with *EcoRI*, (E) EcoRI/ HindIII (E-H) to determine the number of insertions and rearrangements, respectively. Non-transgenic varieties of Palmar (PNT) and Cica 8 (CNT) were used as negative controls, and the constructs as positive controls.

Endogenous gene expression analysis of lip9 and OsDREB1B

This expression analysis aims to determine drought stress conditions for selection of transgenic rice plants based on transgene expression. In the Palmar genotype, *lip9* gene expression was induced by drought shock treatment and the expression reached a plateau at 5 hours with 18 times higher expression than the basal expression at 0 hour (Figure 3). This indicates that the drought stress triggers the expression of the endogenous gene. This result is consistent with previous micro array analysis, which showed that the gene is expressed in response to water depletion, such as drought and high salinity (Ashiq Rabbani et al. 2003).

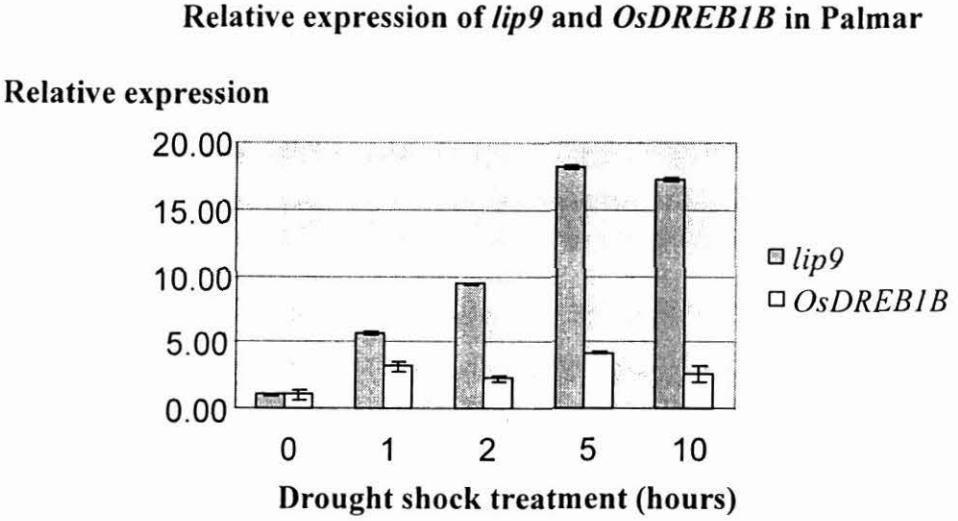

Figure 3. Relative expression of *lip9* and *OsDREB1B* gene in the Palmar. The relative expression level was calculated based on expression level of each gene at 0 hour.

On the other hand, the *OsDREB1B* gene was consistently expressed but the expression was not significantly induced by drought shock treatment. This is consistent with evidence that *OsDREB1B* is cold-responsive, but not drought-responsive (Dobouzet et al. 2003). The CICA8 genotype showed a similar expression pattern for both genes (data not shown) and CT6241 has not been tested. Based on these results, 5 hours of drought shock treatment is enough to induce the lip9 promoter and this condition can be used to select transgenic lines based on the transgene expression.

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2.1.5 Evaluation of resistance RHBV and Selection in the Field in 2005 of Advanced Breeding Generation from Crosses with Transgenic Rice Resistant to RHBV

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Introduction

The Rice Biotechnology Project had generated transgenic rice lines with resistance to RHBV (Lentini *et al.*, 2003) an endemic disease and of the main constrains of rice of Tropical America. Resistant transgenic lines carrying different source of resistance than the one already deployed in commercial varieties would allow increased production to a lower cost by reducing the need of insecticide applications commonly used to control Sogata (the virus vector). This program targeted to transfer the virus resistance by splicing in genes from the RHBV virus into Cica 8 variety, selected by the breeders as potential parent donor of the transgene for future conversion of modern varieties because of its good grain quality, high productivity, and broad adaptation including low soil humidity, and acceptance to large and small resource farmers. However, this variety is not currently grown commercially in spite of its good agronomic qualities because of its high susceptibility to RHBV (Vargas, 1985). The transgenic resistance is RNA-mediated and some plants show hypersensitive reaction when challenged with the RHBV virus (Lentini et al., 2003). Some of the transgenic lines outperform most currently grown commercial rice varieties. Attempts to transfer this resistance into other varieties through regular crossing indicated that the RHBV-N transgene is inherited and expressed independently of the genotype background. Thus this transgenic resistance could be used to complement the breeding resistance that has been deployed so far and does not protect plants when younger than 5 to 20 day-old. Last year we reported the selection based on agronomic performance in the field of 24 lines (F_5-F_6) derived from crosses between selected transgenic lines and commercial rice varieties Oryzica 1, Fedearroz 50 and Iniap 12, and 51 individual transgenic lines (T_5-T_9) selected through pedigree method. This year we report the evaluation of these advanced lines for agronomic traits, RHBV resistance, in addition to other key traits such as grain quality and piricularia resistance.

Materials and Methods

Evaluation of RHBV resistance in the field in 2005. The evaluations were conducted using progeny plants derived from self cross of selected transgenic lines or of crosses between selected transgenic events and Fedearroz 50 or Oryzica 1. The selection of the original lines was based on its agronomic performance in the field in 2004. A row with 40 plants per line with 4 replicates was used for the RHBV evaluation. Eighteen days after planting, plants were infested with two dosages of 0.8 and 1.5 insects per plant using viruliferous insects from the colony "Tolima-CIAT". Five days after the infestation, the insects were killed, and the plants were evaluated for disease symptoms development at 30 and 45 after infestation.

Agronomic evaluation and selection of advanced generations of transgenic events and derived progeny plant from crosses. The same lines evaluated for RHBV resistances were also evaluated for agronomic performance in the field. Plants were transplanted in the field using three rows per lines. Agronomic traits were evaluated throughout the life cycle up to maturity. Agronomic traits were evaluated according the scale IRRI (1996).

Grain quality. Plants selected based on RHBV resistance were characterized by grain size and quality as follows: Grain size (Short, <5.5mm; Medium, 5.6 – 6.5 mm; Long, 6.6 – 7.5 mm; Extra long >7.5mm), absence of white areas and amylose content (Low, <23%; Medium, 24 – 27% and High, >24%).

Results and Discussion

Three dimension analyses were conducted using data from the RHBV resistance evaluations with two dosages of insects per plant and 4 replicated. Cluster analysis using principal coordinates for advanced crosses or transgenic lines are shown in Figure 1. Clusters 1, 6, 7 and 8 from crosses of progeny lines (Figure 1A) and clusters 7, 9, 10 and 12 from transgenic lines (Figure 1B) include the most RHBV resistant plants (score ≤ 5). Plants in cluster 7 of both groups (Figure 1A and 1B) includes the lines with highest RHBV resistance and similar to Fedearroz 2000 (score ≤ 3). The non-transgenic crosses and varieties (controls) were cluster in other groups (Cica 8/Fedearroz 50, score ≥ 7; Cica 8/Oryzica 1, score ≥ 5; Cica 8,, score ≥ 7; Fedearroz 50, score ≥ 6.3; and Oryzica 1, score ≥ 6.5). Based of this evaluation, sixteen F_6 advanced lines derived from crosses between resistant transgenic Cica 8 plants and the commercial varieties Oryzica 1 (63%) and Fedearroz 50 (37%), and twelve pedigree T_8-T_{11} advanced transgenic lines were selected. The crosses with Oryzica 1 showed the highest vigor. No significant differences in other agronomic traits and grain quality were found between the transgenic materials and the non-transgenic crosses and commercials varieties Fedearroz 50, Cica 8 and Oryzica 1 (Tables 1 and 2). Fertility ranges from 81 at 90% likewise the commercial varieties. Grain However, some lines with high agronomic performance were discarded based on the poor grain quality in particular due to its high chalkiness content. The lines shown in Tables 1 and 2 are those with good grain appearance, determined by its endosperm opacity and low amount of chalkiness. The grains of these materials showed medium to long grain size (5.51 to 7.5mm), with a low (CBB) or without (CBE) white center and high amylase contains (AMA, score ≥28%). Several of the lines with highest RHBV resistance level (cluster 7) also showed promissory agronomic characteristics such as high vigor, plant type, plant height, tillering capacity and grain quality similar or outperforming the variety Fedearroz 2000 which is the current commercial variety with highest level of RHBV resistance. In addition line A3-49-60-12-3-20-M-8-M (Table 2) showed additional special characteristics that makes it very promising such as type plant, length of panicle and grains number by panicle among others (data not shown). Analysis of these selected lines for piricularia resistance is in progress.

Future Activity

Subsequent generations obtained from self-cross of the selected advanced breeding and transgenic lines will be evaluated in the field for resistance RHBV, agronomic performance and grain quality to select those lines to be advanced for replicated field trials, and resistance for other traits including sheath blight resistance. These evaluations will determine which of the line(s) have the potential to be deployed as potential commercial transgenic variety once all the biosafety requirements are met.

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Table 1. **Agronomic performance in the field in 2005 of F4 plants (total 16) selected from crosses derived between selected Cica 8-RHBV transgenic resistant events and Fedearroz 50 or Oryzica 1**

Pedigree	Cluster	[2]Vig	[3]DF	[4]DF 50%	Plant Height (cm)	[5]Tiller	F (%)	[6]Grain Quality	[7]Rice blast
A3-49-101-18-19/Oryzica 1-14-M-7-3	1	3	93	98	110	3	85	GM, FWC, AMA	CT
A3-49-60-4-13/ Oryzica 1-13III-6-5-M	7	1	102	107	105	3	90	GM, FWC, AMA	CT,5
A3-49-60-4-13/ Oryzica 1-15A-12-9-M	7	1	93	98	105	63	85	GM, **CBE**, AMA	CT,4,6
A3-49-60-4-13/ Oryzica 1-15A-3-9-M	6	1	87	90	115	3	85	GL, **CBB**, AMA	CT,4,6
A3-49-60-4-13/ Oryzica 1-15A-6-1-M	7	1	91	97	110	3	82	GM, CBE, AMA	CT,6,4,A7
A3-49-60-4-5/ Oryzica 1-15-14-2-M	8	3	93	99	110	3	82	GL, **CBE**, AMA	CT,6,5,4
A3-49-60-4-5/ Oryzica 1-15-14-7-M	1	3	95	101	110	33	80	GEL, **CBB**, AMA	CT,6,5,4
A3-49-60-4-5/ Oryzica 1-232-11-5-M	7	3	97	101	110	3	86	GL, **CBB**, AMA	CT,6,5
A3-49-60-4-5/ Oryzica 1-232-13-7-M	7	3	95	103	100	3	82	GL, **CBE**, AMA	CT,6,5
A3-49-60-4-5/ Oryzica 1-232-13-8-M	7	3	92	103	105	3	84	GEL, **CBE**, AMA	CT,6,5
A3-49-60-4-5/Fedearroz 50-12A-12-11-M	7	3	102	107	98	3	90	GM, **CBE**, AMA	6
A3-49-60-4-5/Fedearroz 50-12A-12-7-M	7	3	103	108	102	3	85	GM, CBE, AMA	6
A3-49-60-4-5/Fedearroz 50-12A-1-6-M	8	3	104	108	105	3	85	GM, **CBB**, AMA	6
A3-49-60-4-5/Fedearroz 50-12A-1-8-M	7	3	103	107	95	3	80	GM, CBE, AMA	6
A3-49-60-4-5/Fedearroz 50-19-11-4-M	7	3	102	108	105	3	84	GM, FWC, AMA	5
A3-49-60-4-5/Fedearroz 50-68-M-5-2	7	3	98	110	115	3	83	GM, FWC, AMA	CT,6,2,1
Cica 8/ Fedearroz 50	6	3	93	102	120	3	82	GM, FWC, AMA	CT,6,5
Cica 8 / Oryzica 1	4	3	96	110	115	3	85	GM, WWC, AMA	CT,6,5,4,1
Oryzica 1	4	3	95	99	105	3	82	GM, WWC, AMA	CT,6,4,2
Cica 8	5	3	98	103	105	3	85	GM, FWC, AMA	CT,5
Fedearroz 2000	7	3	108	116	110	3	87	GL, WWC, AMA	CT,4
Fedearroz 50	1	3	107	113	115	3	84	GM, WWC, AMA	-

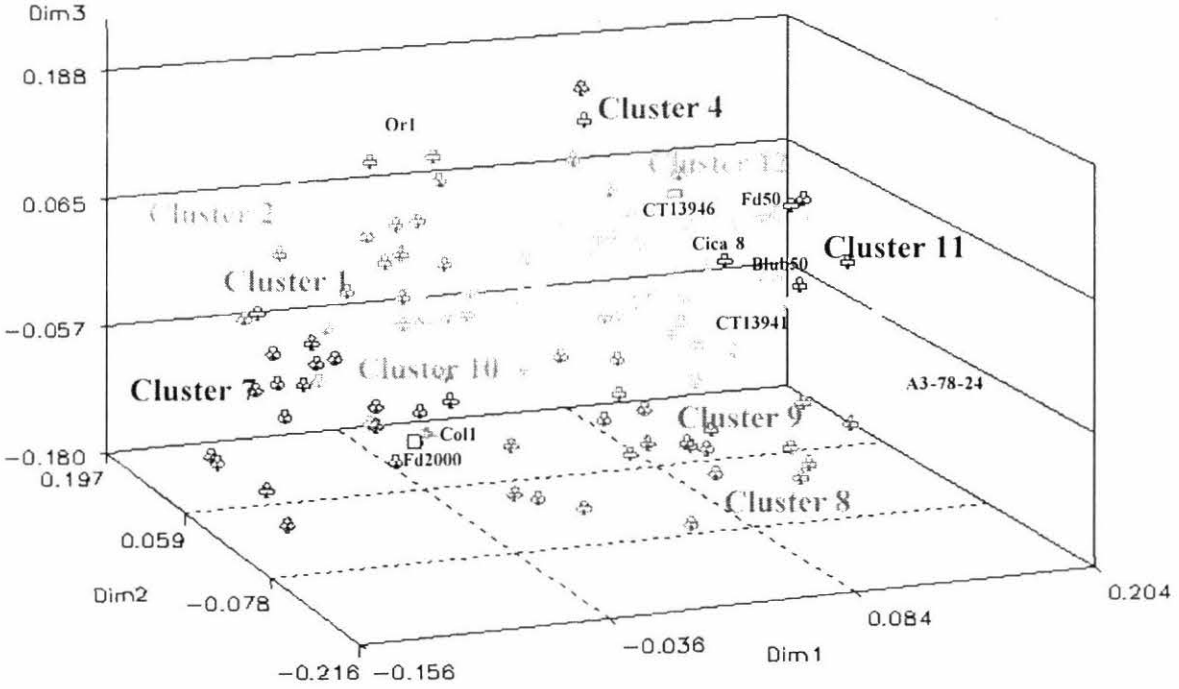
[1] Number of individual plants selected. [2] Plant vigor (1) high vigor and (5) lowest vigor. [3]DF days to flowering initiation. [4] Days to 50% anthesis. F = mean fertility % per line. [5]Tillering ability (1) Extra vigorous and (9) Very weak. [6]Grain quality: GM (L) = grain medium (long); FWC = few white center, WWC = without white center; AMA = high amylase. [7]Susceptibility to specific rice blast lineage

Table 2. **Agronomic performance in the field in 2005 of Cica 8-RHBV transgenic resistant selected (total of 12) progeny plants derived from self cross**

Pedigree	Cluster	[2]Vig	[3]DF	[4]DF 50%	Plant Height (cm)	[5]Tiller	F (%)	[6]Grain Quality	[7]Rice blast
A3-49-60-13-69-M-1-4	ND	3	100	105	115	3	86	GM, WWC, AMA	CT
A3-49-56-15-24-M-M-2-2	7	3	94	99	115	3	80	GM, FWC, AMA	CT,9,6,5,2,1
A3-49-60-12-3-3-20-M-M-1-2	10	3	98	103	110	3	97	GL, FWC, AMA	CT,9,5
A3-49-60-12-3-3-59-M-M-3-5	7	3	98	104	100	3	91	GL, FWC, AMA	CT,9
A3-49-60-12-3-20-M-13-2	7	3	95	100	110	1	90	GM, FWC, AMA	CT,6
A3-49-60-12-3-20-M-13-3	10	3	96	100	100	1	92	GM, FWC, AMA	CT,6
A3-49-60-12-3-20-M-14-3	12	1	96	101	105	3	88	GM, WWC, AMI	CT,6
A3-49-60-12-3-20-M-14-7	10	1	98	104	115	3	87	GM, WWC, AMI	CT,6
A3-49-60-12-3-20-M-8-1	10	3	97	103	110	3	89	GM, WWC, AMA	CT,6,5
A3-49-60-12-3-20-M-8-4	7	3	97	104	110	3	87	GM, FWC, AMA	CT,6,5
A3-49-60-12-3-20-M-8-6	10	3	98	103	105	3	94	GM, WWC, AMA	CT,6,5
A3-49-60-12-3-20-M-M-M2-3	9	3	99	104	105	3	90	GM, FWC, AMA	CT,6
A3-78-24	6	3	105	111	115	3	70.2	GM, CBM, AMA	CT,6,5
Oryzica 1		3	95	100	105	3	87.3	GM, WWC, AMA	CT,6,4,2
Cica 8		3	98	103	105	3	91.2	GM, FWC, AMA	CT,5
Fedearroz 2000		3	110	118	110	3	85.5	GL, WWC, AMA	CT,4
Fedearroz 50		3	106	110	115	3	95.1	GM, WWC, AMA	-

[1] Number of individual plants selected. [2] Plant vigor (1) high vigor and (5) lowest vigor. [3]DF days to flowering initiation. [4] Days to 50% anthesis. F = mean fertility % per line. [5]Tillering ability (1) Extra vigorous and (9) Very weak. [6]Grain quality: GM (L) = grain medium (long); FWC = few white center, WWC = without white center; AMA = high amylase. [7]Susceptibility to specific rice blast lineage

(A)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Dim1
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0.204
Cluster 4
Ori
Cluster 2
CT13946
Fd50
Cica 8
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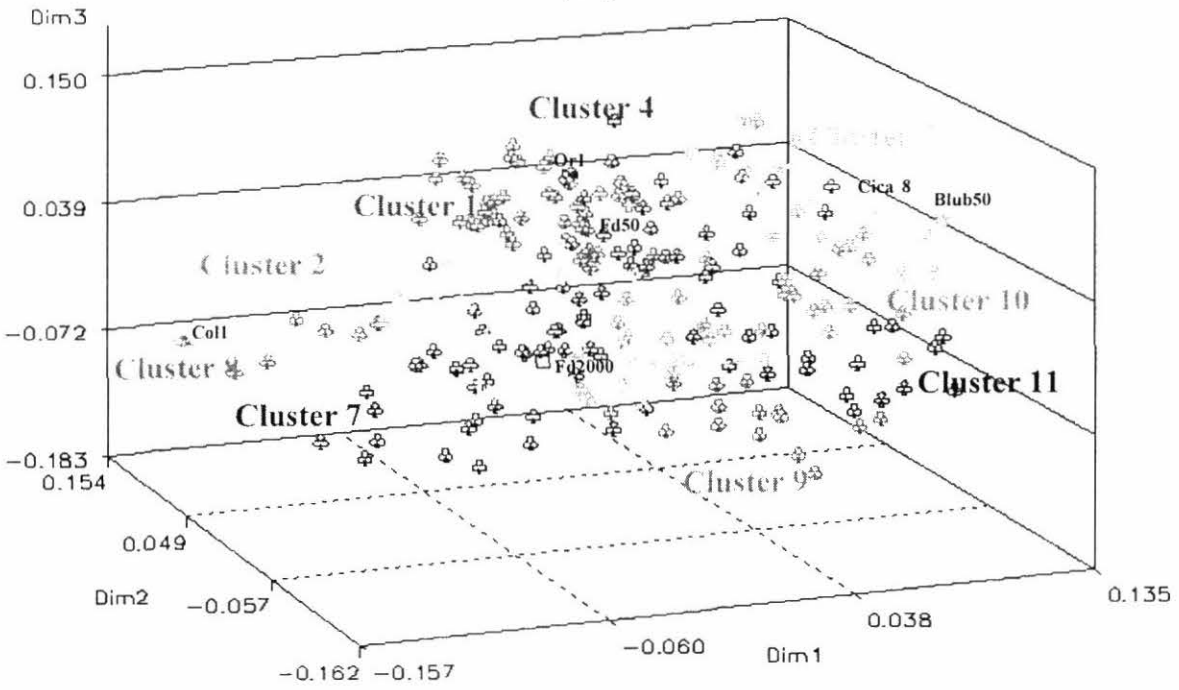
Cluster 8

(B)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Dim1
0.038
0.135
Cluster 4
Ori
Cica 8
Blub50
Cluster 1
Fd50
Cluster 2
Cluster 10
Col1
Cluster 8
Fd2000
Cluster 11
Cluster 7
Cluster 9

2.1.6 Foreign genes as novel sources for increased drought tolerance in rice

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[1]SB2 and [2]IP4 CIAT projects; [3]JIRCAS, Japan.

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 mechanisms 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. Last year we reported the regeneration of about 400 independent transgenic events generated via *Agrobacterium tumefaciens* transformation (E. Tabares *et al.*, 2004, Foreign genes as novel sources for increased efficiency of water use in rice SB2 Annual Report 2004) carrying either *Arabidopsis* or rice DREB genes driven by the *Lip9* stress inducible promoter. This year we report the analyses of selected plants including molecular patterns of gene insertions and preliminary experiments to standardize the analysis of *Lip9* and DREB gene expression.

Materials and Methods

Plant material. A total of 432 independent transgenic events from 726 regenerated plants represented by 118 events of the *Lip9::AtDREB1A* and 228 independent events of the *Lip9::OsDREB1B* gene constructs were used (Figure 1). Transgenic plants were generated using three different rice genotypes adapted to Latin American agro ecosystems, the irrigated varieties Cica 8 and Palmar (*indica* type), and the upland genotype CT6241-1-15-1 (tropical *japonica* type). 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 *Lip9* stress inducible promoter and spliced into the plasmid *pBIG*, carrying the hygromycin resistance gene (JIRCAS). *Lip9* corresponds to a rice promoter whose expression is not affected by ABA. The DREB gene constructs were kindly provided by JIRCAS under material transfer agreement. Rice *Lip9::OsDREB1B* sequence was previously confirmed at JIRCAS. *Lip9::AtDREB1A* was confirmed using 7 primers specifically designed for *Lip9* (sequence provided by JIRCAS) and *DREB1A* (Gene bank accession AB007787). T_0 plants were grown in the greenhouse to maturity, evaluated for agronomic traits and T_1 seeds harvested from each plant.

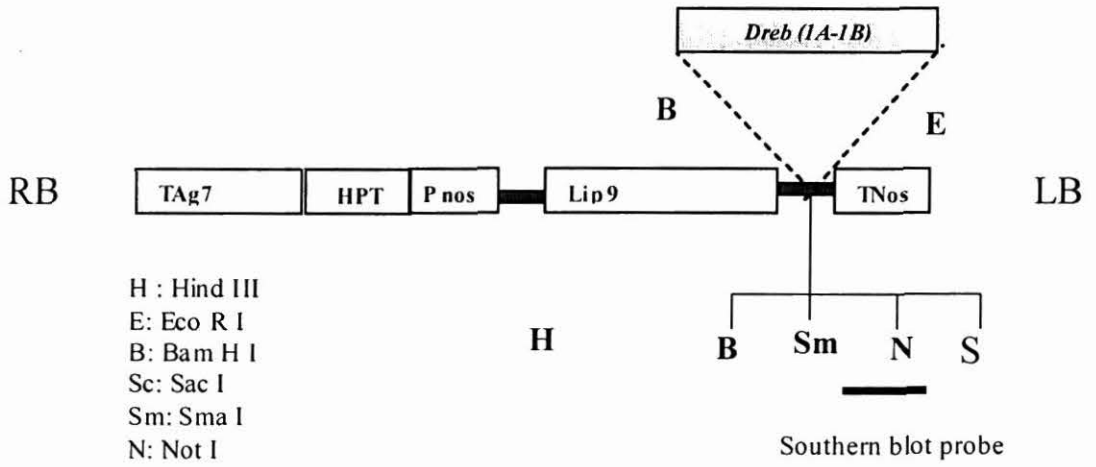

Figure 1. Gene cassette construct map, some restriction sites are showed. HPT hygromycin resistance gene, Pnos promoter, Tag7 terminator, Lip9 promoter, TNos terminator, RB right border, LB left border.

Southern blot analysis of transgenic plants. Genomic DNA was extracted from 1g of rice leaves according to a modified Dellaporta (1983) protocol. Genomic DNA of each T_0 plant was digested with *EcoRI* or *EcoRI/HindIII* (Figure 1) to determine the number and patterns of gene insertions. The digested DNA was fractioned in a 1.0% (w/v) agarose gel. Gels were denatured and neutralized by standard procedures, and DNA transferred onto Amersham N^+ Hybond membrane according to the manufacturer's instructions. Membranes were hybridized using a labeled TNos specific probe (Figure 1) at 60°C (Sambrook *et al.*, 1989). Probe DNA was radio labeled following the Megaprime™ DNA Labeling Systems Amersham Biosciences protocol.

Endogenous gene expression analysis of lip9 and OsDREB1B

To examine endogenous expression of *lip9* and *OsDREB1B*, non-transformed Palmar was subjected to drought shock treatment. 3-week old plants were grown in pots, then removed from the soil, rinsed with tap water, and placed on the lab bench for 0, 1, 2, 5 and 10 hours. Total RNA was extracted from leaves using TRIzol reagent (Invitrogen Inc. USA) by following the manufacture's instructions with some modifications. Residual DNA was eliminated using Deoxyribonuclease I (Invitrogen Inc. USA). Total RNA was reverse transcribed to produce first strand cDNAs using the Superscript III reverse transcriptase (Invitrogen Inc. USA). Real Time PCR analysis was performed in Opticon II (Bio-Rad Inc. USA) using the Dynamo SYBR qPCR kit (Finzymes Inc. USA) with the following PCR conditions: 94°C for 2 min, 45 cycles at 94°C for 30 s, 58°C for 30 s, 74°C for 1 min. The 18s rRNA gene was used for normalization. Relative quantification analysis was conducted as described in Livak et al (2001).

Results and Discussion

Production of DREB transgenic rice plants

A total of 726 rice transgenic plants were regenerated via *Agrobacterium tumefaciens* from which 432 were independent events. These events were evaluated to maturity in the greenhouse. In general, independently of the DREB gene and genotype, transgenic plants were shorter, produced fewer tillers and panicles, and showed delayed flowering and higher sterility respect to the non-transgenic controls (mean values, Table 1). Interestingly, the most affected genotype was the upland line CT6241-1-15-1 drought tolerant, and the least the drought susceptible irrigated variety Palmar. Spite off this problem, some DREB plants performing similarly to the corresponding control plants were identified (see upper range values, Table 1) and selected for future analyses. It is important to point out that in contrast to DREB plants, transgenic plants with high fertility and normal agronomic traits similar to the non-transgenic controls had been routinely generated in our laboratory using a similar protocol and the same genotypes as herein but carrying other transgenes encoding for fungal resistance (PAPy123, Tabares *et al*, 2002 and Fory *et al.*, 2003, Foreign genes as novel sources of resistance for fungal resistance, SB2 Annual Report 2002 and 2003) or virus resistance (Lentini et al., 2003). These results suggest a potential detrimental physiological effect on plant development due to the DREB transgenes, effect that requires a more detailed analysis in the future. Based on the agronomic traits of the DREB plants, a total of 150 T_0 independent events were selected to include plants showing contrasting agronomic performance per genotype and gene construct, but ensuring having enough T1 seeds for future analyses.

Table 1. Agronomic performance in the greenhouse of T_0 transgenic DREB rice plants of the irrigated varieties Cica 8 and Palmar (*indica* type), and the upland genotype CT6241-1-15-1 (tropical *japonica* type).

Genotype	Transgene construct	Events	Height (cm)	Tillers	Panicles	Days to 50% Flowering	Sterility (%)
CT6241-1-15-1 upland	*AtDREB1A*	35	72 (30-95)	17 (2-40)	17 (2-40)	84 (69-113)	36 (5-99)
	OsDREB1B	31	61 (25-90)	17 (3-42)	17 (3-42)	90 (69-159)	45 (10-99)
	Events (A+B)	66					
	None (control)	7	89 (85-100)	23 (13-40)	25 (15-42)	81 (73-89)	38 (5-99)
Cica 8 irrigated	*AtDREB1A*	54	67 (35-90)	20 (6-49)	20 (7-49)	99 (75-117)	42 (5-97)
	OsDREB1B	23	81 (60-100)	19 (7-49)	18 (7-49)	94 (70-136)	35 (5-95)
	Events (A+B)	77					
	None (control)	8	90 (85-90)	24 (10-38)	26 (17-38)	101 (89-108)	25 (2-40)
Palmar irrigated	*AtDREB1A*	128	75 (55-100)	20 (2-48)	20 (2-48)	100 (80-140)	35 (3-100)
	OsDREB1B	161	65 (35-90)	16 (1-45)	16 (1-43)	103 (77-189)	33 (3-98)
	Events (A+B)	289					
	None (control)	8	87 (75-100)	17 (12-30)	17 (12-30)	98 (89-108)	20 (10-30)
	All events	432					

Mean values are followed by minimum and maximum values in brackets.

A total of 150 independent events were selected from the 432 generated based on their agronomic performance in the greenhouse (Table 1). Of the 150 independent events selected, herein we showed the Southern blot analyses of a first set of 43 transgenic events analyzed so far. The analyses of the rest 107 events are in progress. Southern blot analyses were carried out to determine the integration patterns of the DREB transgenes. The interpretation of the results is not simply due to the presence of the *Lip9* promoter and the *OsDREB1B* gene in the rice genome since these sequences were isolated from this crop (Dubouzet et al., 2003). For this reason a NOS terminator probe was used. This region is common in both constructs *Lip9::AtDREB1A* and *Lip9::OsDREB1B* (Figure 1). DNA digestion with *EcoRI* cuts the transgene constructs once outside the DREB coding regions (used herein to determine the number of transgene insertions in the rice genome), and double digestion with *HindIII/ EcoRI* excised the complete *Lip9::AtDREB1A* and *Lip9::OsDREB1B* cassettes (used herein to determine the presence of DREB gene rearrangements) (Figure 1).

Gene copy analysis of the DREB transgenic lines

Southern blot analyses indicated that of the 43 transgenic T_0 events analyzed so far, 28 of them (65%) showed simple transgene insertions (1-2 insertions) and no rearrangements (Table 2), which make these plants ideal candidates for future phenotypic and molecular characterization of

the transgenic trait. It is important to note that a significant higher number of plants (about twice as many) showed simple insertions (1-2 insertions) when using the *Lip9::OsDREB1B* constructs (90% of the plants) than when using the *Lip9::AtDREB1A* construct (41%). However, in most cases few rearrangements (about 12% in average) were noted spite of the number of transgene insertions (Table 2, Figure 2). Likewise, the most complex hybridizations patterns were observed in some plants (all genotypes) when transformed with the *Lip9::AtDREB1A* construct (Figure 2). Three to four apparent hybridization bands were noted in 32 % of plants, and five to seven bands were observed in 27 % of plants. These results indicate that there might be some construct defects or sequence related features that make the *Lip9::AtDREB1A* more prone to complex insertions in the rice genome Similar results had been reported by other groups when the gene encoding C-repeat/dehydration-responsive element binding factor 1 (CBF1/DREB1b) of *Arabidopsis* was introduced into rice. Southern blot analyses showed the integration of one to several copies of the transgene into the genome (Sang-Choon et al., 2004). However is important to note that in our case plants showing simple hybridization patterns of *Lip9::AtDREB1A* were also identified and selected for future analyses (Table 2). Having into account that from 43 T_0 plants analyzed so far 65 % showed a low number of transgene insertions (1 or 2) and without rearrangements, there is a high probability of finding enough number of independent events from the different genotype/construct combinations to elucidate the molecular mechanisms and phenotype underlining by these DREB transgenes.

Table 2. Comparative Southern blot analyses of 43 events of the total 150 events selected on their agronomic performance.

Genotype	Gene	N	1-2 Insertions	>3-4 Insertions	>5 insertions	Events without rearrangements (%)
Cica 8	*AtDREB1A*	8	4	2	2	88
CT6241		6	2	2	2	67
Palmar		8	3	3	2	100
Total events		22	9 (41 %)	7 (32 %)	6 (27%)	85
Cica-8	*OsDREB1B*	7	6	1	0	86
CT6241		6	6	0	0	100
Palmar		8	7	0	1	88
Total events		21	19 (90 %)	1 (5%)	1 (5%)	91
	Total (A+B)	43	28 (65 %)	8 (19 %)	7 (16 %)	88

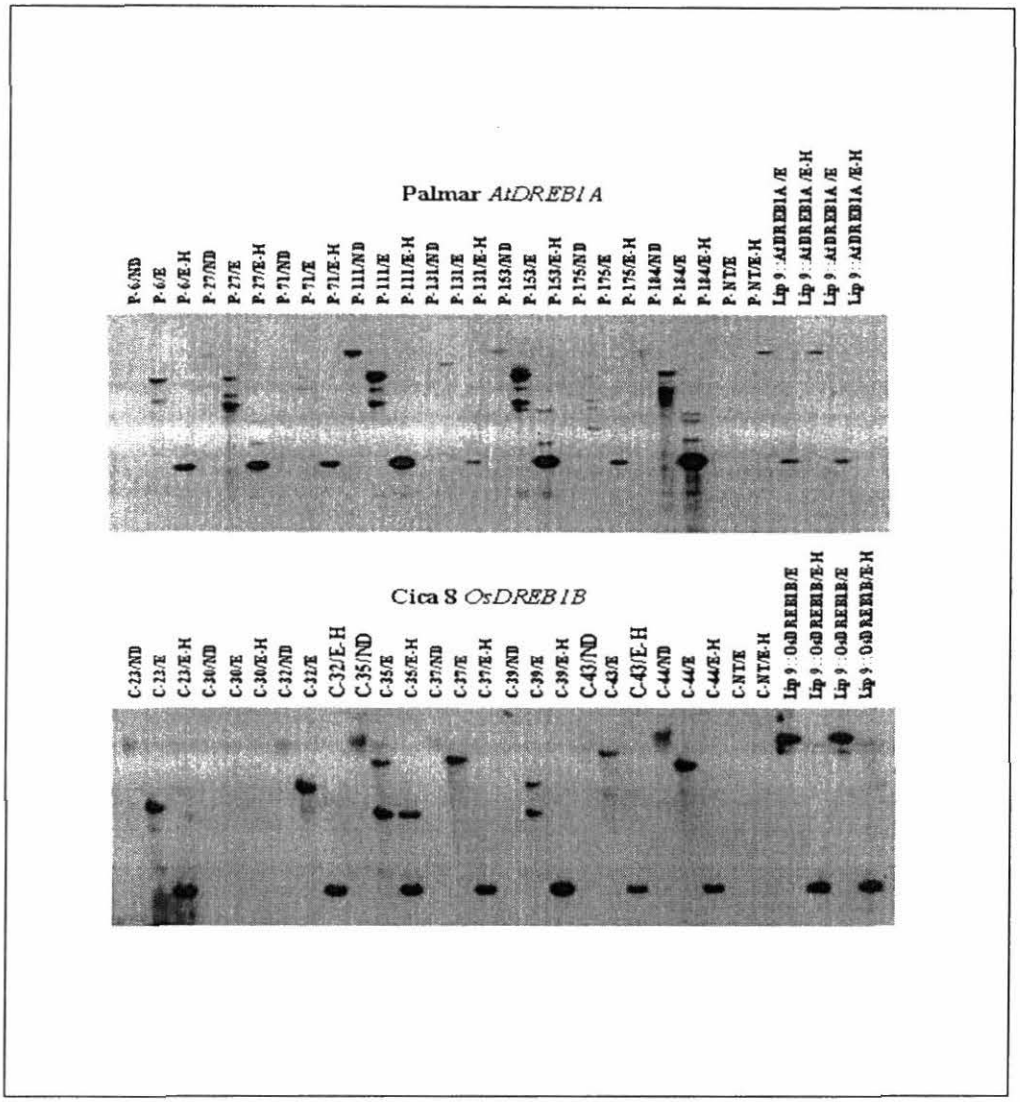

Figure 2. Southern blot analyses of some of the selected Palmar *Lip9::AtDREB1A* (upper) and Cica 8 *Lip9::OsDREB1B* (lower) transgenic events. Genomic DNA was not digested (ND, control) or digested with *EcoRI*, (E) EcoRI/ HindIII (E-H) to determine the number of insertions and rearrangements, respectively. Non-transgenic varieties of Palmar (PNT) and Cica 8 (CNT) were used as negative controls, and the constructs as positive controls.

Endogenous gene expression analysis of lip9 and OsDREB1B

This expression analysis aims to determine drought stress conditions for selection of transgenic rice plants based on transgene expression. In the Palmar genotype, *lip9* gene expression was induced by drought shock treatment and the expression reached a plateau at 5 hours with 18 times higher expression than the basal expression at 0 hour (Figure 3). This indicates that the drought stress triggers the expression of the endogenous gene. This result is consistent with previous micro array analysis, which showed that the gene is expressed in response to water depletion, such as drought and high salinity (Ashiq Rabbani et al. 2003).

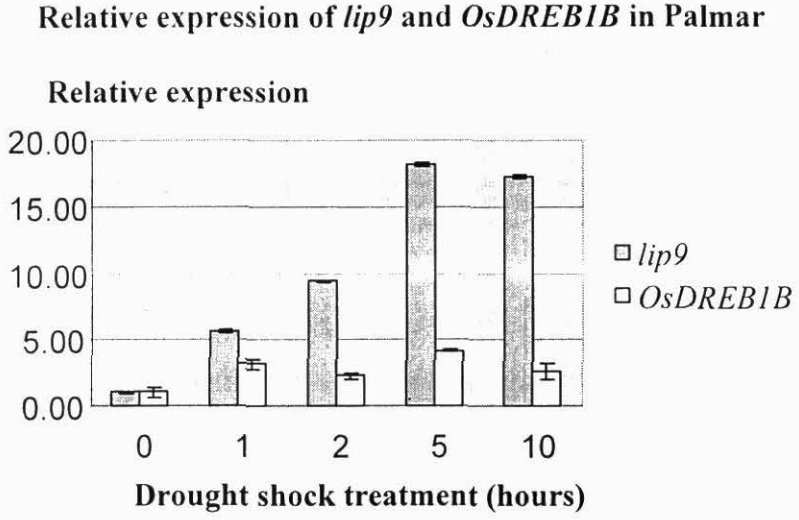

Figure 3. Relative expression of *lip9* and *OsDREB1B* gene in the Palmar. The relative expression level was calculated based on expression level of each gene at 0 hour.

On the other hand, the *OsDREB1B* gene was consistently expressed but the expression was not significantly induced by drought shock treatment. This is consistent with evidence that *OsDREB1B* is cold-responsive, but not drought-responsive (Dobouzet et al. 2003). The CICA8 genotype showed a similar expression pattern for both genes (data not shown) and CT6241 has not been tested. Based on these results, 5 hours of drought shock treatment is enough to induce the lip9 promoter and this condition can be used to select transgenic lines based on the transgene expression.

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2.1.7 Improvement of callus induction and doubled haploid plant regeneration from rice anther and microspore cultures

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[1]SB2, [2]IP4

Introduction

Callus induction and its subsequent plant regeneration are the prime steps in crop plant to be manipulated by biotechnological means. Strategies to improve plant regeneration frequency in cereals, including rice, have been steadily evolving during the last decade. While it has been possible to obtain high plant regeneration frequencies in *japonica* rice varieties, the success for reproducible fertile plant regeneration has been limited in *indica* type. As a result, progress towards the transfer of useful genes in to *indica* rice has been slower. Different reports have shown that many factors affect plant regeneration frequency in rice including: genotype, explant type and developmental stage, medium composition among others (Jain, 1996; Khanna and Raina, 1998). Partial desiccation treatments have been reported to be beneficial for embryogenesis and plant regeneration in several plant species. Tsukahara and Hirosawa (1992) reported that dehydration for 24 h of cell suspension derived callus of *japonica* rice increased shoot regeneration from 5 to 47 per cent. Jain et al. (1996) reported three fold increases in shoot regeneration frequency following partial desiccation for 24 h of suspension cells in *indica* rice. Chand and Sahrawat (2001) carried out partial desiccation of embryogenic callus prior to transfer to regeneration medium and observed increased regeneration frequency in desiccated callus cultures of varieties Safari-17 and Kastur. Saharan et al. (2004) reported increased shoot regeneration frequency from 1.2 to 5.6 fold after 48 h of desiccation of mature seed derived callus of two recalcitrant *indica* rice varieties on different regeneration media. Last year, we reported a comparative analysis of *indica* and *japonica* rice using various PAA concentrations in callus induction medium and aeration treatments (with/without shaker, with/without foam plugs, and RITA system) (Tabares et al., 2004 in SB2 annual report 2004). This year, the work focused in three aspects to improve embryogenesis and regeneration from recalcitrant indica genotypes: 1) to evaluate the interaction between the PAA concentration in the callus induction medium and the aeration treatments (low cost systems; Mini-Rita and Shaker). 2) To improve the shoot regeneration frequency testing different pretreatments (desiccation and callus sub-culture). 3) To test various parameters and protocols for improving microspore isolation, obtaining homogeneous microspores cultures; and maintaining high viable microspore cultures.

Materials and methods

Anther Culture. Anther culture of the indica rice Cica 8, Fedearroz 2000 and CT 11275 were used. Anther donor plants were grown in the field, then the rice panicles were harvested, and anthers cultured according to Lentini *et al.* (1995). Cultures vessels containing the anthers were placed on the shelves (stationary treatment), or with agitation (Shaker or Mini-Rita) in liquid medium contained in baby food jars closed with perforated plastic caps with a foam plug in a hole for aeration.

Plant regeneration. Two preliminary experiments were tested to increase plant regeneration from anther-derived callus. *Partial desiccation (Experiment 1)*: Two stress treatments were tested on callus induction from *indica* rice Cica 8, Fedearroz 2000, and CT 11275 induced on M1 medium (with PAA 10mg/L) in a shaker at 80 rpm. Desiccation was applied by transferring 4 week-old callus into sterile empty Petri dishes containing two sterile Whatman-5 filter papers. The Petri dishes were sealed with Parafilm and kept at 25±1°C in dark for 24 h to obtain the desiccation of callus. Treatment 1, callus was soaked in liquid MS medium for 5 minutes before desiccation (LDCP), and treatment 2, callus were desiccated without previous soaking in liquid medium (DCP). After pretreatment, stressed callus from both treatments were transferred onto MS medium for regeneration and incubated in the light. *Callus subculture (Experiment 2)*: Callus induced in M1 medium (with PAA 10mg/L) in shaker, from *indica* rice Fedearroz 2000, Cica 8 and CT11275 were directly transferred to MS medium and after two weeks of culture only callus showing development of green shoot buds were sub-cultured in liquid or solidified MS medium. Control treatment consisted of transferring callus to MS medium without any pre-treatment as in Lentini et al. (1995). A factorial experimental completely randomized design was used. At least 10 replicates of 20 calluses each was evaluated per treatment per genotype.

Improvement on microspore isolation. Microspore donor plants CT11275 and Fedearroz 2000 were grown in the field, and then the rice panicles were harvested, pretreated and surface sterilized according to Lentini *et al.* (1995). Microspores were isolated by a method similar to the described by Liu *et al.* (2002). Flowers of six panicles were cut and put in a blender cup in 40ml of 0.3M mannitol and blended for 20 seconds (low speed), followed by sequential filtration trough nylon mesh (150, 104, 75 and 20µm pore size). Microspores were washed twice by centrifugation (100g, 1min). Finally, pellet was resuspended in 1ml 0.4M mannitol, and then was fractionated in a Percoll discontinuous density gradient centrifugation of 30%-40%-50% or 50%-60%-70%. Viability was evaluated under a stereomicroscope using 80µg/ml FDA (fluorescein diacetate).

Results and discussion

Two to three fold increases in callus induction was obtained when PAA was added to the induction medium (Figure 1). There is interaction between PAA and different aeration treatments [Stationary (shelf), Shaker and Mini-Rita]. The highest callus induction for Fedearroz 2000 and CT11275 was noted at PAA 10mg/l, and for the recalcitrant genotype Cica 8 at higher concentrations of PAA 30mg/l. PAA also increased embryogenesis up to 28% respect to the control (without PAA) in all genotypes. Similar results were obtained on the regeneration of green plant (Figure 1). After 2 weeks of desiccation treatment, callus was entirely covered by green shoot buds. Plants regeneration was significantly lower in the controls (without desiccation treatment) compared to desiccated callus (Figure 2). Saharan et al. (2004), reported that shoot regeneration frequency was also higher by 1.2 to 5.6 fold in both cultivars in 48 h desiccation whereas in 72 h desiccation treatment regeneration frequency declined. Our results are similar to

those reported by Diah and Bhalla (2000) and Chand and Sahrawat (2001). Significant increase in plant regeneration was observed when callus were sub-cultured (Figure 3). An increase of 3 to 6 fold in shoot regeneration frequency was obtained with retransfer callus as compared to control (Figures 3). When callus were sub-cultured, shoot buds elongated further and multiplied vigorously. Plant regeneration frequencies of 63.0% and 32.7% were seeing when callus were sub-cultured on liquid or solidified medium, respectively (Figure 3). Sub-culturing of callus derived from advanced breeding FLAR lines (ET6941, ET6942, ET6956, ET6976 and TT6963) onto MS free hormone medium increase plant regeneration in about 2 fold (data not shown). These results indicate that callus sub-culturing is a key factor for an optimal plant regeneration of recalcitrant indica rice. Regenerated plants were hardened and transferred to the pot in the greenhouse and then to the field. These plants were fertile and similar to control plants. About 50 – 60% of the regenerated plants were doubled haploids, which are in the range previously reported by Lentini et al. (1995).

Work directed to improve the isolation of high yielding clean microspore suspensions showed isolated microspores should be fractionated by Percoll discontinuous density gradient centrifugation, conserving a large number of viable (80 to 90%) microspores with both Percoll gradients (30- 40-50% or 50-60-70%) for the different genotypes. The microspores suspension was layered on top of a discontinuous Percoll gradient in 15ml test tubes with a screw cap. Follow centrifugation (100g, 3min), each band and pellet formed from each microspore isolation was collected with a pipette and diluted with an equal volume of 0.4M mannitol. In order to obtain a microspores fraction as homogeneous as possible, the two-step Percoll density gradient centrifugation was repeated. The gradient 30- 40-50% gave the most efficient separation of cells by their size, small cells in band No.1 (80 - 90%) and large cells in band No. 2 (88 - 95%). Similar results were obtained in gradient 50-60-70%, but the separation by sizes was as efficient and clear as with the other gradient (Table 1).

Future activities

To study systematically different factors affecting the emission and action of ethylene on *in vitro* culture
To test different times of desiccations and its interaction with hormone medium composition for optimizing green plant regeneration in *indica* rice
To evaluate modifications of culture vessel allowing aeration and/or temporary immersion for reducing current cost for the implementation of the RITA system
To test different conditions and medium cultures for obtaining microspore divisions, induction of micro-callus and plant regeneration.

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Table 1. Microspore separation by size using Percoll gradients 30-40-50% (A) or 50-60-70% (B) using rice genotypes CT11275 and Fedearroz 2000. [1]S = Small cells (range 30-51µm, mean 45µm), B = Large cells (range 51-65µm, mean 60µm)

Genotype	Gradient	Band	Concentration	[1]Size	(%)	Viability (%)
CT11275	A	1	30.4×10^4	S	80	90
		2	33.3×10^4	B	88	96
	B	1	40.0×10^4	S	50	85
		2	27.0×10^4	B	60	70
Fedearroz 2000	A	1	40.5×10^4	S	85	90
		2	43.1×10^4	B	95	86
	B	1	39.1×10^4	S	60	87
		2	30.0×10^4	B	65	80

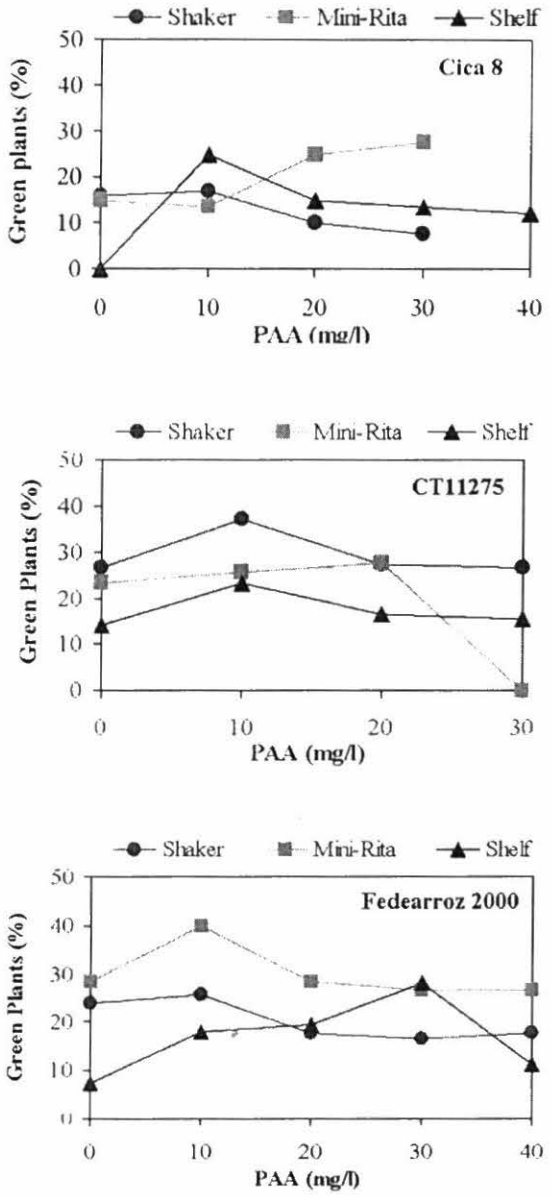

Figure 1. Effect of PPA concentration used for callus induction on plant regeneration of genotypes Cica 8, CT11275, and Fedearroz 2000

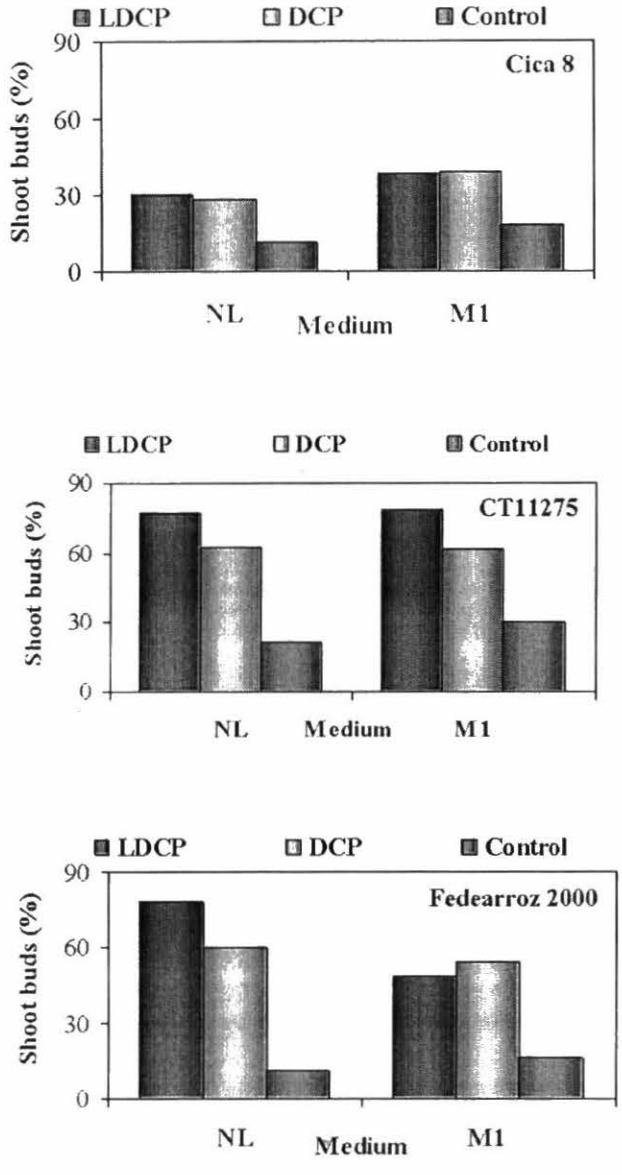

Figure 2. Effect of desiccation pre-treatment (24 hours on Wattman paper) on plant regeneration of 3 indica genotypes. Callus were either previously soaked in MS liquid medium (LDCP) or without soaking treatment (DCP) prior desiccation. Shoot buds refer to callus with green buds development.

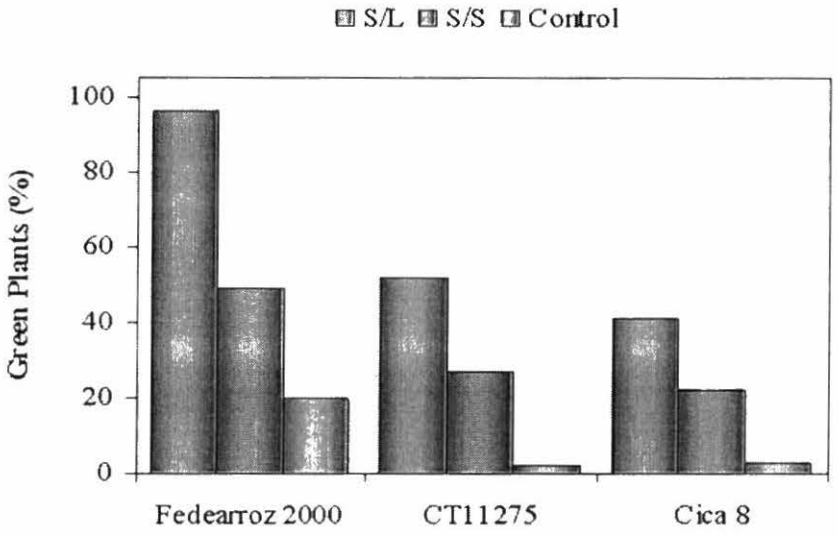

Figure 3. Effect on plant regeneration of callus subculture on fresh regeneration medium. Callus was either subcultured: (S/L) from solid into liquid medium) or (S/S) from solid onto solid fresh medium upon showing the first signs of shoot buds induction; or (control) not subcultured at all.

2.1.8 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 one of the most important calorie-carbohydrate sources in the tropics, adapted to a broad range of environments including tolerance to drought and acidic soils (Kawano, 2003). This important staple food for subsistence farming is also becoming an important raw source for industrial applications worldwide. Cassava breeding is cumbersome and inefficient compared to other crops. Efficient breeding is needed to maintain cassava's competitiveness respect to other commodities. The inefficiency in cassava breeding is mainly due to its highly heterozygous nature and inbreeding depression affecting the selection of early generations of breeding materials in replicated field trials. The *in vitro* production of doubled haploids (DHs, homozygous) lines would serve as a baseline for the development of populations allowing the identification of valuable recessive traits and providing the opportunity for the incorporation of molecular tools. Last year progress was made selecting suitable genotypes for the development and standardization of a protocol to generate doubled haploids in cassava; preliminary work at identifying the various developmental stages of pollen biology with flower bud characteristics; testing various protocols for generating high yield microspore cell suspension; evaluating various pre-treatments and culture conditions to induce microspore division; and corroborating the putative division from isolated microspore culture derived from anthers pre-cultured for five days in 0.3 M mannitol at 26 C in the dark, and cultured at a density of approximately 1 X 10^4 microspores/ ml in B5 liquid medium. In 2005, we directed our efforts mainly to: i) improve further the microspore isolation protocol to obtain homogenous (of similar developmental stage) microspore preparations; ii) optimize conditions for high viability of microspores overtime upon isolation., iii) analyze the effect of field sites on the development of microspores from donor plants; and iv) identify parameters allowing a close association between morphological characteristics of flower buds/ inflorescences and the developmental stage of microspores.

Materials and methods

Plant Material: Plants used in these experiments were planted in the fields of CIAT campus headquarters, ICA experimental station at Palmira, commercial cassava fields at Cabuyal and Buchitolo, Cauca. Only those plants with healthy growth and profuse flowering were used. Immature flower buds were harvested from 8-12 months old for various studies.
Improvement on microspore isolation: Microspores were originally isolated using a similar method adapted from Liu *et al.*, 2002, but modified using filtration, centrifugation and Percoll gradient microspore separation as developed for tobacco by Kyo and Harada (1986), as reported by us in 2004. Flower buds, 2.0-2.9 mm in length, containing a high frequency of tetrads, microspores at various developmental stages and mature pollen were collected (buds < 2.0 mainly contain pollen mother cells), stored a 4°C for 5 days, then immersed in 5% NaOCl for 8-10 min and washed with H_2O for 3 times. Three hundred to 400 flower buds were placed in a blender in 40 ml 0.4 M mannitol and blended for 20 sec (high speed), followed by filtration trough nylon mesh (150, 104, 53 and 40 μm pore size in turn). Microspores were washed twice

by centrifugation (100g, 1 min). Finally, pellets (microspores) were suspended in 1ml of o.4 M mannitol. Microspores were further fractionated by Percoll discontinuous density gradient centrifugation, with the concentrations of 50%, 60%, 70% or 30%, 40% and 50%. The pollen suspension was layered on the top of a discontinuous Percoll gradient in a 15ml test tube with a screw cap. Following centrifugation (150g, 3 min), each microspores population forming bands or a pellet was collected with a pipette and diluted with an equal volume of 0.4M of mannitol. To obtain a pollen fraction as homogeneous as possible, the two-step Percoll density gradient centrifugation was repeated and the microspores remained in the inter-phase were collected in the same manner as described above. Early free spore (EFS) refers herein to those microspores recently released from tetrads, which were transparent or almost transparent under microscope observations, while opaque pollen refers to microspores that were not transparent and with complex cell wall ornamentation.

Monitoring of microspores viability: Anthers containing tetrads or EFS (without thick exine and without auto-fluorescence) from genotypes MTAI-8 and SM1219-9 were isolated under stereomicroscope by squashing them in 15µl of 80µg/ml FDA (fluorescein diacetate) solution on the slide to release microspores, and then incubated in darkness for 5 to 10 min. Observation was made under Leitz fluorescent microscope. For isolated microspores using Blender method, 10µl FDA solution was mixed with 10µl microspore preparation and observed after incubation in darkness for 5 to 10 min. For monitoring the viability loss during microspore isolation, samples were taken from each step and stored at 4°C for further viability analysis.

Analyses of the developmental stages of microspore: Immature inflorescences of genotype "HMC-1" were harvested from Buchitolo, ICA and CIAT field sites. Buds from 1.8mm to 3.1mm in size were dissected. Ten anthers (and at least three replicates) were divided into two groups according to their filament length and squashed to release microspores. The sizes of buds, anthers (with short or long filaments) and microspores were measured under the microscope, the microspore developmental stage was determined.

Results and discussion

Improved separation of the different microspore developmental stages was achieved when using Percoll gradient 30-40-50% when compared with gradient 40-50-60% for all genotypes tested (Figure 1). By using 30-40-50% Percoll gradient, it was possible to obtain a rich band (1st band after centrifugation) composed mainly of tetrads (70-80%) and EFS (15-25%) for a total of >95% of tetrads + EFS, and very few microspore of advanced developmental stages (opaque pollen cells <5%). The second band contained relatively more EFS (30-50%) and less tetrads (15-45%) for a total of 55-80% tetrads + EFS, but also relatively higher percentage of pollen (20-45%). The interaction between genotype and Percoll gradient on the distribution and yield of microspores with different developmental stages after centrifugation was analyzed by ANOVA (Table 1). The proportion of tetrads was significantly affected by Percoll gradients and significant difference was observed between the 1st and 2nd band. It seemed that the microsporocyte amount was more or less independent to the invested factors while the amount of mature pollen was greatly influenced by gradient that has given rise to significant difference between bands. Percoll gradient 30-40-50% allowed a better separation than the gradient 40-50-60% also use efficiently in tobacco (Kyo and Harada, 1986) perhaps because cassava microspores are larger (76 to 123µm) than those of other species cassava.

Table 1. ANOVA analysis of the interaction between genotype and Percoll gradient on the distribution and yield of microspores with different developmental stages after centrifugation.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Tetrads					
Genotype	2	0.03289409	0.01644705	0.30	0.7395
Gradient	1	0.42891930	0.42891930	7.93	0.0075
Genotype*Gradient	2	0.28154957	0.14077478	2.60	0.0866
Band	1	2.17382858	2.17382858	40.18	<.0001
Genotype*Band	2	0.25963464	0.12981732	2.40	0.1037
Gradient*Band	1	0.00000106	0.00000106	0.00	0.9965
Genotype*Gradient*Band	2	0.08628596	0.04314298	0.80	0.4575
Microsporocyte					
Genotype	2	0.34528451	0.17264225	2.47	0.0976
Gradient	1	0.00017308	0.00017308	0.00	0.9606
Genotype*Gradient	2	0.21821111	0.10910556	1.56	0.2228
Band	1	0.11001650	0.11001650	1.57	0.2171
Genotype*Band	2	0.05233092	0.02616546	0.37	0.6904
Gradient*Band	1	0.18162575	0.18162575	2.60	0.1150
Genotype*Gradient*Band	2	0.05268763	0.02634381	0.38	0.6886
Opaque pollen					
Genotype	2	0.26475247	0.13237623	1.87	0.1669
Gradient	1	0.43222029	0.43222029	6.11	0.0178
Genotype*Gradient	2	0.08818752	0.04409376	0.62	0.5410
Band	1	2.20020572	2.20020572	31.13	<.0001
Genotype*Band	2	0.07859714	0.03929857	0.56	0.5779
Gradient*Band	1	0.09070271	0.09070271	1.28	0.2641
Genotype*Gradient*Band	2	0.00178393	0.00089197	0.01	0.9875

The thick exine-ornamented wall on the surface of cassava microspore auto-fluoresce at the same wavelength used for FDA analysis, which hinders the use of this staining for the analysis of viability in older microspores. In contrast, tetrads and EFS cells (which don't auto fluoresce) can be stably stained with FDA in cassava. Therefore, we only monitor tetrads + EFS viability as indicator to evaluate the effect of various treatments. A protocol for FDA staining was optimized using tetrads from fresh materials. Different solvents including water, 20% sucrose solution, MS and B5 culture media, and PBS (phosphate buffered saline, pH6.2) were tested. PBS gave the best results maintaining high cell viability for longer time and giving stronger fluorescence signal, followed by MS and B5 and then 20% sucrose (Table 2). This buffer was then used to assess viability in the rest of experiments.

Table 2. FDA staining on different solvents to determine viability of tetrads isolated from freshly harvested flower buds *

Fluorescence	**H_2O**	**Sucrose**	**MS**	**B5**	**PBS**
Duration/sec	15	23	30	30	68
Signal strength	+	+++	+++	+++	+++++

*Two of the ten anthers from the same bud were used for different solutions.

Results showed that the microspore viability is not affected by the surface sterilization treatment with 30% commercial bleach (equivalent to 2% NaOCl) for 10 min. Blending process, however, caused a drastic loss of viability up to 0.52% of tetrads at room temperature. Results suggested that severe mechanical damage can be caused by blending (Warring Blender) at high speed with respect to low speed for 20 seconds. Temperature plays an important role in maintaining the viability. As Zheng et al. (2003) suggested a relative low temperature should be used during the whole process of microspore isolation. In order to understand the relative long-term effect of low temperature on maintaining the viability of tetrads, samples using 0.3M or 0.4M mannitol solutions or B5 liquid medium were tested to evaluate viability over time at 0h, 3h and 16.5h

after blending. In all cases, viability decreased at 3h after isolation and then increased to the original level seen right after isolation (at 0 h) or above at 16.5h (Figure 2). In all cases, viability was higher when the blending and incubation process was kept at 4C respect to room temperature (Figure 2). Using cold box for materials storage, pre-chilled equipments and containers, as well as cold solution used in microspores isolation will favor the microspores in fighting with the *in vitro* stresses. Moreover, the addition of a washing step following blending increased the viability of tetrads, indicating that some substances released from broken tissues could be toxic to tetrads. Additionally, higher viability was maintained with 0.3M mannitol (327 mOsm/kg H_2O) and B5 medium than with 0.4M mannitol (437 mOsm/kg H_2O) (Figure 2).

In some cases, there was noted not synchrony in the developmental stages of microspores from anthers of the "inner" layer (with long filaments) and "outer" (with short filaments). Microspores from the "inner" layer sometimes developed faster (more advanced stage) than those from the "outer" layer. But most cases microspores were at the same developmental stage independently of its origin thus it is unnecessary to isolate microspore from "inner" and "outer" anthers separately.

Likewise to other species, specific flower bud size corresponds to certain developmental microspore stages. However, variation in the range of bud size with the same developmental stages occurred within the same genotypes between the three fields investigated, indicating there was a strong environmental interaction involved. Flowers buds derived from Buchitolo field showed broader range of variation in microspore developmental stages respect to buds from CIAT and ICA fields (Figure 3). Flower buds from the ICA experimental station showed the most discrete and clear cut differences: 2.0 mm flower buds contain pollen mother cells; 2.1-2.2 mm tetrads; 2.2-2.4 mm EFS; and 2.3-2.9 mm mature pollen. This analysis was conducted using one genotype. Therefore, a "case by case" strategy has to be used to elucidate the appropriate developmental stage of microspores from different locations, at different seasons and from different genotypes and determine if there is a general pattern. The quality-control step should be adopted in future woks by sampling the buds prior to microspore isolation.

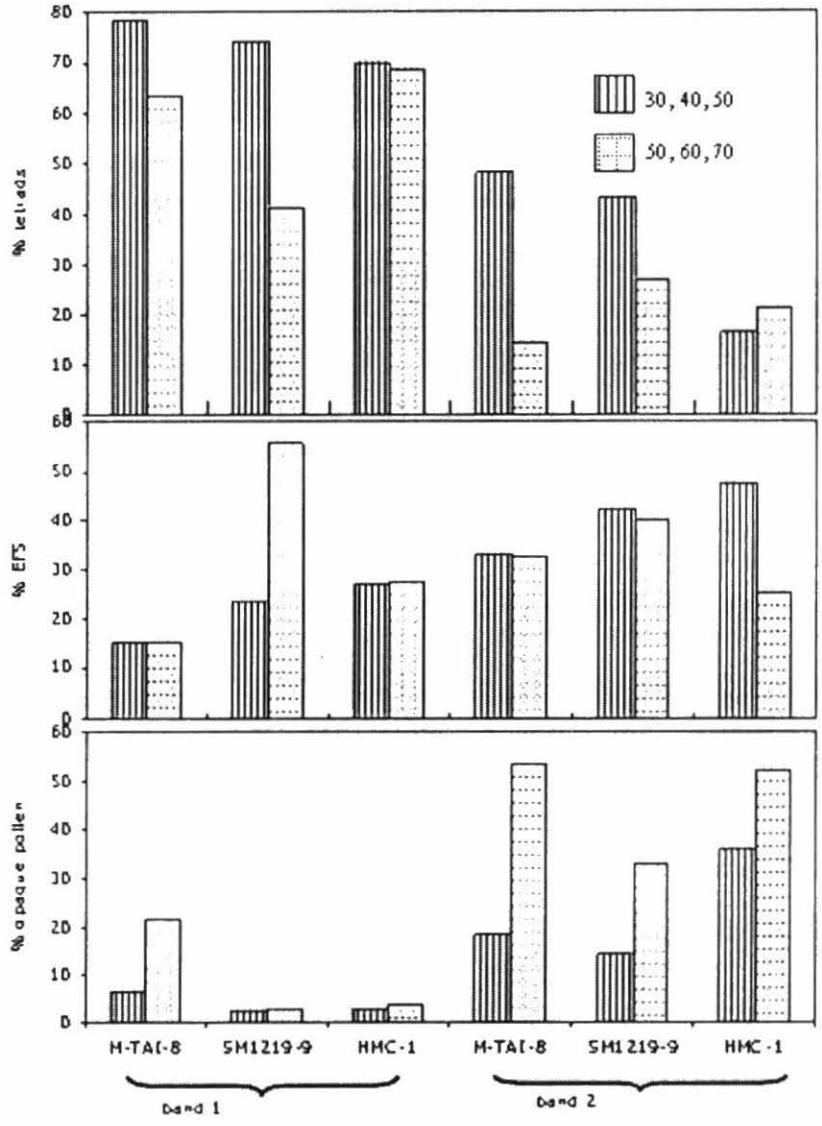

Figure 1. **Effects of the Percoll gradients on yields of the tetrads, EFS and opaque pollens**

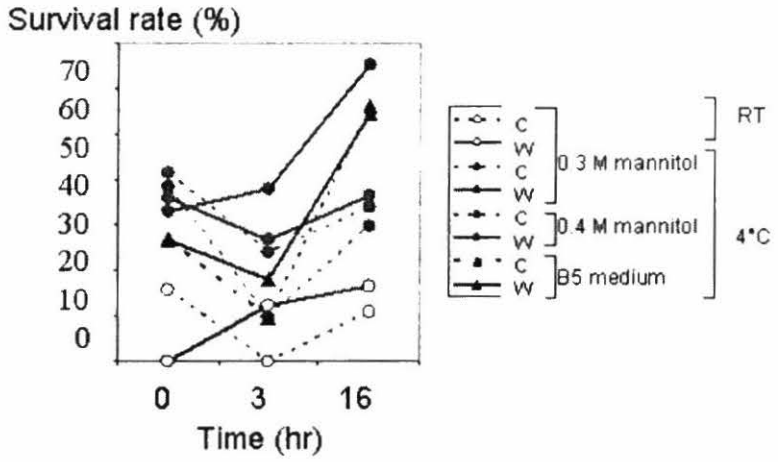

Figure 2. Survival rate of tetrads after isolation with blending using different treatments

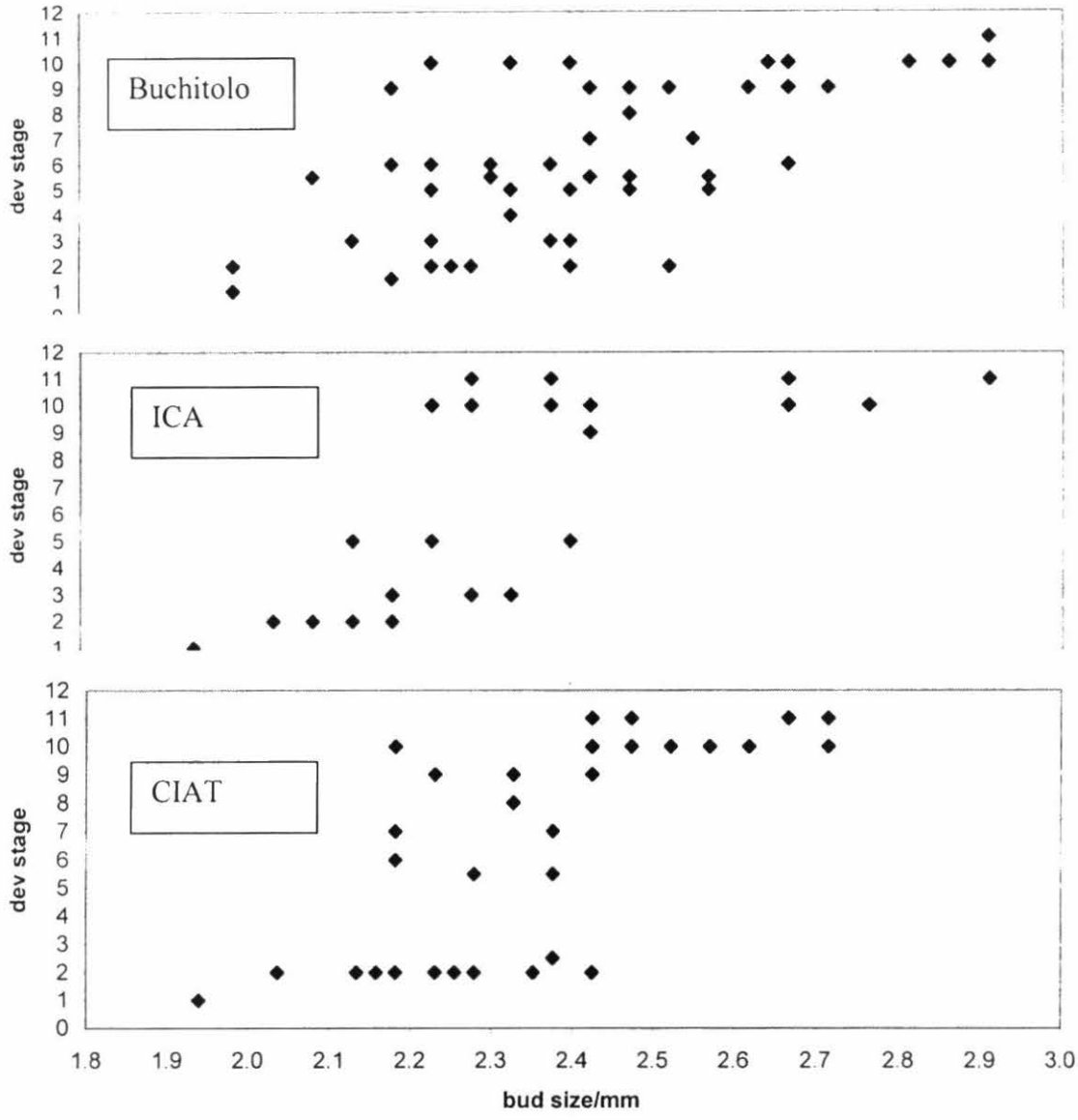

Fig.3 Relationship between bud size and developmental stage of microspores

Y-axe: 1.pollen mother cell; 2.tetrads; 3.old tetrads; 4.small transparent microspore; 5, transparent microspore; 6-11. microspores in the color from light to heavy black.

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2.1.9 Detection, copy number and expression of transgenes in cassava and potato assessed by Real Time PCR

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Introduction

In transgenic plants, transgene copy number can greatly influence the expression level and stability of the target gene, making estimation of copy number relevant. typically transgene copy number is estimated by *Southern blot* analysis, which is very sensible, although laborious and time consuming, and requires relatively large amounts of plant materials (Ingham et al 2001) and radioactivity. To overcome these limitations fast, sensitive and effective quantitative PCR techniques for determining transgene copy number in transformed plants have been developed (Ingham *et al* 2001, Mason *et al* 2002). However, to date, the use of Real Time PCR for estimating transgene copy in Genetically Modified (GM) cassava has not yet been investigated. We report here an assay for detection, fast and accurate relative estimation of transgenes, and verification of transgene expression in cassava using Real Time PCR.

Methodology

Two sets of transgenic cassava plants were evaluated. The first set was transformed with the plasmid pBIGCry to evaluate the *Gus* reporter gene and selection with the antibiotics Geneticin or Kanamycin, conferred be the expression of the gene *npt* II. A second group of lines was transformed with plasmid pCAMBIA 1305.2, evaluating *Gus plus* reporter gene and of selection gene *hpt* II. The general methodology is summarized in figure 1.

Transgene detection Real-time PCR reactions were carried out in a fluorometric thermal cycler *Opticon 2* (Mj Research) and whit the *DyNamo®* SYBR® *green* kit. The specific amplification of transgenes was detected by a *melting* curve analysis using *Opticon monitor 2* software.

Calculation of transgene copy number *To calculate copy number we used a modification of the relative quantitative method, initially reported by Weng* et al *2004, in which the single copy gene* G3pdh *of cassava (Olsen and Schaal 1999) was used as internal control.*

Relative standard curves of PCR reactions for the endogenous gene *G3pdh*, and four transgenes, *Gus*, *npt* II, *Gus plus* and *hpt* II, were prepared whit DNA of transgenic lines, which were confirmed to contain the transgenes by *melting* curve analysis.

Transgene expression Real Time RT-PCR. *cDNA was synthesized from RNA of each transgenic line and used for Real Time PCR amplifications whit specific primers. The expression of the target transgene was confirmed by* melting *curve analysis.*

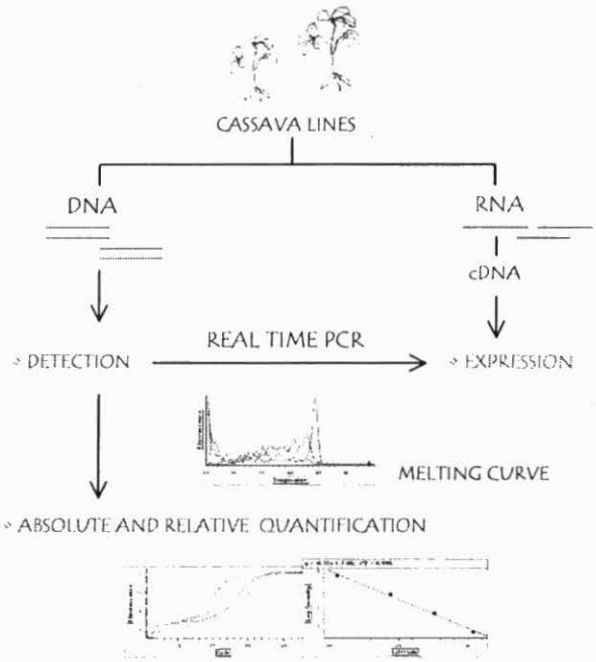

Figure 1. Schematic representation of molecular analysis of cassava transformed plants using Real time PCR.

Results and discussion

Table 1. Transgene detection by *melting* curve analysis contrasted with conventional PCR.

Plasmid	Line	PCR		Real Time PCR	
		Gus	***npt* II**	***Gus***	***npt* II**
	92	+	+	+	+
pBIGCry	80	-	+	-	+
	27	-	+	-	+
		Gus Plus	***hpt* II**	***Gus Plus***	***hpt* II**
pCAMBIA 1305.2	52	+	+	+	+
	54	+	+	+	+

The presence, or absence, of transgenes was confirmed by conventional PCR. Both methodologies, Real Time and conventional PCR, were concordant, indicating that to the former may be used for the detection of trangenes with good confidence. Twenty more lines were confirmed as being transgenic for the transgenes *Gus plus* and *hpt*II (Hygromycin phosphotransferase) by *melting* curve analysis (data not shown).

Figure 2 shows representative curves for the Real Time PCR of the *Gus plus* gene, as well as the correlation coefficient (r^2) of the standard curve for *Gus plus*. In this case r^2 was 0.998. For the remaining four genes r^2 ranged between 0.993 and 0.999.

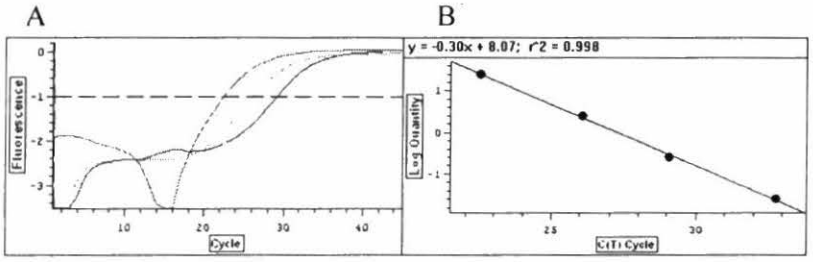

Figure 2. (A) Real-time PCR amplification of the *Gus plus* gene: logarithmic plot resulting from the amplification of four serial dilutions of DNA from line 52. (B) The relative standard curve, obtained with the threshold cycle (*CT*) value versus the log of each initial concentration.

The results of copy estimation are shown in the Table 2. These results suggested the integration of one or two copies, consistent with transformation systems that use *Agrobacterium tumefaciens* as vector. Compared with classical PCR, one of the main advantages of Real-Time PCR is its rapidity to provide reliable data. In addition, recording the amplification in Real-Time avoids collecting samples at different steps of the PCR experiment, making the process less tedious and time-consuming.

Table 2. Copy number of transgenes in cassava assessed by Real time PCR

Plasmid	Line	Copies	
		Gus	***npt* II**
pBIGCry	92	2	2
	80	*	1
	27	*	<1
		Gus plus	***hpt* II**
pCAMBIA	52	2	2
	54	2	1

* No detected.

Expression analysis with Real Time PCR demonstrated active transcription of transgenes *Gus, Gus plus* and *hpt* II, whereas for *npt* II it was not detected. All the results of Real Time RT-PCR were identical to those of conventional PCR (data not shown). The perspective is to develop a system of quantification of transgene expression.

Estimation of copy number for the cry1Ac gene in transgenic potato plants.

The methodology was adapted to evaluate potato transgenic lines developed by the Corporation for Biological Research (CIB, Medellín, Colombia; unpublished results). We analyzed the number of copies of the cry1Ac gene in eight independent transgenic lines. The results indicated between 1 and 4 copies per plant (Table 3).

Table 3. Number of copies of the gene cry 1Ac, estimated by Real Time PCR, in potato transgenic lines

Potato Transgenic Line	2(Xo/Ro)*	Estimated copy number
paz40.4D2	1.13	1
DC28.10.A	0.78	1
pp40.20A	0.38	1
DC40.5	1.01	1
pp40.10C	2.53	2-3
paz 40.1	1.57	2
DC40.61	1.67	2
DC40.7A	4.37	4

(*) Relationship between transgene (Xo) and endogenous gene (Nitrate Reductase) Real Time PCR amplification products

Conclusion

This study indicates that Real-Time PCR is a fast and simple method to detect transgenes and to analyze their expression. It could be easily automated and applied to a larger number of samples. The sensibility is greater than with conventional PCR. Using Real Time PCR, a transgene can be detected in 0.1 ng of cassava DNA, and one transgenic individual can be identified out of a bulk of 400 individuals (on a DNA mixture basis; data not shown). The technology offers major advantages over other methods, including a large dynamic range of quantification, no post-PCR

manipulations (greatly reducing the risk of carryover contamination), use of small amounts of starting material, and high-throughput capacity. In spite of all its advantages, to be on the safer side, the detection of gene copy number with Real Time PCR may need to be confirmed with standard techniques like Southern blots, at least in cassava, and/or at least until the Real Time PCR becomes the standard technology of preference worldwide.

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2.1.10 Gene expression analysis of the genes *psy*, *pds* and *zds* involved in the carotenoid biosynthetic pathway, and carotene quantification in three varieties of cassava

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Introduction

In humans and animals, the health benefits of dietary carotenoids are becoming increasingly apparent (Zimmerman & Hurrel, 2002). The widespread occurrence of Vitamin A deficiency is well documented and emphasizes the requirement for staple foods enhanced in carotenoids exhibiting provitamin A activity (Ye *et al*, 2000). Provitamin A activity is the best established function of caroteniod; and in particular, all carotenoid with the β-ring end group such as the accessory pigment β-carotene.

Many genes encoding the enzymes in the carotenoid biosynthetic pathway have been sequenced and cloned from several species (Cunningham, 2002); however, the sequence of these genes is still unknown in cassava and no studies of gene regulation have been achieved in this staple food. Fraser and Bramley (2004) suggested three levels of regulation of carotenoid biosynthesis in higher plants: Transcriptional regulation, post-transcriptional regulation and carotenoid sequestration.

Aiming to establish relations between gene expression levels and accumulation of carotenes in cassava roots, in three different varieties, relative quantification of three key genes in the pathway, *PSY, PSY* and *ZDS,* was performed using *Real Time* PCR, accompanied with total carotenes and β-carotene quantification in leaves and roots of three varieties of cassava with evident differences in root color. Both measurements were determined in samples of 3, 6 and 8 months old plants.

Materials and Methods

Plant material

Three varieties of cassava: MBRA 253 (**Y**ellow), CM 2772-3 (**C**ream) and CM3306-4 (**W**hite) were grown under the same conditions at CIAT fields. Young leaves and roots were collected from five plants per variety at 3, 6 and 8 months of age. The material was separated to perform carotenoid and RNA extraction.

Carotenoid content

For total carotene measurements, an extraction of total carotenes was performed followed by a determination by visible Spectrophotometry (*DU 640 Beckman* Spectrophotometer). β-carotene was determined by HPLC (*High Performance Liquid Chromatography, 1050 Hewllet Packard*) with a YMC-C30 carotenoid column. β-carotene was detected by monitoring absorption at 450 nm. Identification and measurement was performed by comparing retention times and visible spectra with a β-carotene (Aldrich 85,555-3) standard.

Real Time PCR

Primers and probes were designed to evaluate partial sequences of putative *Manihot esculenta PSY, PDS* and *ZDS* genes. For *PSY* and *PDS,* the primers were designed using partial sequences from cassava, sequenced in a previous work at CIAT (Salcedo, 2001). For *ZDS* primer design we used an EST found in the Cassava EST database of the University of Perpignan, with high homology to *ZDS* genes from other plant species.

Total RNA was extracted from each sample (leaf and root); DNA digestions were performed followed by a reverse transcription of single strand cDNA using random hexamers as primers. Amplification of the samples was carried out in a DNA Engine Opticon® 2 System (MJ Research), with the Brilliant SYBR® Green QRT-PCR Master Mix kit Stratagene®, and then confirmend by TaqMan® real time PCR in an ABI® 7000 Prism System, using the ABI TaqMan® reagents. A relative quantification was performed based on the standard curve method, described by Livak, 2001. The Ribosomal 18S gene was used as a reference gene.

Results and Discusion

In leaves of cassava plants from the three different varieties, no correlation? was observed between carotenoid content and expression levels for PSY, PDS and ZDS, respectively?. Figure. 1 shows the mean values for all three varieties at different developmental stage.

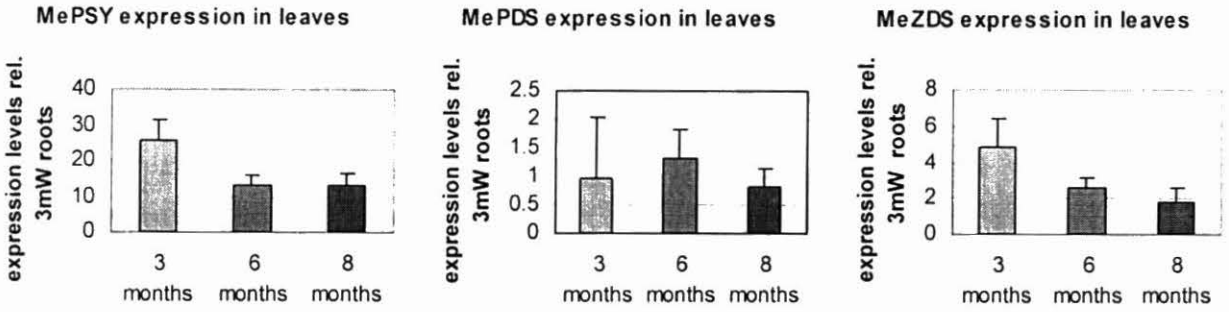

Fig. 1. PSY, PDS and ZDS expression levels in cassava leaves. Expression levels were calibrated to the corresponding expression level detected in a 3 months old root sample white? (W). The data represent the mean of 6 samples (2 samples each variety).

Comparing the expression levels of these three genes in leaves with the expression levels observed in roots, the strongest discrepancy was found for MePSY which showed between 14 times (leaves from 6 and 8 months old plants) and 25 times (leaves from 3 months old plants) higher levels compared to 3 month old roots (W). MeZDS expression levels were about 2 – 5fold higher in leaves than in roots (3 mr W), while the MePDS expression levels remained unchanged and showed tissue-independent expression level.

The differences in carotenoid content measured in roots of the three cassava varieties included in this study could not be explained by differences in the expression levels of the genes investigated (Fig. 2). MePSY expression levels showed almost no differences within root samples of 3 and 6 months old plants. In 8 months old roots, MePSY expression levels in roots of the white variety showed a slight decrease, while the MePSY expression levels in roots of the cream and yellow-coloured varieties showed a slight increase, compared to the values obtained for younger roots.

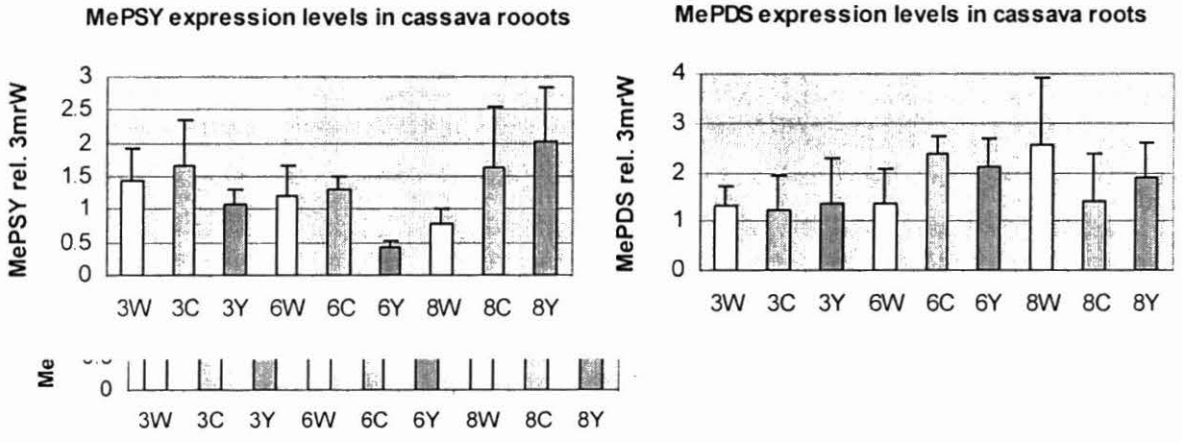

Fig. 2. MePSY, MePDS and MeZDS expression levels in cassava roots. Expression levels were calibrated to the correspondent expression level detected in a 3 months old root sample (W). The data represent the mean of 3 to 5 samples each variety. CM3306-4 (W), CM2772-3 (C) and MRBA 253 (Y).

While MePDS expression levels remained quite unaffected from developmental stage and variety, MeZDS expression levels were highest in young, 3 month old roots and decreased by 60% in 6 and 8 month old roots in all varieties.

Table 1, summarizes the pattern of carotenoid distribution in the root of adult cassava plants. The three varieties showed different distribution pattern of the carotenoid compounds; however ANOVA (<0.05) analysis revealed statistical differences in the root total carotenoid and β-carotene content among the varieties (3 and 6 months old plants); where the Y variety showed the higher values of accumulation of these compounds followed by C and finally W variety. Leaf contents showed a more homogeneous distribution and no correlation between leaf and root content inside the varieties was observed.

Table 1. Carotenoid accumulation pattern of cassava roots from adult plants. DW: dry weight

Variety	CAROTENOID CONTENT *relative [%]*								
	Absolute [µg/g DW]	**phytoene**	**phytofluene**	**□-caro**	**□-caro**	**□-caro**	**lutein**	**violaxan**	**other xanthophylls**
CM3306-4	2,38	--	--	--	91,74	--	1,62	6,64	--
CM2772-3	11,76	2,18	2,18	--	80,61	0,79	11,22	3,02	--
MBRA 253	36,68	2.04	2.04	--	47,52	2,14	2,89	31,45	11,93

Conclusion and future plans

The differences in carotenoid content accumulated in the roots of three cassava varieties can not be explained by different expression levels of MePSY, MePDS and MeZDS. Roots of 3, 6 and 8 month old plants of the three coloured varieties showed statistical significant differences in carotenoid content, while the expression levels of the three genes investigated showed no differences. Transcript level of MePSY in roots showed the lowest level of the three genes investigated, when it was compared to the transcript level in leaf tissue.

It has been proposed to seek at different levels of regulation that could be responsible for the differences in carotenoid accumulation in the cassava roots, such as modification of protein, carotenoid degradation, carotenoid storage and others. Consequently, measure of the volatiles degradation compounds in the three varieties seems to be the next step of the investigation.

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2.1.11 CIAT's field trials of transgenic Cassava plants in 2005-2006

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Introduction

In 2004 the first set of transgenic cassava plants, carrying marker genes of no commercial value, were planted in the field at CIAT to increase seeds to perform agronomic evaluation, with enough repetitions so the findings would have statistical support. At the end of 2005 those experiments continued and they are described below.

This year CIAT received permits from the Colombian authorities, the National Biosafety Technical Committee (NBTC), to plant in the field new transgenic cassava lines that carry genes of potential commercial interest. These new transgenics contain genes for reducing the amount of cyanide produced throughout the plant, or genes that help in the retention of green leaves for longer periods, or genes that alter the quality of the starch accumulated in the roots. All the field trials in the Center follow compulsory biosafety regulations demanded by the Colombian government to perform safe tests with transgenics in open fields.

Materials and Methods

Trails are being carried out on a 1700 m^2 plot that has been divided into smaller plots to fit different number of plants, depending on the trial, from four cassava clones, for a total of 177 plants. A live fence of elephant grass surrounds the entire plot, and it is distant over 500 m from the nearest cassava plantation (Figure 1). Transgenic plants are not allowed to flower to prevent pollen movement. Moreover, the plot is immersed in sugar cane plantations that separate it from other areas where cassava is grown in CIAT. This year represents the second growing cycle for 120 plants of clones CM3306-4, SM1219-9 and 60444, which carry marker genes, and for which a Complete Randomized Block Design was set up to evaluate their agronomic performance. Mr Danilo López, as part of his MSc thesis, is performing this work. Plants will be harvested later this year, so complete results were not available for this report.

Results

A total of 177 plants are currently growing in the field.. The results of the field trials for agronomic evaluation of clones CM3306-4, SM1219-9 and 60444 will be available after the harvest in April 2006. Table 1 summarizes the characteristics of the field trials being carried out at CIAT.

Table 1. Clones of transgenic cassava growing in the field in 2005-2006.

Trial number	Clone (lines)	Character(s) of interest	# of plants in the field*	Purpose of trial	Collaborators
I	60444 Lines 529-28 and 529-48	"Staygreen", plants matain green leaves longer	48	Increase seed for agronomic evaluation trials	Swiss Federal Institute of Technology
II	Mcol2215 Lines 5'2, 6'2 and 9A1	Reduced cyanide content	9	Same	Ohio State Univ Univ of Puerto Rico-RP
III	60444 At least ten lines GBSSS-sense and antisense	Increase amylopectin content in starch	Plants are still in the biosafety greenhouse	Same	Cassava Breeding Program-CIAT
IV	CM3306-4 Lines 001, 048, 023, 061, 027 and 014	Marker genes	60	Agronomic evaluation trials	The Cassava Breeding Program collaborates with the assessment of agronomic evaluations
V	SM1219-9 Lines 004, 005, 014 and 015	Marker genes	48	Agronomic evaluation trials	Same
VI	60444-270400	Marker genes	12	Agronomic evaluation trials	Same

(*) only transgenics are counted; non-transgenic controls, and border plants are not included.

Figure 1. Watering of recently planted transgenic cassava plants. The youngest plants correspond to clones with reduced cyanide content or increased longevity of green leaves. Taller plants in the background belong to three different clones with marker genes, which are being evaluated for agronomic performance. Among the characters that will be measured in April of 2006 there is yield. On the right of the picture the fence of Elephant Grass is visible, next to the natural fence of *Swinglia* (tallest trees) that surrounds CIAT's headquarters in Palmira.

Perspectives

Field trials of transgenic cassava at CIAT are increasing in area. For the second semester of 2006, it is anticipated that at least 124 plants will be planted for agronomic evaluation of trials I, II and III. If there is need of other trials, for example to test for drought tolerance of plants that retain leaves longer, then, another set of 36 will be necessary. Besides, all transgenic lines, authorized for field testing, will be maintained in the field, at least by triplicate, even if there are not tests running on

them, so the numbers go up. Therefore, the need for land and funds to maintain the field site is increasing, so one of the priorities is seeking for more land and new sources of funding.

2.1.12 Somatic Embryogenesis and Plantlet Regeneration of Mango (*Mangifera indica* L.)

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Introduction

In the last 15 years there has been a tremendous increase in areas dedicated to mango production in the tropics and subtropics. Mango production is appealing because the fruit is nutritionally important and constitutes an attractive option to increase income and reduce poverty in the rural sector of developing countries.

Despite its importance and worldwide distribution, mango suffers from a long juvenile period, erratic flowering and alternate bearing habits. In fruit crops, control of flowering is a critical aspect in the production system, since it determines the seasonality of fruit supply to the market. One of the great advantages of the tropics is the possibility of producing during the whole year, nevertheless fruit producers and markets face major challenges to supply fruits of high quality throughout the year. Biotechnology can potentially be used to manipulate existing cultivars by targeting specific genetic traits, such as flowering behavior. If farmers were to gain the ability to control flowering in fruits, they could better target their produce to markets with narrow windows of opportunity.

This research aims to manipulate the expression of a target set of developmental genes known to modulate flowering. An efficient protocol for genetic transformation is needed in order to splice in genes for flowering control and other valuable traits of interest. However, to apply these tools, highly efficient embryogenesis and regeneration protocols are required.

Materials and methods

As preliminary work, published protocols for somatic embryogenesis induction, proliferation of proembryogenic masses, embryo maturation and plantlet regeneration were tested (DeWald et al., 1989a-b; Pateña et al., 2002; Rivera-Dominguez et al., 2004 and Xiao et al., 2004). Immature fruits (40-50 days after anthesis) were colleted from commercially grown mango plots from various regions in Colombia. Four monoembryonic Florida cultivars (Keitt, Tommy Atkins, Kent and Irwin), and nine polyembryonic Colombian cultivars (Magdalena River, Yulima, 505, Jobo, Azúcar, Arauca, Manzano Vallenato and Sufaida) were chosen. The Florida cultivars were selected because are commercially important in most developing countries including those in Africa, the main target region of this project.

Immature fruits were washed and disinfected with commercial bleach 20% (v/v) for 20 min, supplement with 3 drops of Tween 20, then washed three times with sterile, distilled water. The length of each immature fruits was determined before dissection, and the length of the zygotic

embryo within each ovule was determined prior to its removal. Ovules were bisected symmetrically and the nucellus was separated from each ovule and transferred to the induction media. The embryogenic response was evaluated as number of nucellus producing proembryogenic masses or somatic embryos directly.

Results and Discussion

Nucellar tissues cultivated on Pateña et al., 2002's formulation exhibited the best embryogenic response. All of the cultivars tested showed some level of somatic embryogenesis induction. Thus apparently the response was independently of cultivar used, in contrast to previous studies (Litz et al., 1998; Rivera-Dominguez et al., 2004).

The highest embryogenic response was observed in Keitt (48%), followed by local cultivars 505 (34%), Jobo (31%) and Magdalena River (20%), and Kent (12%). On Keitt, proembryogenic masses were observed on about 2-3 weeks after culture (Fig. 1 k). Polyembryonic cultivars, such as Magdalena River, induced somatic embryos directly from the nucellar tissue approximately four weeks after culture on induction medium (Fig. 1 f). Somatic embryos at different developmental stages of Magdalena River, Yulima, Keitt and cultivar 505 have been obtained (Fig. 1). Somatic embryos of Magdalena River reached cotyledonary stage in about three months (Fig. 1 h). At present, some somatic embryos developed roots (germinated) (Fig. j). Embryogenic cell suspension cultures of Keitt and Magdalena River are being established.

Future Works

- To establish protocols to improve plantlet acclimatization of mango developed *in vitro*.
- To initiate genetic transformation tests.

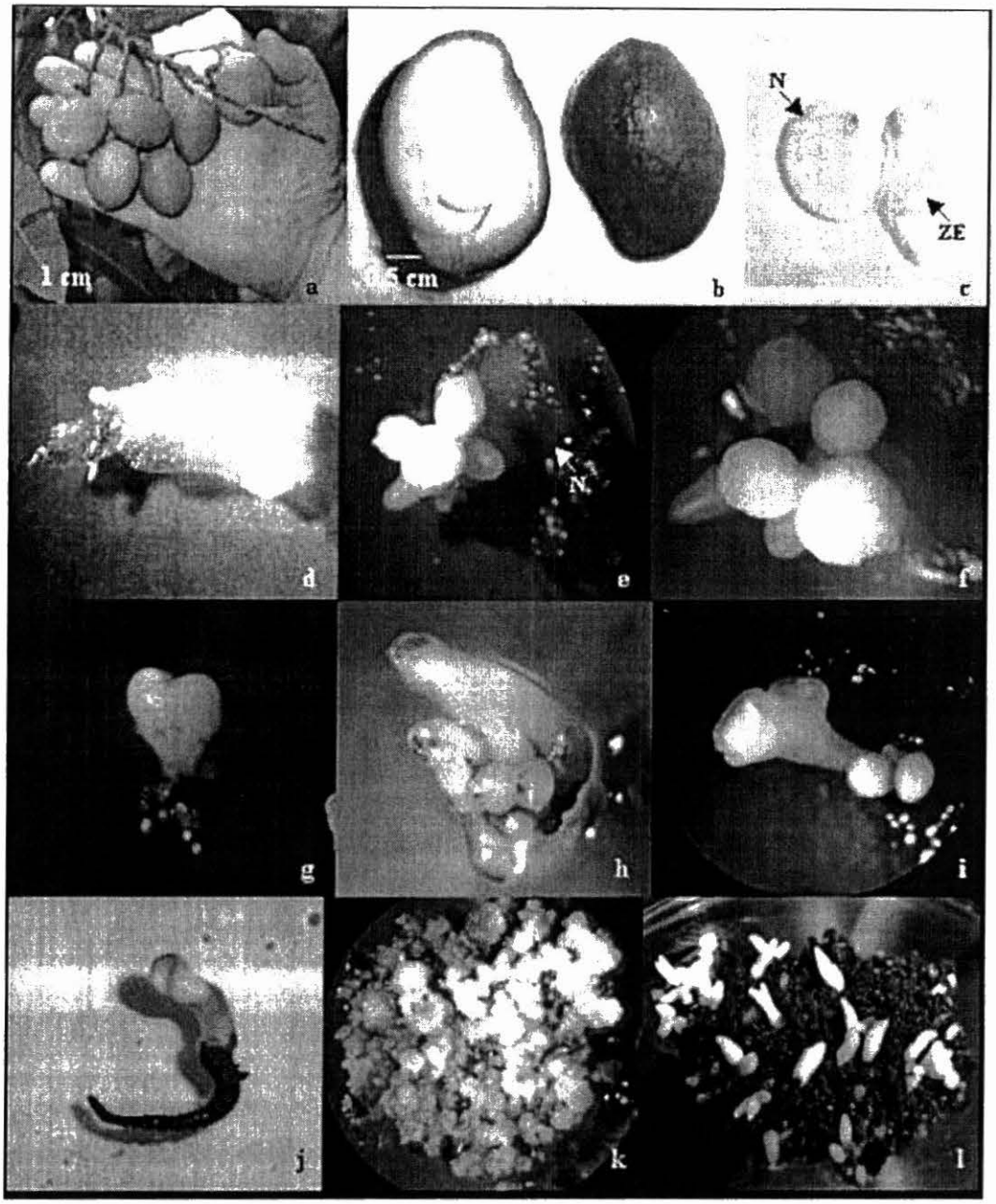

Figure. 1. Somatic embryogenesis from nucellar tissues of Keitt and Magdalena River cultivars of mango. *(a-j)* Somatic embryogenesis of Magdalena River Mango. *(a)* Immature Fruits, source of nucellar explants; *(b)* immature fruits cut longitudinally; *(c)* ovules cut open lengthwise showing nucellus and zygotic embryo; *(d)* nucellar tissue isolated; *(e-f)* globular somatic embryos; *(g)* somatic embryo at heart stage; *(h)* torpedo somatic embryos; *(i)* cotyledonary somatic embryo; *(j)* germinated somatic embryo *(k)* proembryogenic masses from nucellar tissue of Keitt; (l) somatic embryos of Keitt at different developmental stages. N: nucellus; ZE: zygotic embryo(s).

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2.1.13 *In-vitro* plants production's to support CIAT's and other partners research agenda's

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[1]Agrobiodiversity and Biotechnology Project; [2] FIDAR; [3]CIAT-MADR; [4]Virology Unit. CIAT.

Introduction

Material and Methods

In-vitro propagation on 4E medium as Roca (1984) suggest was followed. In some cases SB-2 made hardening phases on greenhouses facilities, but when the *end-users* has this facilities they do it.

Results

Table No1 summarize the total *in vitro* plant produced during 2005, for different users. The clone produce during this period of time has different quality depending of the project objectives (FSD tolerances, FSD testing, farmers local clones, whitefly resistances, etc). For the next year, FIDAR discount from the propagation scheme the MBRA 383 clone, for its highly FSD susceptibility.
One plot of clone CG489-31 has been produce for Tolima to supply clean cutting for that area. It's important to stick out that this clone was develop for that area conditions but nowadays, farmers not allow them with enough clean planting material for renew plantations.

Table 1: Consolidated *In vitro* plant production by SB-2 project during 2005.

Institution	Clones	No. Plants	Use of plant for
FIDAR	HMC-1	1220	*End-user* diffusions in Cauca.
	CM523-7	1180	(Fresh consumption and starch production).
	M Bra 383	900	
	M Per 183	990	
	CM 7951-5	740	*End-users* diffusion. Material with FSD tolerance.
*	37 Local clones	535	Replace of farmers' local seed bank.
CIAT-MADR	CG 489-31	1100	*End-users* diffusion in Tolima. Material with Whiteflies resistances.
CIAT	TMS 60444	511	Double haploids project.
	M Col 2063	200	For FSD grafting test.
Total plants		7376	

To replace first plot of material generated, due by animal attack.

Conclusions

The SB2 project holds a good laboratory facilities and manpower that it could cover *in vitro* planting demand of different *end-users*. However it is necessary that they make its requirement with time that allow us make a working plan, that let us cover this demand. A coordinated join effort, with a previous planning allows be more efficient in input and manpower uses. It's necessary that new proposal consider, as part of the project, the cost and time duration of generation of planting materials in its project schedule before start it.

Perspectives and Future activities

It is necessary that *end-users* (CIAT and others partners) make a join agenda that allow us to project our capacity for plant production. This let us to know a real demand of planting material *in-* and *out-site* CIAT. For example, if CIAT (entomology, pathologist, virology and breeding group among other users) needs to establish a continuous FSD testing, it is necessary to make a realistic plan to produce it's mother clean plants. Besides, *in-vitro* activities could support refresh clone for renewal field and to support planting material of new delivered clones. All these works need its to be on budget plan.

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2.1.14 Implementation of Cassava's Cryopreservation Protocol Using the Core Collection as a Model

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Introduction

During period's 2003-2005, different activities was putting into practices to know logistical aspect involved in the management of an IVBG cassava collection. It will give us a general idea about critical points, how much effort and manpower will be necessary for the implementation of the cassava cryopreservation process's with the entire collection.

Material and Methods

The encapsulation-dehydration methodology was implemented (Annual Report, 2000) using *in-vitro* plants supplied by the GRU. It was necessary to recover a copy of core collection coming from clones maintained at BRU to make a Cryo-core II

Results

Actually, we maintain under L.N. conditions 621 clones of the core collection. Per each clone it maintain 6 tubes with 10 beads. At this year, we initiate to duplication of the Cryo-core, but high contamination and logistical problems in the lab with agar lots and autoclave it, was not allowed to us to go farther with any of the copies (Cryo- core I and II) (Table 1).

Table 1: Groups of response and copies established under L.N. conditions using the cassava core collection as a model.

Collection Type			No Clones	% of response based on
Cryo-Core I		Frozen	621	98.57% of core collection
		Evaluated	599	96.5% of frozen clones
	Group of response*	Lowest	191	Lest than 30% plant recovery
		Intermediate	245	Between 30-70% plant recovery
		Highest	163	Up to 70% plant recovery
Cryo-Core II		Frozen	84	

* Clones are distributed into response's groups based on n=599 clones evaluated.

Based on frozen clones, 96.5% of them have been recovered and grouped according its responses in low, intermediate or highest group. More than 68% (intermediate and highest group) of the clones showed up to 30% considered as base line for maintaining one clone into the cryo-tank (Figure 1).

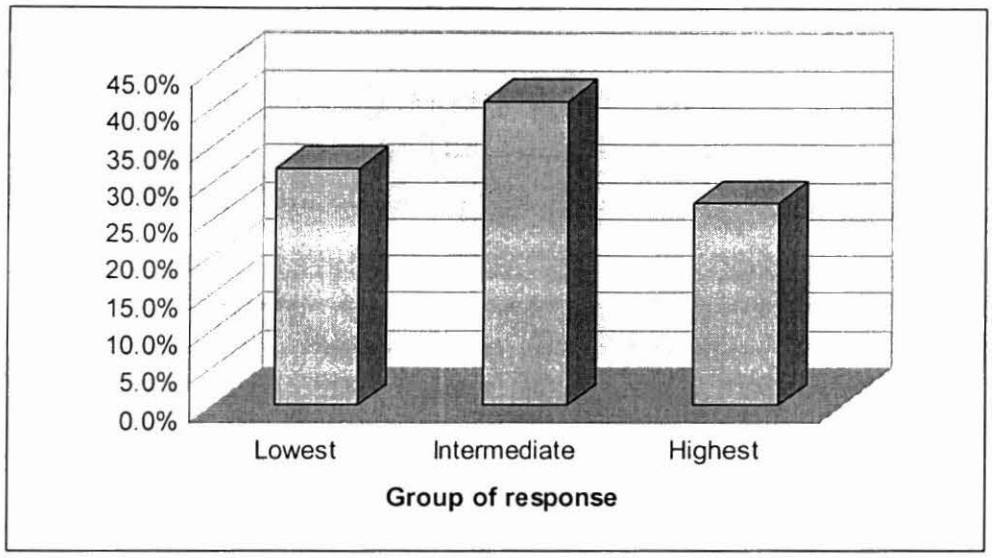

Figure 1: Grouping of clones after freezing procedure (<30%, 30<x>70%, >70% of response's of lowest, intermediate and highest group respectively). n = 599 clones tested.

We consider that a critical point during the process is the propagation schema that produces explants for cryo, because is time consuming and require a lot of manpower. In the same sense, encapsulation-dehydration has a lot of steps that are not practical for implementation with a big collection as that of cassava (based on number of clones conserved per month).

For that reason we initiated activities with Vitrification protocol using buds as explants. Probably, these procedures allow us make all freezing activities in one day and put it more clones into the tank per month. Panis *et al* (2005) at INIBAP adjust it a droplet-vitrification protocol and tested with *Musa* and *Ensete* genera being possible its applicability to all banana accessions.

Conclusions

- We conserved under L.N. condition 98,6% of core collection (Cryo-core I). As duplicated copy of Cryo-core II, it was included 84 clones during 2005 period.
- Based on core collection, 68% of clone tested it showed plant formation up-to 30% (245 and 163 clones as intermediate and highest responding groups respectively)
- Propagation activities and contamination aspect occurring during process could be a critical point for routine of cryopreservation. It could delay the process.
- It necessary frozen 22 clones to complete an entire core collection copy under L.N. conditions.

Future activities

- Establish a critical point scheme for monitoring cryopreservation activities. HACCP analysis will be useful to make control activities.
- Adjust procedure to use nodes as explants that allow us reduce propagation activities.
- Adjust Vitrification procedure with lowest responding clones.

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2.1.15 Optimization of methodologies for massive production of clonal planting material of yellow passionfruit (*Passiflora edulis* f. flavicarpa Degener) free of diseases

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Funding: Ministry of Agriculture and Social Development of Colombia – MADR

Collaborators: Corporación Colombiana de Investigación Agropecuaria - Corpoica

Introduction

The juice of the yellow passionfruit (*Passiflora edulis* f. flavicarpa Degener) is the third most important exotic juice in the world market. Colombia's productivity of passionfruit is considered the highest average yield with around 20 tons/ha; Colombia is the third largest producer of passionfruit concentrate after Brazil and Ecuador (Anonymous, 2005), and consequentely, the central government is promoting the establishment of new plantations as a strategy for the replacement of illegal crops.

A disease known as the "Secadera" is considered to be caused by a complex of soil borne fungi with predominance of *Fusarium* (Torres *et al.* 1999). Secadera is the main disease of the yellow passionfruit in Colombia, ana affects the most important producing regions of the country. This disease reduces de longevity of the plantations from 36 to 19 months (Rodriguez and Marmolejo, 1999) causing the loss of more than a half of production potential.

To minimize losses induced by "Secadera" one could identify commercial cultivars with tolerance to this disease. However, this is a long term task and requires a sustainable breeding program, as tolerant material might not have commercial attributes that make them appealing. A short term strategy to solve this lack of tolerance and to keep using the existing culvitars is to identify rootstocks tolerant to soil borne diseases. Rootstocks could be found in other other *Passiflora* species resistant to them like *P. nitida* and *P. giberti* (Roncatto *et al.* 2004) or resistant selections of the same species (Newet, 2005).

Plantlets produced by seeds of resistant cultivars may be genetically unstable, or produce too weak stems to be used as rootstocks (Costa *et al.* 2004); therefore, the most appropriate tissues for the production of rootstocks are vegetatively propagated plants obtained from rooted cuttings.

Production of rooted cuttings will also be useful to fix other traits such as the productivity or fruit quality, if superior clones are vegetatively propagated and grafted onto resistant rootstocks.

Methods for the vegetative propagation of the yellow passionfruit have been developed using greenhouse based (Molina-Meletti *et al.*, 2002; Salomao *et al.*, 2002; Chamhum *et al.*, 2002) and tissue culture technologies (Gonzalez, 2003). In Colombia tissue culture derived plants have been commercialized in the past. However the adoption of this technology for establishment of plantations has not been as expected, possibly due to the high costs of the clonally propagated plants or the little agronomic advantage of the propagated clones.

During 2005, as part of a project funded by the Ministry of Agriculture and Social Development of Colombia – MADR, and in collaboration with the Colombian Corporation for Agricultural Research, CORPOICA, we investigated the development of fast and inexpensive methodologies for clonal propagation of yellow passionfruit and other *Passiflora* species that could be applied for the massive production of disease tolerant grafted plants .

Methodology

Plants of the yellow passionfruit and of different *Passiflora* species included in this study were maintained in a greenhouse at room temperature. Stem segments with two or three nodes were cut from actively growing branches for producing cuttings. All leaves of the cutting with exception of that of the most upper node were cut away and discarded. The cuttings were treated with two concentrations of naftalenacetic acid (NAA; 10 and 100 mg/L) dissolved in water, for different periods of time (0, 10, 120, 180, 360 and 1440 minutes or 30 min in a second experiment). Then they were planted in sterile sand and placed under intermittent mist (from 6 to 18 hours, every hour for 1 minute) in a greenhouse (modified after Chamhum *et al.* , 2002)
The following variables were evaluated 45 days after planting: number of buds and roots developed, length of the longest root and plant recovery efficiency (PRE, the percentage of treated cuttings which produced plants). Means were analyzed with the Ryan-Einot-Gabriel-Welsch test of variance at a level of 5% of probability.

Results

Plant recovery from yellow passionfruit cuttings
Forty five days after experiment initiation most of the cuttings showed prolific adventitious root formation. With exception of the longest treatment with the highest NAA concentration (100 mg/l NAA for 1440 minutes, Table 1) which produced less than 60% recovered plants, all other treatments yielded over 80% plant recovery. Dipping the cuttings in a solution of 10 mg/l NAA for 120 minutes was the best treatment, yielding 100% plant recovery. However, for our surprise, also the control treatments in which no growth regulator was used, yielded plant recovery values over 98.6%, indicating that the growth regulator NAA is not necessary for an efficient root induction of cuttings with three nodes of the yellow passionfruit. Practically all of the rooted cuttings could be established in the greenhouse as a new plant, which served later on as a mother plant for production of further cuttings.

Table 1. Average number of buds and roots produced, length of the longest root and plant recovery efficiency of cuttings of the yellow passionfruit treated with different periods of time and concentrations of NAA (average followed by the same letter indicates a statistical similarity within each column to 5% of probability).

Exposure time (min)	Number of buds			Number of roots			Length of the longest root (cm)			Plant recovery efficiency* (%)		
	0 mg/L	10mg/L	100mg/L	0 mg/L	10mg/L	100mg/L	0 mg/L	10mg/L	100mg/L	0 mg/L	10mg/L	100mg/L
0	1,8 bcd			8,6 bc			13,1 c			98.6		
10		3,7 a	3,1 ab		14,9 bc	24,7 ab		18,1 bc	17,4 bc		83,3	87,5
120		3,0 ab	2,3 abc		14,0 ab	40,8 a		21,2 b	20,8 b		100	92,5
180		1,9 bcd	1,4 cd		12,1 bc	18,7 ab		17,6 bc	16,3 bc		89,5	90,7
360		1,9 bcd	1,4 bcd		16,3 ab	23,5 ab		16,4 bc	16,2 bc		84,6	96,4
1440		2,9 ab	0,6 d		25,5 ab	16,4 c		19,9 b	28,8 a		94,9	52,6

* % treated cuttings which produced plants

Plant recovery from cuttings of different *Passiflora* species

In other set of experiments, cuttings of two or three nodes from different *Passiflora* species, were treated with one or two concentrations of NAA for 30 min and then placed under mist conditions. In a first experiment with three node cuttings plant recovery efficiency ranged between 43 and 56%. In a second experiment with two node cuttings plant recovery efficiencies increased to values between 89 to 100% (Table 2). This increased plant recovery efficiencies of the second experiment do not seem to be related to the number of nodes of the explants, but to the physiological conditions of the mother plants from which the cuttings were isolated. The mother plants from the second experiment were younger than plants used in the first experiment. Thus, it seems likely that plant recovery efficiencies of cuttings of *Passiflora* species may be improved by maintaining the mother plants in optimal conditions.

Table 2. Plant recovery efficiency from cuttings of different *Passiflora* species treated for 30 min with one or two NAA concentrations

Species	Number of Nodes	Plant recovery efficiency (%)	
		10mg/L	100mg/L
P. alata	3	56.7	43.3
P. maliformis	3	50	50
P. mollisima	3	51.5	48.5
P. alata	2	100	
P. edulis	2	89.8	
P. ligularis	2	100	
P. quadrangularis	2	90.9	

Additionally to the above mentioned *Passiflora* species, several other tested with the developed propagation methodology (*P. cincinnata, P. subpeltata, P. lehmani, P. foetida* and *P. popenovii*) could be cloned with efficiencies over 50% (data not shown).

Determination of the number of nodes needed for efficient clonal propagation

To be able to use cuttings with less than two or three nodes for propagation will allow one to produce more propagules from a single mother plant.

Analysis of the buds of two node cuttings participating in the formation of the new cloned plant, indicates that the first (the upper) bud participates in the formation of most of the recovered plants, while the second stayed dormant. This suggests that it may be possible to reduce the number of nodes of the cuttings to single node ones, and increase by this way the propagation efficiency. Experiments are underway to confirm this.

The methodology developed is much simpler than that reported by Molina Meletti *et al.* (2002) who used hydroponic cultures with complex recycling forms of nutrient solutions. Also, with the possibility of using single node cuttings as initial explant, our propagation methodology may be applied with higher efficiencies than that developed by Chamhum *et al.* 2002, who used only selected portions of the stem of the mother plants as source of cuttings of three or more nodes.

Taking the necessary measures for maintaining mother plants in good health in the greenhouse, this propagation methodology may represent a simple alternative for tissue culture propagation of selected cultivars of the yellow passionfruit or other *Passiflora* species.

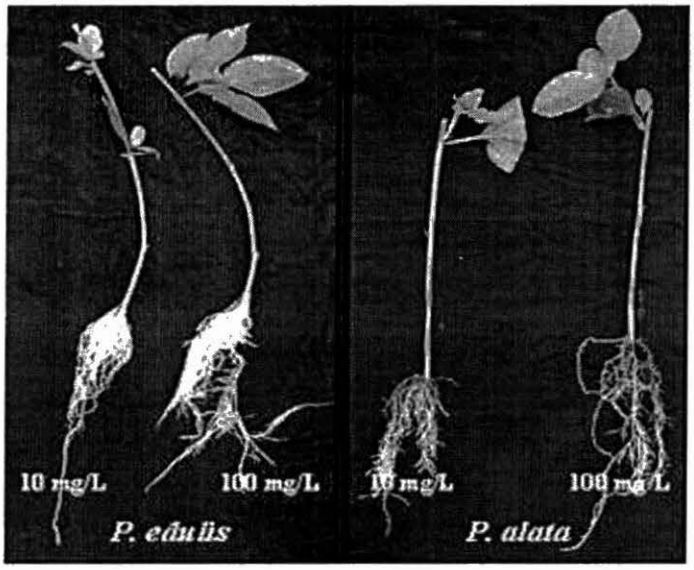

Figure 1. Rooted cuttings of *the yellow passionfruit (P. edulis* f. flavicarpa Degener*)* and of the sweet passionfruit (*P. alata* Dryand.) after 45 days of treatment with NAA solution and maintainedeunder intermittent mist conditions.

Conclusions

An efficient and inexpensive methodology has been adapted and optimized for the clonal propagation of the yellow passionfruit and other *Passiflora* species. This methodology can be used for the propagation of selected cultivars or for the production of rootstocks tolerant to pests or diseases.

Future plans

To test the propagation efficiency of single node cuttings. To produce grafted yellowpassionfruit plants using rootstocks of *Passiflora* species reported as resistant to *Fusarium* wilts, and test its tolerance to Secadera in the field.

This project will be continued with minimal core funding until a proper donor is found,

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2.1.16 *In vitro* Propagation and Regeneration of *Solanum quitoense* (Lulo) Plants and their Use as Elite Clones by Resource Farmers

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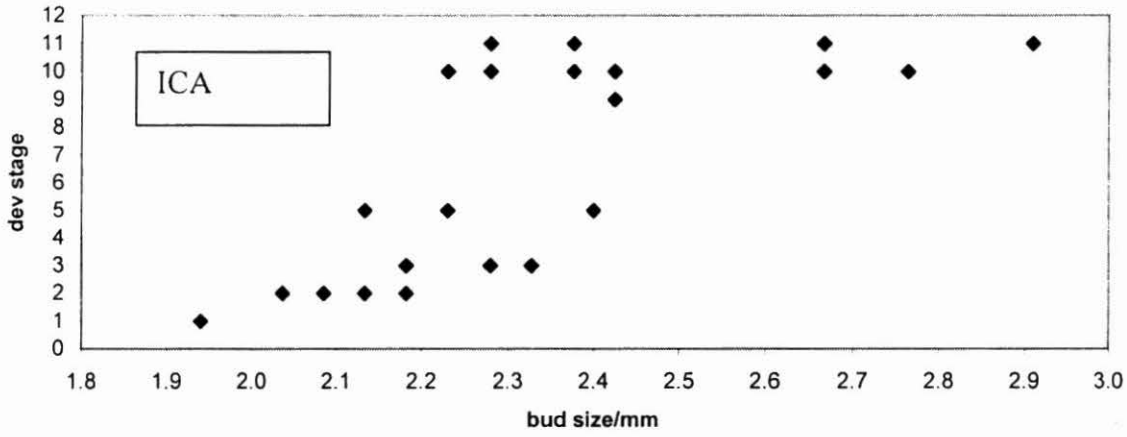
Introduction



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 for local fresh consumption to become an industrial high value crop as ingredient of juices, yogurt, flavoring and processed food 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. The objective of this work is to develop *in vitro* protocols that facilitate *(a)* the conservation of germplasm, *(b)* the multiplication and distribution of healthy elite clones selected by farmers, *(c)* the evaluation of *in vitro* propagated plants vs. sexual seeds propagated plants in farmer's fields and *d)* the genetic transformation of this crop. This year report summarizes the progress attained in the field evaluation of *in vitro* propagated elite clones selected by experienced farmers in two commercial regions of Colombia using participatory research approaches; with the aim of comparing in the field the performance of the *in vitro* plants with those conventionally propagated materials through seeds. The current report also describes enhancement of plant regeneration protocol suitable for high efficiency genetic transformation.

Materials and methods

Plant Evaluation by Farmers in the field. The *in vitro* propagated plants, from original elite clones selected by farmers in their fields, 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 were taken to the field experimental 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 were planted following a randomized split-plot design. Farmers from two commercial production areas of Colombia (Cauca and Huila) evaluated the plants performance from planting to fruit harvest. Each field-testing orchard was considered as a block and the global test had a split-plot design. Under each experimental site, the propagation systems (*in vitro* and seed) and the genotypes constitute the split-plots or sources of variation. This design allows farmers to compare the genotype and propagation system in a more objective form. The traits evaluated included plant survival upon transplanting in the field, days to flowering and fructification, as well as fruit productivity and quality.

Plant Regeneration. Experiments were performed using *in vitro* propagated plants. Shoots were obtained from plants of a clone selected in the experimental field; this particular clone has shown good response to plant regeneration (Ruiz *et al.*, in SB2 Annual Report 2003). Petioles were identified as the most responsive explant for plant regeneration as compared to leaves (Segovia, 2002). Petiole explants were cultured, without and with a sonication treatment for 1 minute, using a Branson 450 Ultrasonic Corporation apparatus, followed by vacuum at 500 mmHg for 15 minutes. Eight petioles explants were cultured per Petri dish. A total of 120 explants were sonicated and 120 were cultured without sonication per experiment. Four experiments were conducted. Explants were placed axial side down on solid regeneration media (Ultzen, *et al.*, 1995).

Results and discussion

With the participation of farmers a process was initiated to evaluate the advantages of using *in vitro* propagated plants as planting materials to establish new crops. The potential advantage of the *in vitro* source is the supply of pathogen-free, homogenous plants, maintaining the selected traits of the elite materials. During the first month, 100% of plant material (*in vitro* and seed) was grown successfully in 3 of 4 testing orchards at the farms. In this report, we have available information from two different locations in Pescador, Cauca that were selected to conduct this study (Ruiz *et al.*, in SB2 Annual Report 2004). According to the farmers, the *in vitro* generated plants showed higher vigor, earlier development and rooting respect to seed propagated plants. These observations were corroborated by the statistical analyses. Significant differences in plant development and performance were found between the *in vitro* and conventionally propagated plants. *In vitro* propagated plants initiated flowering and fructification earlier than seed-derived plants (Figure 1). Crop productivity was affected by water stress during the dry season period between July to October 2005, however, we have available production dates of 10 *in vitro* clones and 5 seed-derived clones (Figures 2 and 3). There are not statistically significant differences between production and number of fruit, per plant. It was also found that the *in vitro* clone JY-E1 showed the highest productivity. This clone was selected by farmers from Tierradentro-Cauca and then was evaluated in a different location (Pescador-Cauca). Farmers from Pescador-Cauca are very interested in having *in vitro* propagated material in their fields and they think that with the appropriated weather conditions, they will have a good development.

Earlier work by our group reported increased plant regeneration when only using sonicated petioles from clones with thorns in contrast to leaf segments. Field evaluation of regenerated plants indicated that lulo plant growth and development appears not to be affected by the organogenesis process (Segovia, 2002). Recent results indicate that it is possible to increase the efficiency without sonication. These results seem to indicate that the physiological condition of the donor plant has major influences on the explant response to plant regeneration. A high efficiency of plant regeneration was obtained when juvenile material was used increasing the response near to 80% without sonication (Figures 4 and 5). Not statistically significant differences in the number neither of explant response nor in the number of plants regenerated are seen with or without sonication. (Figure 6). Approximately 50% of explants regenerated after 4 weeks of culture, and the highest regeneration rate from petioles was observed at 6 weeks (Figure. 5). It was also found that petioles are the best explants. Results suggest that it is possible to use this protocol without sonication for genetic transformation avoiding the physical damage that sonication causes to the explants (Segovia, 2002).

Conclusions

A dedicated, enthusiastic and self-motivated group of farmers are very interested to grow *in vitro* propagated plants in their fields. The application of *in vitro* propagation technology reduced flowering and fructification time in about 1 month with respect to plants propagated through botanicals seeds used by farmers without affecting crop productivity and fruit quality. An optimum direct plant regeneration procedure via organogenesis using petiole explants without sonication was established. This protocol is suitable for the development of gene transfer technologies for this species, and is being considered on the future development of an *Agrobacterium*-mediated transformation protocol for lulo.

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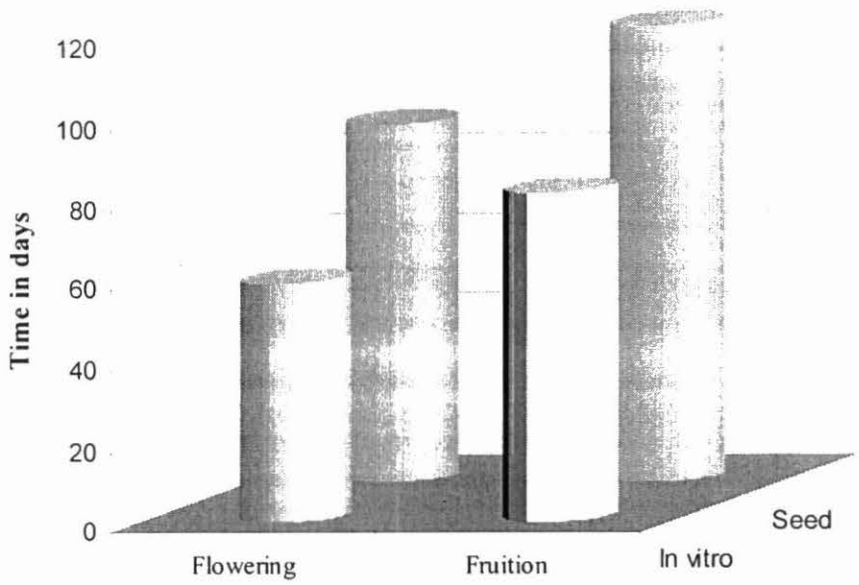

Figure 1. Days to flowering and fructification in the field (Pescador, Cauca) of plants propagated *in vitro* or from botanical seeds

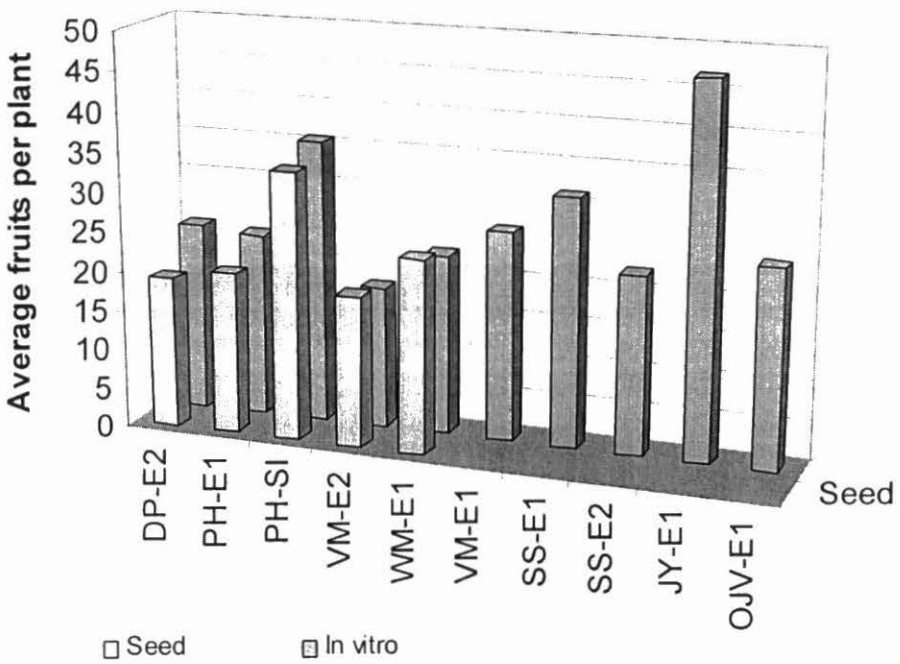

Figure 2. Average of number of fruits per plant from *in vitro* propagated plants and seed derived plants (Pescador, Cauca)

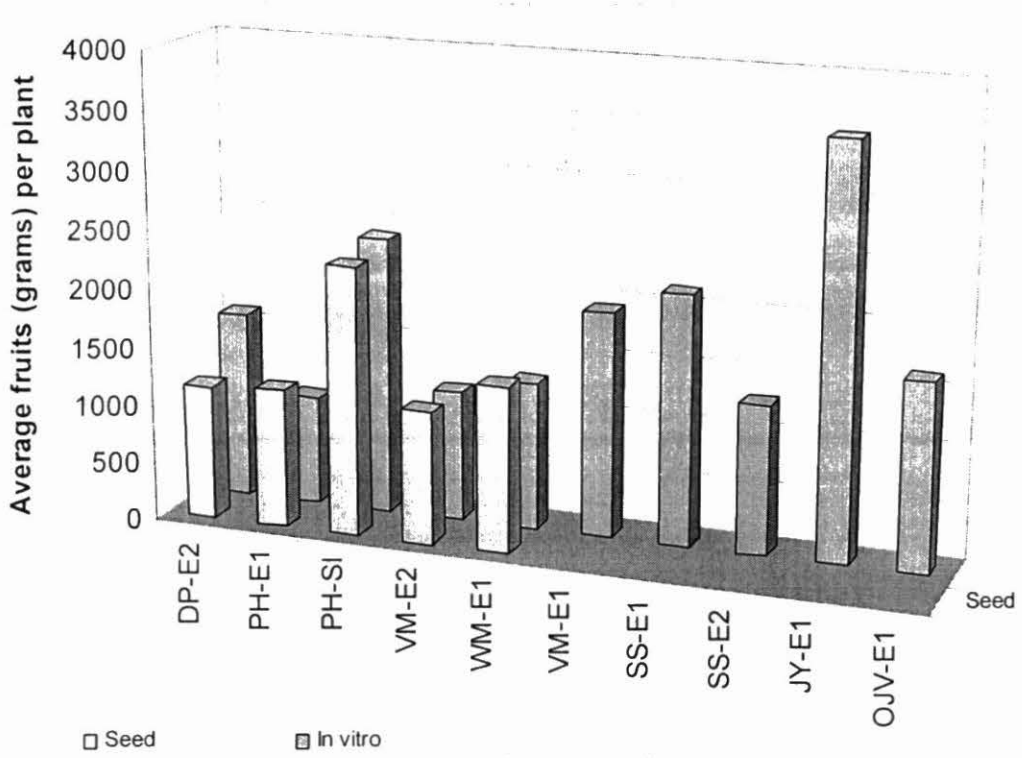

Figure 3. Average of production per plant from *in vitro* propagated plants and seed derived plants (Pescador, Cauca).

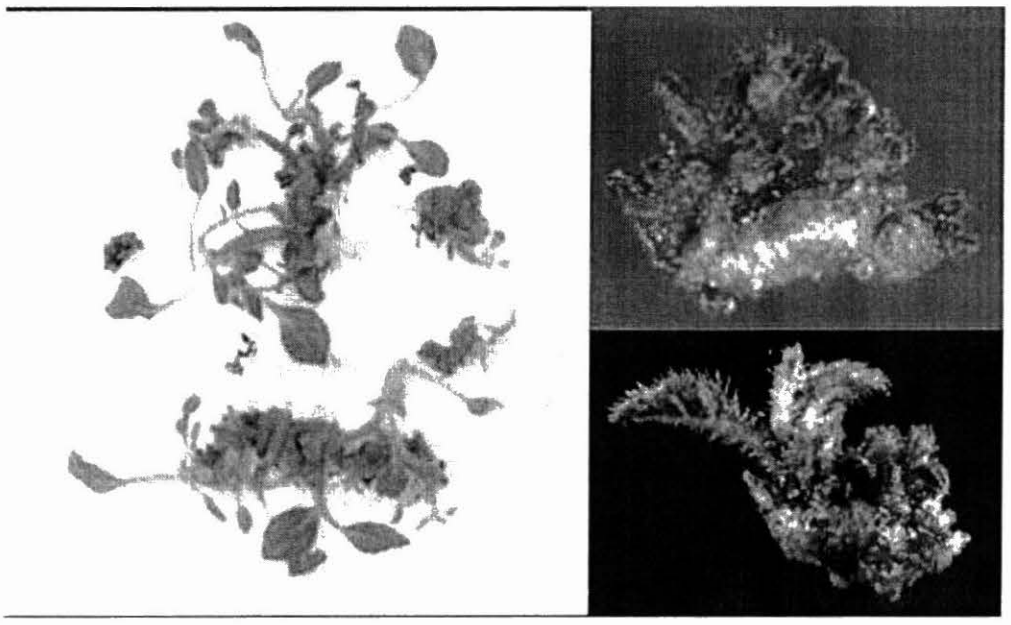

Figure 4. Plant regeneration from petioles without sonication. Multiple shoots formation throughout the explants.

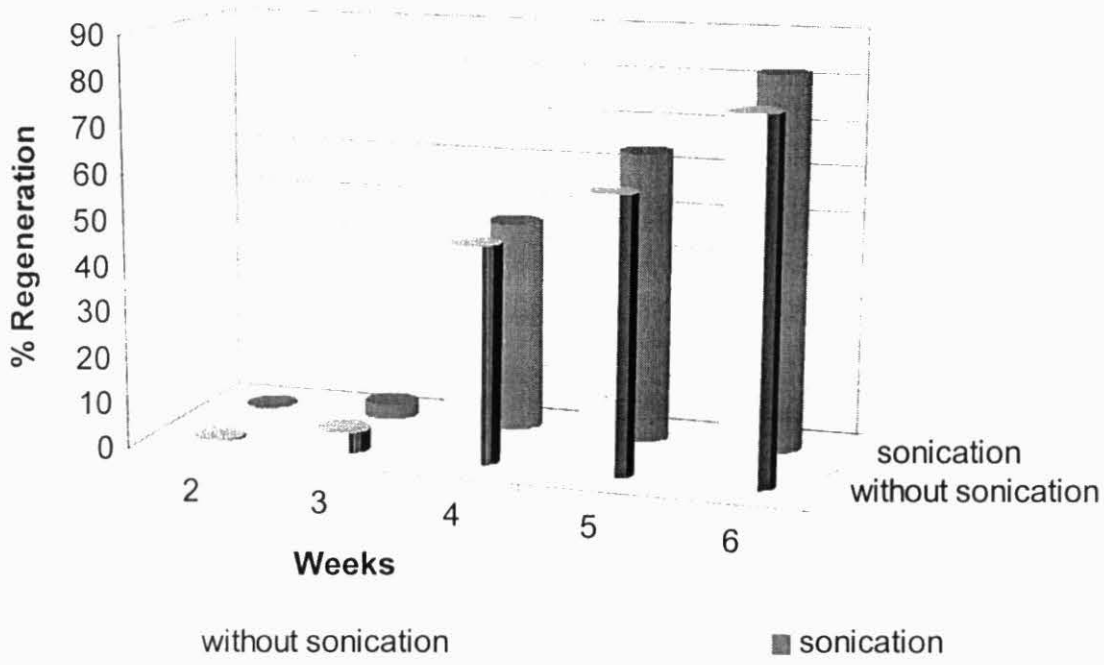

Figure 5. Plant regeneration from petioles of lulo (*Solanum quitoense*) with and without sonication

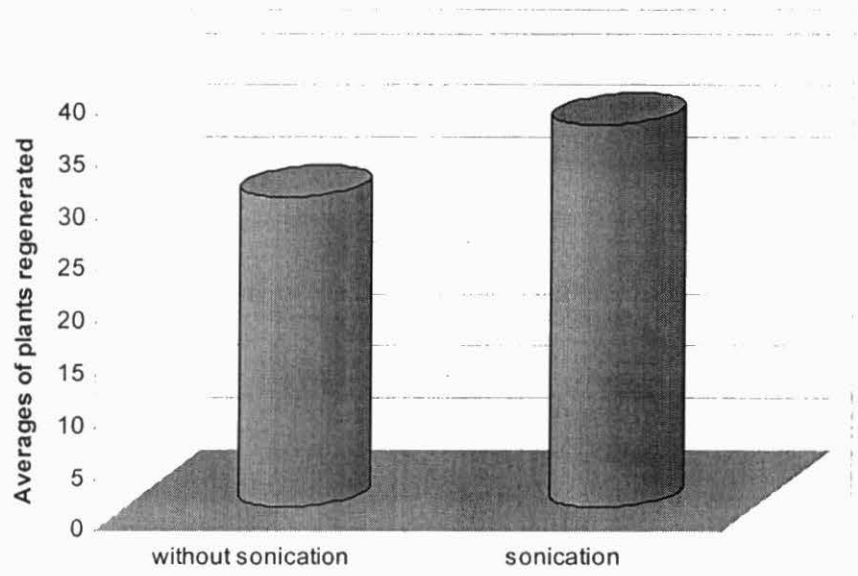

Figure 6. Plants regenerated from petioles of lulo (*Solanum quitoense*) with and without sonication

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 methodologies for the efficient production of transgenic common bean cultivars

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Agrobiodiversity and Biotechnology Project, SB-02

Introduction

In the past twelve years different agronomic important transgenes have been introduced to common bean cultivars through particle bombardment (for a review see Svetleva et al 2003). However, the fact that there is worldwide no transgenic marketed common bean cultivar or breeding line produced through this technology, may indicate that it is not ready for routine application. Low transformation efficiency, failure of transgene expression (Azzam *et al.* 1996), or transgene elimination (Romano et al. 2005) are among the constraints of the available methodologies for the production of transgenic common bean plants through particle bombardment.

Compared to particle bombardment, *Agrobacterium*-mediated transformation (*Agro*-transformation) offers different and unique advantages for transforming plant cells, such as the possibilities to: (i) transfer only one or few copies of DNA fragments carrying the genes of interest (ii) transfer of very large DNA fragments of a size as large as 150kb, (iii) produce transgenic plants with fragments of foreign DNA of the desired size, and (iv) produce more efficiently transgenic plants free of antibiotic marker genes.

Yet, also with *Agrobacterium* as vector the genetic transformation of common bean has been difficult. In the genus *Phaseolus* only the tepary bean (*P. acutifolius*) has been reproducibly *Agro*-transformed (Dillen et al. 1997; Mejía et al. 2000; De Clerck et al. 2003; Zambre et al. 2005).

Since traits of tepary bean have been introgressed stably into common bean cultivars, and breeding lines through interspecific crosses, the use of tepary bean accessions competent to *Agro*-transformation as bridge for the production of transgenic common bean cultivars has been proposed (Dillen et al 1997). However, this strategy has not produced common bean transgenic plants so far. The principal constraints of such strategy are two:

1. Most of the tepary and common bean accessions tested so far have been incompatible when crossing directly, producing lethal or self sterile F_1 hybrids (Waines et al.1988)
2. Only a relatively small proportion of the genome of tepary bean is transferred to common bean through recurrent or congruity backcrosses (an average of 5.2% in recurrent backcrosses and 8.8% in congruity backcrosses; Muñoz et al. 2004). This presupposes that a transgene should integrate into a tepary bean locus that can be introgressed into common bean.

Instead of using tepary bean accessions as target for the *Agro*-transformation we propose the development of interspecific common x tepary hybrids that are competent to *Agro*-transformation and cross-compatible with common bean cultivars, for using them as bridge to efficiently produce transgenic common bean plants. Once the transgenic hybrid is produced, two or more

backcrosses to a desired cultivar of common bean, would be enough for transferring the transgenes.
During 2005 we continued with our effort to develop such hybrid lines. We also made investigations on the optimization of the transformation protocol.

Methodology

Hybrid progenies involving the wild tepary bean accession NI576, which have been competent to *Agro*-transformation, and the common bean cultivar Bayo Madero (among others used as facilitators of the interspecific cross) were generated through double congruity backcrossing (http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf).
Whole mature seeds without one cotyledon or cotyledonar nodi were used as explants in the transformation experiments. The cointegrate strain of *Agrobacterium* LBA4404 pTOK233 (Hiei et al., 1994), and the binary strains C58C1 pTARC B1B (Dillen et al., 1997) and C58C1 pCambia1305.2 (http://www.cambia.org.au) were used for transformation, following the previously described protocol (Mejía-Jiménez et al., Annual Reports 2000 and 2001). In the second half of 2005 the transformation methodologies were modified after Olhoft et al. (2003).

Results

Attempts to transfer a transgene from an intraspecific tepary bean hybrid to common bean through sexual crosses
A transgenic plant obtained after *Agro*-transformation of intraspecific tepary bean hybrids (G40022 x NI576, event 23A; Mejía *et al.* 2000), which shows a strong expression of the GUS transgene, was used to test the possibility of transferring a transgene from tepary to common bean through sexual crosses.
The transgene could be easily transferred to other accessions of tepary bean or interspecific tepary x common hybrids with the cytoplasm of tepary bean. However all the attempts to transfer the same transgene to common bean cultivars or interspecific hybrids with the cytoplasm of common bean, failed. No fertile or cross-fertile hybrids carrying the transgene could be produced (Table 1). Even after hundreds of backcrosses to interspecific A-DCBC or V-DCBC hybrids aimed to break possible linkages between the transgene and interspecific incompatibility alleles, the production of cross fertile hybrids with the cytoplasm of common bean was not possible.
Thus this transgene might be linked to an interspecific incompatibility allele that prevents the production of fertile hybrids with common bean.
This example illustrates the difficulties that may arise by trying to introgress transgenes introduced into tepary bean accessions to common bean, as has been proposed (Dillen *et al.* 1997).
Only transgenes which integrate into chromosomal loci of tepary bean that are readily introgressed to common bean, will be efficiently introgressed to common bean through sexual crosses.
This supports our strategy of using interspecific common x tepary hybrids that are competent to *Agro*-transformation, instead of using tepary bean accessions themselves as bridge for the introgression of transgenes into common bean. The chance of insertion of a transgene in a locus that can be readily introgressed to common bean will increase proportionally to the amount of chromosomal segments of common bean present in the hybrid.

Table 1. Attempts to transfer a GUS transgene from an intraspecific hybrid of tepary bean (G40022 x NI576 event 23A) to other accessions of tepary or common bean, or to interspecific hybrids between both species. The production of fertile progeny carrying the transgene was only possible after crossing with accessions or hybrids with the cytoplasm of tepary bean.

FEMALE PARENT	TRANSGENIC GUS POSITVE MALE PARENT	RESULT OF THE CROSS
Common bean Cultivars	Tepary bean hybrid G40022 x NI576-23A	No viable or self-sterile hybrids
Tepary bean accessions	Tepary bean hybrid G40022 x NI576-23A	Fertile hybrids carrying the transgene
Interspecific tepary x common bean DCBC hybrids with tepary bean cytoplasm (A-DCBC)	Tepary bean hybrid G40022 x NI576-23A	Fertile hybrids carrying the transgene
Interspecific common x tepary bean CBC hybrids with common bean cytoplasm (CBC-7)	Tepary bean hybrid G40022 x NI576-23A	Viable but sterile hybrids carrying the transgene
Interspecific common x tepary bean DCBC hybrids with common bean cytoplasm (V-DCBC)	Interspecific tepary x common bean DCBC hybrids with tepary bean cytoplasm (A-DCBC)	Viable but sterile hybrids carrying the transgene

Optimization of the transformation protocol

In the last two years none of the *Agro*-transformation protocols developed for tepary bean could be successfully applied to common bean cultivars or common x tepary bean hybrids, for the production of transgenic plants. It is possible that the two plant species, which have quite different growth habitats in the wild, require also different growing conditions when cultured *in vitro* and that the conditions developed for the transformation of tepary bean are not the optimal for common bean.

The most critical step in the genetic transformation of grain legumes through the cotyledonar node approach seems to be wounding and inoculation of the pre-existing meristems in the explants (embryo axes or cotyledonar nodes). In soybean, the most investigated grain legume regarding genetic transformation, the transformation efficiencies could be increased through the use of thiol compounds during inoculation. It is believed that this occurs through the inhibition of enzymes involved in plant pathogen and wound response (Olhoft et al. 2003).

In the second half of 2005 we started a reevaluation of the different important parameters during inoculation and coculture with *Agrobacterium*. We tested the combined use of thiol compounds, reduced mineral salt concentrations, increased concentration of osmotic active substances, among other parameters during co-culture (Table 2). For measuring the gene transfer, the standard transient expression assay of the GUS-Intron transgene was used, three days after inoculation.

Compared with the methodologies used before, a clear increment of the transient GUS-expression is being achieved with the modified inoculation methodology (Table 2). Levels of 100% transient expression are routinely being obtained with selected interspecific common x tepary hybrid lines (Fig.1). It is still necessary to determine which one of the modifications made to the protocol is responsible for the increase of the transient GUS expression. It is expected that this high transient expression of the GUS-Intron gene results in increased transgenic plant recovery.

These experiments have not concluded and will be continued during 2006.

Table. 2 Comparison of the methodologies used for wounding and inoculation of mature embryos of *Phaseolus* bean before co-culture with binary *Agrobacterium* strains

	Previously used methodology*	Modified methodology
Wounding method	Sonication of the embryo axes	A cut along cotyledonary node and apical meristem
Basal salt solution	AB salts +1/4 MS salts	B5 salts 1/10 of the original concentration
Sugar-Osmotica:	Glucose 0.5%	3% mannitol+ 3% sucrose
MES	20 mM (3.9g/L)	20 mM (3.9g/L)
Thiol compounds**	-	L-cysteine 10mM ,dithiothreitol 1mM, and sodium thiosulfate 1mM**
Growth regulator	TDZ 25mg/l and NAA 0.5 mg/l	BAP 2 mg/l (8.8 µM)
pH	5.7	5.4

*Mejia et al. 2000
**Olhoft et al. 2003

Fig. 1. Transient expression of the GUS intron reporter gene in explants of advanced common x tepary DCBC hybrids with common bean cytoplasm after three days of coculture. The explants were inoculated with a binary Agrobacterium strain and with a modified inoculation methodology (Table 1) The dark blue spots, mostly in the hypo- and epicotyl of the half embryo explants correspond to the expression of the transfered GUS intron transgene

Production of fertile interspecific common x tepary bean hybrid lines using parental accessions which show competence to *Agro*-transformation, or good response to methods for *in vitro* culture and plant regeneration

Different accessions of tepary bean such as the wild genotype NI576 (Dillen et al. 1997; Clercq et al. 2002), the cultivated ones TB-1 and PI440795 (Zambre et al. 2005) and the intraspecific hybrid G40022 x NI576 (Mejia et al. 2000) have been *Agro*-transformed, while with similar or slightly modified methodologies, the *Agro*-transformation of wild and cultivated accessions of common bean has not been possible

In other crops such as rice and maize it has been demonstrated that the response to *in vitro* tissue culture methodologies for plant regeneration or genetic transformation is influenced by genetic traits. It would be therefore possible to improve the response of low responsive accessions of common bean to Agro-transformation through classical breeding.

In order to breed common bean genotypes which are responsive to the *Agro*-transformation, in the year 2000 we started interspecific crosses in which we included the tepary bean genotype NI576, as a putative source of competence genes for *Agro*-transformation. We also included tepary bean genotypes, G40022 and G40065, which were selected as the best in forming regenerable, meristematic callus (a trait that can be useful during selection of transformed tissues), and the best-identified genotype of common bean Bayo Madero, which also forms similar callus type. The development of fertile interspecific hybrids involving NI576 and Bayo Madero was possible only through a complex series of backcrosses we called Double Congruity Backcrosses (DCBC; see http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf). These crosses have yielded in the past years hundreds of fertile interspecific hybrid lines with the cytoplasms of common or tepary bean.

For accumulating alleles that may play an important role in competence to *Agro*-transformation, hybrid-lines, which show a good response to Agro-transformation methodologies, are selected and used again as parentals in the next DCBC.
During 2005, 3 generations of interspecific and 10 of intraspecific DCBC with the common bean cytoplasm, and 3 generations of interspecific and 9 of intraspecific DCBC hybrids with the tepary bean cytoplasm succeeded and more than 500 fertile hybrid lines were produced (See annual report on interspecific crosses). The number of crosses performed and the embryos rescued have not been yet evaluated to calculate and compare hybridization efficiencies among the different DCBC generations.
It is expected that the level of recombination between the genomes of common and tepary beans increases with every new full cycle of DCBCs.
The fertile populations produced during 2005 are being multiplied and will be screened during 2006 with the improved inoculation methodology.

In planta inoculation of *Agrobacterium*, a novel transformation methodology for common bean?
At the end of 2005 an *Agrobacterium* mediated transformation protocol for common bean was published (Liu et al. 2005). This protocol uses no *in vitro* tissue culture steps for producing transgenic plants at high efficiencies (approx. 6% of the treated explants in the best treatments). Seedlings of commercial cultivars were treated with sonication and vacuum during co-culture with the binary *Agrobacterium* strain LBA4404 and then planted in the soil. No chemical selection was applied. Putative transgenic plants were identified through PCR reaction and confirmed through Southern blot of the T2 progeny.
We will test during 2006 the reproducibility of this methodology.

Conclusions

Current *Agro*-transformation methodologies developed for tepary bean have not been effective for the production of transgenic common bean cultivars.
The use of interspecific common x tepary interspecific hybrids competent to *Agro*-transformation, and cross compatible with common bean cultivars, in combination with improved transformation methodologies may represent an alternative for the production of transgenic common bean cultivars.

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2.2.2 Embryo rescue of gene tagging and MAS breeding populations

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Funding: CIAT

Important Outputs

1. A total of 7,222 sexual seeds made up of mapping and MAS breeding populations were established from embryo axes of which 2,780 have been multiplied and 5785 plants were transferred to the screen house and subsequently to the field.

Introduction

Embryo rescue of matured sexual seeds have become a key technique for the establishment of MAS breeding populations *in vitro* for ease of distribution, and for the safe keeping and sharing of gene tagging populations. As the MAS for breeding CMD resistance project gathers speed and additional gene tagging projects are added the volume of embryo axes culture has also grown and become key activity of the cassava tissue culture facility. A new growth room facility to handle the new materials was added this year. We describe below activities on the establishment of gene tagging and breeding populations in vitro this year.

Methodology

Embryo rescue of 7222 mature seeds of cassava from F_1, BC_1, and S_1 populations corresponding to 50 families was carried out this year following a protocol developed earlier at CIAT (CIAT 2003). The seeds represented either gene tagging populations for high protein, QTLs controlling traits that differ between cassava and wild relatives (wild QTLs), delayed post-harvest physiological deterioration (PPD), resistance to hornworm, or breeding populations for MAS of CMD resistance. Plants recovered were immediately multiplied to obtain between 6 and 8 plantlets for the gene tagging populations or subjected to molecular analysis with markers closely linked to CMD resistance before multiplication for the MAS breeding populations. After multiplication, plants belonging to gene tagging populations were sent to the screen house for hardening, while those of the MAS populations were shipped to partners in Africa and Brazil, a small sub-set was also transferred to the screen house for hardening and subsequent evaluation here at CIAT.

Hardening of *in vitro* plantlets in screen house followed a slightly modified protocol this year. A soil:sand mixture of 2:1 was used rather than the traditional 3:1 mixture to provide for stronger root system of the plantlets. A stronger fungicide solution of Banrot, 1 gr/l was applied to the seeding the day of transfer to the soil. Fertilization using a solution 1.5 g/l of Plantex (NPK 10:52:10) remained the same. On some occasions it was necessary to make applications against some fungi such as *Oidium* or against mites using Elosall (fungicide and acaricide) at a concentration of 0.5 ml/l or Vertimec at a concentration of 1 ml/l (acaricide). After 60 to 90 days in the screen house and plants now had well lignified stems, they were placed outside the screen house in the shade for 1-2 weeks and then planted in the field that had previously been irrigated. Plantex an a organic fertilizer (Teravite) was applied to plants in the field on a weekly basis for the first month. The above modifications led to very high recovery of hardened plants in the screen house (>85%) and of plants established in the fields (90%).

Results

A total of 7222 seeds harvested from controlled crosses made up of mapping and MAS breeding populations were established *in vitro* from embryo axes. As at the time of submission of this report, 2780 genotypes have formed plants and have been multiplied for transfer to the screen houses or molecular analysis. A total of 2954 seeds representing crosses for mapping of high content of protein from wild relatives were planted of which 1577 genotypes were obtained, a plant recovery of 71% (Table 1). From these mapping populations, 225 each genotypes were chosen from the B1P2, B1P5 and B1P6 families and micro-propagated and a total of 5985 plants transferred to the screen house for hardening, These plants have now been hardened and transferred to the field.

Table 1. Summary of the establishment of mapping populations for high protein content from embryo axes

CODE	MOTHER	FATHER	Delivered No. Seeds	No. Sem. Shallow/Perd.	Planted No. Seeds	Formed No. Plants	% Formation of Plants
B1P1	CW178-2	SM1219-9	204	5	199	166	83%
B1P2	CW198-11	MTAI 8	654	176	478	328	69%
B1P3	CW 205-7	MTAI 8	132	59	73	68	93%
B1P4	CW187-222	MTAI 8	374	157	217	167	77%
B1P5	CW205-2	MTAI 8	508	122	386	348	90%
B1P6	CW201-2	MTAI 8	659	142	517	437	85%
B1P7	CW 208-183	MTAI 8	75	36	39	19	49%
B1P8	CW 208-183	OW 280-1	348	47	301	44	15%
TOTAL			2954	744	2210	1577	71%

Additional large crosses processed this year were several S_1 families derived from inter-specific hybrids between cassava and its wild progenitor for genetic mapping of QTLs that control traits which differentiate cassava from its wild progenitor (Table 2). Others include mapping populations for delayed post-harvest deterioration (CW429-1) and high cassava protein crosses. Of 1,773seeds planted only 678 plants were obtained, a plant recovery of 39%, this low percentage of formation of plants could be due to the fact that selfing F_1s from parents of wild origin may release a large genetic load that lead to severe inbreeding depression.

Conclusion and perspectives

A total of 7222 sexual seeds of cassava were established from embryo axes this year and 2780 of these have been multiplied and plantlets transferred to the screen house. There was obtained a low percentage of formation of plants for some populations, this could be due to the fact that there was many parental of wild origin. Perspectives include transfer of all micropropagated materials to screen house and plants in the screen house to the field. Additional seeds pending for rescue of embryos will also be established in vitro.

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Table 2. Summary of the establishment of gene tagging populations for wild QTLs, delayed post-harvest deterioration, and resistance to hornworm from embryo axes

CODE	MOTHER	FATHER	Delivered No. Seeds	No. Sem. Shallow/Perd.	Planted No. Seeds	Formed No. Plants	% Formation of Plants
CW188-1	CW 188-1	CW 188-1	514	89	425	212	50%
CW188-12	CW 188-12	CW 188-12	545	144	401	138	34%
CW188-14	CW 188-14	CW 188-14	369	213	156	59	38%
CW188-18	CW 188-18	CW 188-18	306	45	261	53	20%
CW188-9	CW 188-9	CW 188-9	73	30	43	19	44%
CW184-4	CW 184-4	CW 184-4	94	44	50	15	30%
B1PD274	MGUA 79	TAI 8	17	7	10	6	60%
B1PD275	MGUA 76	TAI 8	11	11	0	0	0%
B1PD276	TAI 8	COL 678	57	12	45	0	0%
B1PD277	SM 673-1	COL 678	3	1	2	1	50%
B1PD278A	TAI 8	SM 673-1	34	5	29	4	14%
B1PD278B	SM 673-1	TAI 8	76	18	58	9	16%
B1PD279	TAI 8	SM 1406-1	21	7	14	12	86%
B1PD280	OPEN POLLINATED	CW 429-1	82	21	61	28	46%
B1PD281	C-19	CW 429-1	16	1	15	0	0%
B1PD282	C-4	CW 429-1	12	12	0	0	0%
B1PD283	C-39	CW 429-1	6	2	4	1	25%
B1PD284A	TAI 8	CW 429-1	88	21	67	10	15%
B1PD284B	CW 429-1	TAI 8	20	0	20	20	100%
B1PD285	C-4	SM 678	17	2	15	15	100%
B1PD286	TAI 8	AM320-145	6	6	0	0	0%
B1PD287	C-4	AM320-145	14	4	10	8	80%
B1PD288	C-4	SM 1406-1	6	5	1	1	100%
B1PD289	CW 429-1	SM909-25	103	28	75	60	80%
B1PD290	CW 429-1	AM320-145	6	3	3	2	67%
S1CH	NGA 11	NGA 11	11	5	6	3	50%
S1CW	CW 429-1	CW 429-1	2	0	2	2	100%
TOTAL			2509	736	1773	678	39%

2.2.3. Sharing results of 30 years of cassava breeding: shipments of improved germplasm to Africa, Asia and Latin America

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Funding: CIAT

Important Outputs

Shipment of 316 genotypes consisting of CIAT improved varieties and advanced breeding lines to countries in Africa, Asia and Latin America
Transfer of 27 accessions of wild *Manihot* species from *in vitro* to the screen house for evaluation for pest and disease resistance

Introduction

The cassava tissue culture facility was set up to facilitate easy and rapid sharing of improved varieties and advanced breeding lines from the genetic resources unit (GRU) and the cassava project to collaborators in Africa, Asia, and Latin America. This year, CMD resistant lines bred via MAS at CIAT and improved varieties were shipped to 9 countries. A member of the tissue culture laboratory also visited National collaborators in Nicaragua and Mexico to train their staff in rapid tissue culture multiplication, and screen house hardening of tissue culture plants. We describe below germplasm shipment and training activities undertaken by the cassava tissue culture laboratory this year.

Methodology

After request for germplams is received, improved varieties or advanced breeding lines held by GRU or the cassava tissue culture facility are micro-propagated to obtain the number of plantlets per genotype requested. Micro-propagation using meristems or axillary nodes is as described by Roca et al. (1984) in17N (only apexes) for screen house hardening or 4E for conservation or screen house hardening. Following a request by the GCP project working on the identification and pyramiding of useful genes for pest and disease resistance from wild relatives a total of 27 accessions representing 8 *Manihot* species were requested for from GRU multiplied and transferred to the screen house for hardening and eventual establishment in the field for pest and disease evaluations. Hardening of the wild accessions was as described earlier (This report, Activity 12.27)

Results

A total of 316 genotypes consisting of CIAT improved varieties and advanced breeding lines were shipped to 4 African countries, 1 Asian, 3 Latin America, and Austria. A list of the countries, shipments, and genotypes are shown in Table 1 and Table 2 Between 6 and 10 plantlets per genotype were shipped

Table 1. List of shipments and destination of improved varieties and advanced breeding lines shipped from the cassava tissue culture facility between Nov 2004 and October 2005

COUNTRY	DATE	FAMILY	No. GENOTYPES
Nigeria	November 25/04	Material Elite	38
Suriname	December 06/04	Material Elite	5
Mexico	December 10/04	Material Elite	10
China	December 23/04	Material Elite	1
South Africa	May 24/05	Material Elite, CR, and AR	32
South Africa	September 12/05	Material Elite, CR, and AR	35
Austria	September 20/05	TME -3	1
South Africa	September 23/05	Material Elite, CR, and AR	35
South Africa	September 30 /05	CR	24
Kenya	October 13/05	Material Elite, CR, and AR	48
Brazil	October 14/05	Material BRA, COL, Elite	39
Ethiopia	October 21/05	Material Elite, CR, and AR	48

Twenty accessions of 8 wild *Manihot* species received from the GRU were multiplied *in vitro* to give between 2 and 15 plantlets per genotype, the large variation in the *in vitro* growth rates of the wild accessions explains the widely differing number of plantlets per genotype. A total of 155 plants representing 27 accessions were planted in the screen house and out of this 106 plants or 68% could be successfully hardened (Table 3).

Other activities conducted this year by the cassava tissue culture facility include multiplication of *in vitro* material from the Bank of Germplasm for cassava breeding (Nelson Morante, Teresa Sánchez, Fernando Calle, and Juan Carlos Pérez), multiplication of elite varieties requested by CLAYUCA for shipments to other countries, and multiplication of 146 genotypes of the cassava genetic map population (CM 7857). Drought tolerant and susceptible genotypes to be evaluated in the generation challenge program were also multiplied as transferred to the screen house for hardening and transfer to the field (Fig 4). These materials will be evaluated for parameters of drought tolerance in the generation challenge program project on genetic mapping of the trait.

Table 2. Improved varieties for the sub-tropics and highlands and CMD resistant genotypes sent to South Africa, Kenya, and Ethiopia

ITEM	Genotype	Delivered No. Plants	Planted No. Plants	No. Live Plants	Percentage of Survivors (%)
1	BRA895	5	5	4	80%
2	MCOL 2261	11	11	11	100%
3	SM 998-3	6	6	6	100%
4	MTAI16	5	5	5	100%
5	SM1433-4	5	5	5	100%
6	CM523-7	5	5	5	100%
7	CR25-4	5	5	5	100%
8	CR27-20	5	5	4	80%
9	CR43-3	5	5	5	100%
10	CR43-12	6	6	6	100%
11	CR43-13	5	5	3	60%
12	CR45-10	5	5	3	60%
13	AR9-18	6	6	6	100%
14	AR14-3	5	5	5	100%
15	AR15-1	5	5	4	80%
16	AR15-5	5	5	4	80%
17	AR17-25	10	10	9	90%
18	AR30-3	5	5	3	60%
19	AR32-3	5	5	3	60%
20	AR37-1	5	5	2	40%
21	AR37-38	5	5	5	100%
22	AR37-89	6	6	6	100%
23	AR37-99	5	5	1	20%
24	AR40-3	10	10	10	100%
25	AR40-15	5	5	4	80%
26	AR42-3	5	5	4	80%
27	AR42-4	5	5	5	100%
TOTAL		155	155	133	86%

Renewal of genotypes being conserved *in vitro*, including genotypes of the CR and AR families (resistance to CMD), the mapping population for cyanogenic potential and dry matter content (AM320), the core collection and a group of 38 elite varieties, 60444 (the model cassava regeneration genotype being used to produce FECs for genetic transformation), TME-3 (source of CMD resistance), and a number of other materials including high protein content varieties and 33 wild *Manihot* genotypes were also carried out by the cassava tissue culture facility. The above materials were renewed during the year.

Table 3. Accessions of Wild *Manihot* sent to the screen house for hardening

ITEM	GENOTYPE	No. Plants multiplied	No. Plants planted in the screen house	No. of surviving Plants	Percentage of Recovery (%)
1	ALT-4	5	5	5	100%
2	BLO-1	4	4	4	100%
3	CHL-10	5	5	5	100%
4	CTH-96	1	1	1	100%
5	CTH-113	4	4	1	25%
6	CTH-115	2	2	1	50%
7	CTH-326	3	3	1	33%
8	FLA-15	11	11	4	36%
9	FLA-19	15	15	15	100%
10	FLA-21	6	6	4	67%
11	FLA-25	10	10	7	70%
12	FLA-33	5	5	5	100%
13	FLA-52	6	6	4	67%
14	FLA-57	2	2	1	50%
15	FLA-61	17	17	13	76%
16	FLA-75	11	11	8	73%
17	FLA-80	7	7	5	71%
18	FMT-2	4	4	2	50%
19	FMT-3	5	5	1	20%
20	FMT-5	3	3	2	67%
21	FMT-6	3	3	1	33%
22	PIL-1	4	4	2	50%
23	TST-3	3	3	1	33%
24	TST-18	5	5	5	100%
25	TST-26	5	5	4	80%
26	TST-61	4	4	3	75%
27	SAW-1	5	5	1	20%
TOTAL		155	155	106	68%

Table 4. Drought tolerant and susceptible genotypes sent to the screen house for hardening, establishment in the field and evaluation of parameters of drought tolerance

ITEM	GENOTYPE	Delivered No. Plants	Planted No. Plants	No. Live Plants	Percentage of Survivors (%)
1	BRA 114	20	19	14	74%
2	BRA 116	5	5	5	100%
3	BRA 134	15	15	10	67%
4	BRA 179	10	10	10	100%
5	BRA 200	40	38	23	61%
6	BRA 201	31	26	18	69%
7	BRA 209	16	16	13	81%
8	BRA 216	26	26	18	69%
9	BRA 249	31	31	20	65%
10	BRA 253	41	33	15	45%
11	BRA 255	29	28	24	86%
12	BRA 264	21	18	13	72%
13	BRA 293	35	26	16	62%
14	BRA 346	20	18	17	94%
15	BRA 534	8	7	7	100%
16	BRA 835	23	23	23	100%
17	BRA 846	51	46	25	54%
18	BRA 849	25	24	11	46%
19	BRA 878	34	34	20	59%
20	BRA 879	49	44	24	55%
21	BRA 886	26	26	23	88%
22	BRA 969	39	36	30	83%
23	BRA 974	13	9	9	100%
24	BRA 997	9	9	9	100%
25	BRA 1142	15	15	15	100%
26	BRA 1204	33	32	26	81%
27	BRA 1342	30	27	20	74%
28	BRA 1346	26	25	21	84%
29	BRA 1394	37	35	31	89%
30	BRA 1400	5	4	4	100%
31	TAI 8	36	29	20	69%
32	TAI 16	34	33	22	67%
33	CM 3306-9	15	14	14	100%
34	CM3306-9	15	14	14	100%
35	SM1438-2	9	9	9	100%
36	COL 2215	15	9	9	100%
37	COL 949	3	2	2	100%
38	COL 948D	3	3	3	100%
39	COL 1734	10	10	10	100%
40	COL 1725	3	3	3	100%
41	COL 1719	3	3	3	100%
42	COL 1468	10	10	10	100%
43	VEN 77	3	3	3	100%
44	COL 2066	3	2	2	100%
45	COL 1522	13	10	10	100%

46	AM313-1	5	5	1	20%
47	AM313-3	5	5	3	60%
48	AM313-4	5	5	1	20%
49	AM313-5	5	5	1	20%
50	AM313-6	3	3	3	100%
51	AM313-7	6	6	1	17%
52	AM313-8	5	5	5	100%
53	AM313-10	5	5	4	80%
54	AM313-12	4	4	2	50%
55	AM313-17	3	3	3	100%
56	CWR271-1	16	14	12	86%
57	WC1-1	19	16	16	100%
58	WC1-2	12	12	12	100%
59	WC2	5	5	5	100%
TOTAL		1036	952	717	75%

Training

Another principal activity of the tissue laboratory is training, this year the following training activities were carried out:
Oral presentations and hands-on laboratory and greenhouse training for breeders attending the "Advanced cassava breeding Course for NARs breeders from Brazil and Africa."

Training of Ms Sandy Lorena Molina from Venezuela in rapid multiplication.
Adriana Alzate of the tissue culture laboratory visited NARs collaborators in Nicaragua and Mexico to help train technicians and trouble shoot problems being experienced.

Training of Ms Tatiana Ovalle, intern of the University of Tolima, Colombia.
The above training focused on the following points:

Proper management of equipment, implement, and safety cabinet in a tissue culture lab.
Precautions that should be taken into account upon entering the tissue culture work area and growth rooms.

Importance of keeping an aseptic environment at all times
Multiplication spread of *in vitro* material, embryo rescue, transfer of screen house plant *in vitro.*

Preparation of tissue culture media and stock solutions.
Handling massive micro-propagation and flow chart of the process
Safe practices in a tissue culture laboratory
Screen house hardening of *in vitro* plants.
References and support material for tissue culture manipulation of plants

Conclusion and perspectives

The cassava tissue culture laboratory has continued to provide invaluable services of micro-propagation and shipment of elite materials to partners, propagation of germplasm required by CIAT projects as well as conservation of a large group of materials being held for cassava breeding and genetics. Members of the laboratory have also trained a large number of people this

year. Future perspectives include micro-propagation and distribution of elite material, conservation of useful germplasm, and screen house hardening of *in vitro* materials.

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2.2.4 Cleaning of materials from offspring CM-8996 (population for whitefly resistance) affected by frog skin disease (FSD) using thermotherapy and *in vitro* cultivation

A. Bohorquez, B. Arias, A. Bellotti, and J. Tohme

Introduction

Whiteflies, especially in the Neotropics, cause direct damage to cassava by feeding on the phloem of the leaves. The most important species causing direct feeding damage in the Northern region of South America, is *Aleurotrachelus socialis*. In Colombia cassava field yield losses as high as 79% are reported caused by *A. socialis* (Bellotti, 2002).

The CIAT cassava and IPDM project (IP-3 and PE-1) have placed a special emphasis on our ongoing efforts to develop whitefly resistant cultivars in cassava. More than 5,000 cassava genotypes have been evaluated systematically (Bellotti and Arias, 2001) and have identified resistant genotypes and through a comprehensive breeding scheme, develop commercial hybrids containing whitefly resistance. The resulting progeny, approximately 700 genotypes, are being systematically evaluated at the two sites with the objective being to study the genetics and inheritance of whitefly (*A. socialis*) resistance in cassava and root yield data was recorded at harvest by harvesting the central three plants of each genotype/row. Evaluations for whitefly populations and damage levels were carried out during the course of the crop cycle at Santander de Quilichao (Cauca) and at CORPOICA Nataima, El Espinal (Tolima) during the years 2002-2005.

The results of these evaluations indicate the possible yield potential of some of the genotypes and the production capacity of this family (CM 8996), yielded until 50 T/ha. These results further indicate that it is possible to combine good whitefly resistance with high yield and excellent commercial qualities. However, during the last harvest season (2005) the occurrence of Frog Skin Disease (FSD) was detected in both locations, this disease is a serious phytosanitary problem that affects cassava production in Colombia. The causes of FSD are still uncertain and diseased cassava plants do not produce marketable roots. Propagation by stems cuttings often exposes the cassava crop to diseases like a FSD. Due to the importance of these materials, it is necessary to recovery of healthy clones from diseased varieties by meristem and shoot tip culture methods. The plants were harvested and in process of certification as FSD free, using a tissue culture multiplication scheme, which allow us to certify to quality of material each cycle and support seed releases or renewing materials (Roca, 1984)

Methodology

The disease materials collected in the field, at Santander and Nataima were planted in greenhouse and in laboratory at CIAT for seed cleaning using the protocol according to Roca (1984). The protocol used for this cleaning was the following:

1-Stakes were planted in pots containing sterilized soil and place them in a growth chamber at a temperature of 38-40°C (thermotherapy) during three weeks.
2- Extraction of meristems and *in vitro* growth.
3- Shoot tip culture and *in vitro* thermotherapy (30°C, 2 cycles).
4- Induction of roots and transfer to greenhouse conditions.
5- Grafting with a indicated clone (clone "Secundina") in greenhouse and PCR tests.
6- If the above mentioned tests are negatives (FSD-free) then propagation of healthy plants certified FDS-free. If tests are positives, repeat since step 2.

Results

The main results for this work were:

93 collected clones (several stakes per clone) planted and it put under thermotherapy, all survivor to treatment.

190 meristems extracted (4E cultivation medium) (Fig. 1).

80% of these meristems grown.

124 shoot tips culture and *in vitro* thermotherapy (first cycle).

86 total clones recovered using *in vitro* propagation system (Table 1).

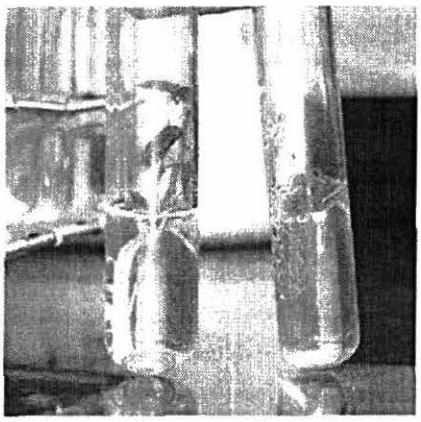

Figure 1. Detail of two meristems recovered from diseased materials of family CM8996, growth on 4E cultivation medium.

Table 1. List of clones affected with FSD and # of meristems recovered of the family CM 8996 collected at two sites during 2005.

Clone	# Meristems recovered	Clone	# Meristems recovered	Clone	# Meristems recovered
CM 8996-1	2	CM 8996-157	2	CM 8996-289	2
CM 8996-5	2	CM 8996-160	2	CM 8996-290	1
CM 8996-10	2	CM 8996-165	2	CM 8996-322	2
CM 8996-15	1	CM 8996-166	1	CM 8996-324	0
CM 8996-19	2	CM 8996-167	2	CM 8996-326	2
CM 8996-20	2	CM 8996-175	2	CM 8996-353	2
CM 8996-37	2	CM 8996-181	2	CM 8996-359	1
CM 8996-42	2	CM 8996-184	2	CM 8996-400	2
CM 8996-46	3	CM 8996-189	1	CM 8996-442	2
CM 8996-47	2	CM 8996-193	2	CM 8996-451	2
CM 8996-54	2	CM 8996-198	2	CM 8996-464	2
CM 8996-60	2	CM 8996-199	2	CM 8996-471	1
CM 8996-71	2	CM 8996-206	2	CM 8996-472	1
CM 8996-73	2	CM 8996-208	2	CM 8996-477	1
CM 8996-76	1	CM 8996-211	2	CM 8996-479	2
CM 8996-80	2	CM 8996-215	2	CM 8996-481	1
CM 8996-85	4	CM 8996-223	1	CM 8996-493	1
CM 8996-88	2	CM 8996-229	2	CM 8996-520	1
CM 8996-93	1	CM 8996-230	1	CM 8996-537	1
CM 8996-97	1	CM 8996-240	2	CM 8996-554	2
CM 8996-99	1	CM 8996-243	2	CM 8996-581	3
CM 8996-107	2	CM 8996-255	2	CM 8996-592	2
CM 8996-121	2	CM 8996-251	1	CM 8996-596	2
CM 8996-122	2	CM 8996-263	2	CM 8996-602	2
CM 8996-134	1	CM 8996-269	2	CM 8996-635	2
CM 8996-136	1	CM 8996-280	2	CM 8996-662	2
CM 8996-149	2	CM 8996-281	4	CM 8996-668	2
CM 8996-152	4	CM 8996-282	1	CM 8996-671	2
CM 8996-153	2	CM 8996-284	2	CM 8996-757	2

Ongoing activities

- Second cycle of *in vitro* thermotherapy.
- Induction of roots (17N cultivation medium) and transfer to greenhouse conditions.
- Grafting and PCR tests.
- Propagation of certified plants FDS-free

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2.2.5 Isolation and Expression of Cassava Root-specific ESTs - (Expressed Sequence Tags)

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Introduction

The progress on cassava transformation has been outstanding during the last decade (Taylor et al, 2004). Unfortunately, the lack of tissue-specific promoters has delayed the research towards pest resistance and increasing the nutritional content (carotenoid, protein, starch). Relatively little research has been done in order to characterize the differential expression of the most economically important organ of this plant, the root (Zhang, et al, 2003, Carvalho, et al, 2002). By characterizing the expression of the specific root genes, we will be able to isolate useful specific promoters to target gene expression directly to the root.

Objectives:

- Isolate and characterize root-specific ESTs from cassava.
- Perform an expression analysis of selected ESTs by Quantitative PCR (qPCR).

Materials and Methods

In order to isolate genes of high and restricted expression in the root, we acquired a significant genetic pool under the different conditions. Plant material was collected from five different varieties (SM1219-9, MCOL2215, MPER183, MTAI8, CM523-7) under stress and unstressed conditions. The samples were mixed into two pools: aerial parts, and root and treated as described before (Sarmiento, 2005).

To obtain the differentially expressed root ESTs we used the PCR-Select cDNA Subtraction kit (Clontech™, 2001). The clones were successfully inserted into bacteria to create a root-specific EST library. 132 Clones were sequenced, leading to 61 singletons and 16 contigs. A general pipeline was developed and used for the bioinformatic analysis (Sarmiento, 2005). VectorNTI 9.0.0 suite (Informax, 2003) and the BLAST algorithm (Altschul *et al*, 1990) against the NCBI database (Wheeler *et al,* 2004) and a cassava EST database (Verdier *et al*, 2004) were used to select putative root-specific coding sequences. These sequences were further analyzed using the Interpro database (Kulikova *et* al, 2004) and the Aracyc tool (Rhee *et al,* 2004) to identify gene families and relate each sequence to a metabolic process.

Parallel to the construction of the subtractive library, a database search was done for genes reported as root-specific. The sequences were taken from the NCBI database and compared by BLAST (Altschul *et al*, 1990) against the cassava EST database (Verdier *et al*, 2004). The sequences with high similarity to root sequences were selected for the bioinformatic analysis previously described (Sarmiento, 2005)

Preliminary Expression Analysis

Real Time PCR was used to observe relative abundance of each transcript in cassava leaves, stems and roots. Material from four varieties (CM 3306-4, CM 2772-3, SM 1219-9, MPER 183) was collected and divided in two sets. Total RNA was extracted from root tissue using TRIzol reagent (Invitrogen®) under manufacturer specifications with some modifications, and the kit SV total RNA Isolation (Promega®) was used for RNA isolation from leaves and stems. Two different extractions from pooled material were done and each repetition was organized in each set. Residual DNA was eliminated using Deoxyribonuclease I (Invitrogen). Total RNA was reverse transcripted into first strand cDNA using the Superscript III reverse transcriptase (Invitrogen).

A relative quantification methodology, according to ABI Prism user bulletin #2 (Livak, 2001), was undertaken by using Brilliant® SYBR® Green QPCR Master Mix (Stratagene La Jolla, Ca 92037) in 20 ul reactions. 100 ng of template has been used for each PCR experiment, and 18S was used as an endogenous control for product amplification.

Results and Discussion

Subtractive Library

130 clones have been analyzed from a 400-clone library. The sequences obtained were examined by a bioinformatic workflow based on similarity searches (Sarmiento, 2005). Six sequences were selected as possible root-specific (Table 1)

Table 1. Results from root-specific sequence search

Clone	Result	Functional Group
F12	putative ADP-glucose pyrophosforylase large subunit	Sugar biosynthesis/metabolism
D3	putative Galactinol synthase	Sugar biosynthesis/metabolism
G2	putative Annexin I	Cellular cycle
H7	Putative 26S proteasome regulatory subunit (RPN12)	Cellular cycle / protein metabolism
B1	putative Alanine aminotransferase	Amino acid biosynthesis/metabolism
F2	Putative allene oxide synthase (AOS)	Signaling, defense

Three sequences were selected from the database search. These sequences are shown in the table below (Table 2)

Table 2. Blastn (Altschul *et al* 1990) results against cassava database from the selected orthologous genes (López *et al*. 2004).

Gene	NCBI accession	Result	Tissue	E-value	Score
Glycine max, similar to extensin	AF520576	cn366	Root	3.00E-07	28
P. banksiana, root specific S-adenosyl methionine synthetase	U38186	c.09.I7.5	Root	4E-61	234
N. tabacum, mRNA for root-specific gene (possible porin)	X54855	m.12.F16.5	Root	3E-14	8

Real Time PCR Analyses

Real Time analyses were done to selected sequences after initial RT-PCR results (data not shown). Two sequences were selected for these analyses.

Extensin Expression Analysis.

A histogram of the calibrated data is shown in figure 1. Samples were calibrated against leaf amplification, which had the lowest level of expression. Considerable over expression of the extensin sequence was found in roots of cultivar CM3306-4, compared to leaf. There was some variation between clones. The data also indicated lower expression of the extensin gene in stems, which varied between clones, and which has been observed for the expression of other root-specific promoters isolated from cassava (Zhang et al 2003).

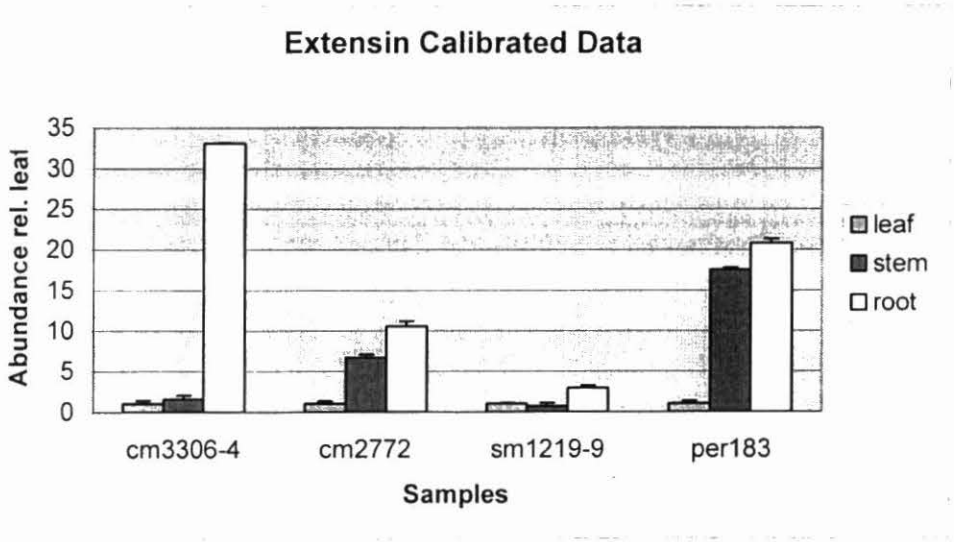

Figure 1. Relative abundance of Extensin transcript in four varieties.

GolS Expression Analysis

The histogram of the calibrated data is shown in fig 2. Samples were calibrated against leaf amplification in each variety, which had the lowest level of amplification. Only in the variety cm2772 shows over expression of the sequence. The rest of the varieties show a 2-fold increase compared to leaf and stem. The evidence presented in this report states that, probably, this gene is a rare transcript, not related to carbohydrate storage, and possibly has a function in biosynthesis and transport. A more sensible methodology could be used to corroborate the data from this sequence, such as hybridization probes (Taqman, molecular beacons, scorpions), due to the late amplification curves obtained with SYBR-green method.

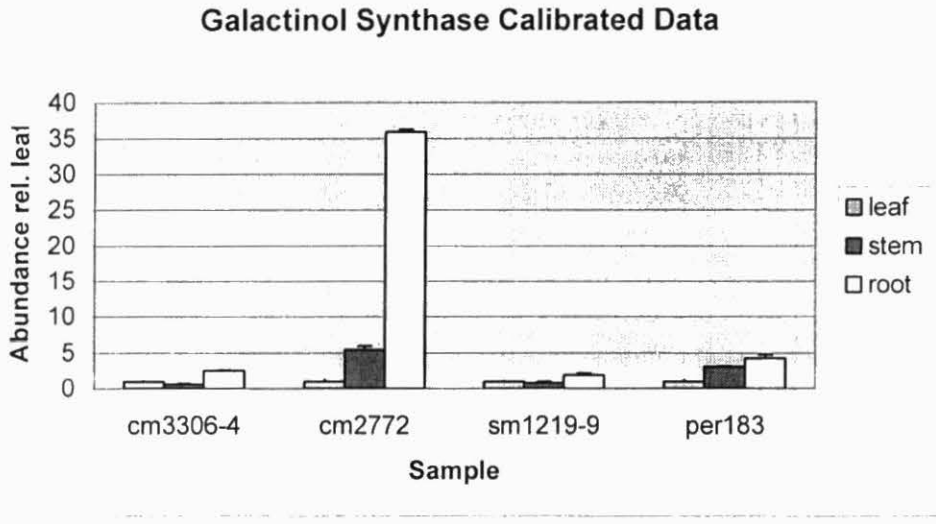

Figure 2. Relative abundance of GolS (clone D3) transcript in four varieties.

Further Activities

1. Although these results are promising towards root-specific expression, it is necessary to confirm the EST's expression by monitoring expression in time. A scheme of four varieties and 5-10 plants per variety grown at the same time and conditions will be suitable for this test. By collecting pooled samples from all plants at similar time intervals (3-6-9 months), a more robust analysis can be done, and possible background noise due to variation between plants can be minimized.
2. Overall methodology could be modified to enhance rare transcript amplification, by changing endogenous control to a housekeeping gene (e.g. G3PDH), purifying messenger RNA from total RNA pools, priming the RT reaction with oligo dT primers to enhance production of cDNA derived from mRNA, or changing the detection chemistry to a more sensitive detector (e.g. Taqman, beacons).

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2.2.6 Single sequence repeat (SSR) markers to confirm possible genetic duplicates in the cassava germplasm collection of Colombia

C.H. Ocampo (CIAT, GRU), G. Mafla (CIAT, GRU), A. Bohórquez (CIAT, BRU) and D.G. Debouck (CIAT, GRU).

Introduction

Ex situ conservation plays a major role in the preservation of genetic diversity of cassava. The collection held at CIAT *in vitro* is by far the largest in the world. A large percentage of duplicates was identified using passport, morphological and isozyme characterization (Ocampo *et al.* 1993; Jiménez, 1994; Sumarani *et al.* 2004). Such a redundancy makes the maintenance and management of the existing collection expensive, and slows down the introduction of new germplasm. CIAT has implemented a model to detect possible groups of genetic duplicates using morphological descriptors and aß-esterase isozyme patterns (Hershey *et al.* 1991). The combination of molecular markers with morphology/isozymes can give a high degree of confidence to identify duplicates (Ocampo *et al.* 1995). A research project is under way using DNA fingerprinting to confirm groups of possible genetic duplicates; that is, to detect genotypic differences among these groups that otherwise appear identical in their morphology and isozyme-banding patterns. In this study we have the following objectives: (1) to develop a description of each accession based on its molecular pattern (fingerprinting) as a criterion to avoid genetic duplicates when new germplasm is introduced in the cassava world collection held at CIAT, (2) once we know the level of redundancy, to study the distribution of the resulting genetic diversity in the different agroecological zones of Colombia.

Materials and Methods

Plant material. This work has been initiated on the cassava germplasm collection of Colombia maintained at CIAT as FAO Designate Collection, consisting of 1,986 accessions (the largest collection by country). The *in vitro* Cassava Laboratory (GRU) provided the accessions for characterization according to morphological and isozymatic similarities. Priority has been given to groups including three and more accessions, resulting in more efficient procedure to identify duplicates.

Molecular Markers. One type of molecular markers that may be suitable for cassava germplasm characterization are the microsatellites (SSR). Microsatellites are considered more sensitive in detecting genotypic differences as compared to morphological and isozymatic descriptors. Microsatellites, like RFLPs, are considered codominant markers. Their high polymorphism makes microsatellites suitable markers in order to identify redundancies in the cassava world collection (Chavarriaga *et al.* 1998).

Results and Discussion

Four SSR markers [SSR59 (249), SSR105 (506), SSR100 (498), SSR109 (521)] showed identical patterns in 31 evaluated groups (94 accessions) (Table 1). Polymorphisms were displayed among different groups, but there are no differences among accessions within these groups. The mean number of alleles in the identical groups was 7. This level of polymorphism is low due to similarity in morpholgy among accessions within the same group. In addition, they have identical isozyme patterns.

In conclusion, the fact that these groups of possible genetic duplicates were confirmed by four SSR markers suggests that the model developed at CIAT to detect these duplicates is reliable. However, more SSR markers are necessary to confirm with high level of confidence these possible genetic duplicates.

Table 1. Possible genetic duplicates in the Colombian cassava collection showing identical fingerprints with a set of four SSR markers [SSR59 (249), SSR105 (506), SSR100 (498), SSR109 (521)].

Group No.	Accessions
1	COL 25 and 896
2	COL 45, COL 948C, COL 1008, COL 1431
3	COL 61, COL 978
4	COL 70, COL 78B
5	COL 76B, COL 912A, COL 927, COL 1962
6	COL 81, COL 647, COL 1067, COL 106, COL 1538
7	COL 93, COL 1044
8	COL 134, COL 138
9	COL 137, COL 140, COL 145
10	COL 207, COL 1485
11	COL 240, COL 281
12	COL 261, COL 547
13	COL 376, COL 380
14	COL 436, COL 2617
15	COL 437[a], COL 1934
16	COL 467, COL 1720
17	COL 942, COL 958, COL 1955
18	COL 1043, COL 1057, COL 1065
19	COL 1092, COL 1602, COL 1616, COL 1821
20	COL 2239, COL 1830, COL 1828A, COL 1518, COL 1516, COL 151
21	COL 1601, COL 1990,
22	COL 2282, COL 2297, COL 2375, COL 2390
23	COL 2286, COL 2292, COL 2298, COL 2300, COL 2313
24	COL 1672, COL 1673, COL 1678
25	COL 1711, COL 1764A, COL 1764B
26	COL 1772, COL 1777, COL 1781, COL 1895
27	COL 1786, COL 1879, COL 2023
28	COL 1889, COL 1893, COL 1894
29	COL1896, COL 1900, COL 2062
30	COL 1901, COL 1902, COL 1903
31	COL 2358, COL 2362, COL 2407

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2.2.7 Isolation and characterization of candidate genes for Al tolerance in *Brachiaria*

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SB-2 CIAT project

Introduction

Previous results demonstrated that there is pronounced difference in aluminum (Al) resistance between *B. decumbens* (resistant) and *B. ruzisiensis* (susceptible). The objective of this work is to identify candidate genes responsible for high level of Al resistance in *B. decumbens* using PCR-based technology. Identification of these candidate genes would enhance our understanding of molecular mechanisms of Al resistance, thereby contributing to genetic improvement of both forage and field crops for Al resistance.

Materials and Methods

Plant material and measurement of root length. Seeds of *B. decumbens* and *B. ruziziensis* were germinated in 200 μM $CaCl_2$ (pH 4.2) for 4- 5 days in the greenhouse. Homogeneous seedlings with root lengths between 4 and 5 cm were transferred to continuously aerated solutions, of 200 μM CaCl2 (pH 4.2) with and without $AlCl_3$. Seedlings were left to grow in the greenhouse. Root length was measured at 0h, 3h, 6h, 24h, and 72h after Al treatment.

Isolation of differential expressed genes. Root tips (1 cm length) from *B. decumbens* and *B. ruzisiensis* were collected at 0h, 3h, 6h, 24h, and 72h after Al treatment. Total RNA was isolated from the root tissue using Trizol® reagent by following manufacture's protocol (Invitrogen, USA). The RNA samples from each time point were pooled to isolate poly(A) RNA using PolyATtract® mRNA Isolation Systems (Promega, USA). mRNAs (300 ηg each) were used for cDNAs synthesis using SMART cDNA synthesis kit (Takara-Clontech, USA).
Differential expressed genes between *B. decumbens* and *B. ruzisiensis* were selected by PCR-Select cDNA subtraction kit (Takara-Clontech, USA) according to manufacture instructions. The cDNA fragments obtained from a forward subtractive library (cDNAs from *B. decumbens and B. ruzisiensis* as tester and driver, respectively) were directly cloned into a T/A cloning vector pGEMT-easy (Promega, USA) in *Escherichia coli* DH5α.

Screening for differentially expressed genes In order to obtain candidates for differentially expressed genes in *B. decumbens* the subtracted library was screened using a PCR-Select Differential Screening Kit (Takara-Clontech, USA) according to the manufacturer's instructions.
Individual colonies that showed the presence of inserted DNA were picked, and grown in a 384 microplate with LB-ampicillin freezing solution, (yeast extract 5 g, tryptone 10g. NaCl 10g, $K_2HPO_4.3H_2O$ 63 g, KH_2PO_4 18 g, $MgSO_4$ 0.43g sodium citrate 4.4 g, $(NH_4)_2SO_4$ 9g y 349 ml of glycerol autoclave until 1L). The colony array was blotted onto nylon membranes (Amersham, USA) resting on LB-agar and then cultured overnight at 37°C. Cloned DNA then was denatured, neutralized and affixed to the membranes by using UV (120mJ)
Probes for hybridization were derived from the forward- and reverse-subtractive libraries as described in the PCR-Select Differential Screening Kit (Takara- Clontech, USA). Radio labeled probes with (α-^{32}P)-dATP were purified by spin filtration (Takara-Clontech, Chroma-spin 400 columns). Hybridization was carried out by using a Hibridiser HB-1(Techne). Membranes were pre-hybridized for 6 hours at 70°C in a solution of 1% BSA, 1 mM EDTA, 7% SDS, and 0.25 M sodium phosphate. Denatured probes were added and were hybridized to the cloned DNA's overnight at 70°C. After hybridization, membranes were washed two times for 20 minutes in a low-stringency washing buffer [2× standard saline citrate (SSC)/0.5% SDS] and one time for 20 minutes in a high-stringency washing buffer (0.2× SSC/0.5% SDS). Membranes were exposed to film (Kodak MXG-1) for 16-72 hours.
Positive clones were sequenced and homology analysis was undertaken using the BLAST-X algorithm of GeneBank NCBI, (http://www.ncbi.nlm.nih.gov/) and Gramene (www.gramene.org) databases.

Results and Discussion

We tested root growth of two *Brachiaria* genotypes, *B. decumbens* and *B. ruziziensis* under Al stress conditions used in this work to understand phenotypic responses to Al toxicity of the genotypes. As shown in Figure 1, root growth of both genotypes was not significantly different under control conditions (-Al). Under Al treatment (+Al) conditions root growth was inhibited for both genotypes. However, the Al resistant genotype, *B. decumbens* had less inhibition of root elongation compared with the Al sensitive genotype, *B. ruziziensis*. The small difference in root elongation with Al treatment between the two genotypes appeared at 24 hours after treatment. Since the root phenotype was present at the measured time point, gene expression related to that phenotype must occur prior to the expression of the phenotype.

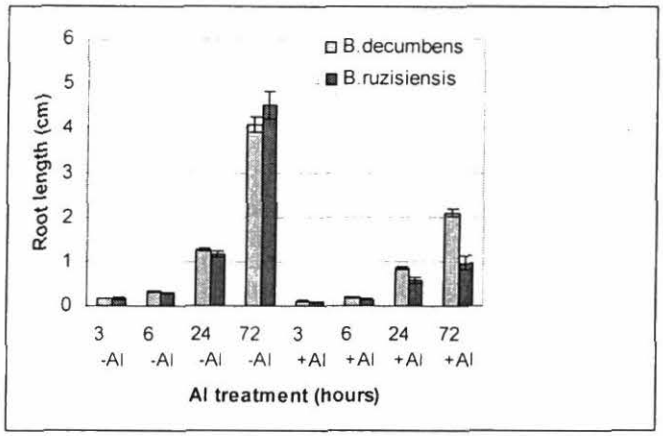

Figure 1. Comparison of root elongation of *B. decumbens* and *B. ruziziensis* exposed to 0 uM (-Al) aluminum (Control treatment), and 200 uM (+Al) aluminum.

To identify genes involved for Al tolerance in roots, differentially expressed genes were isolated using a Clontech PCR-Select cDNA subtraction kit followed by PCR-Select Differential Screening Kit. Figure 2 shows clones derived from this process.

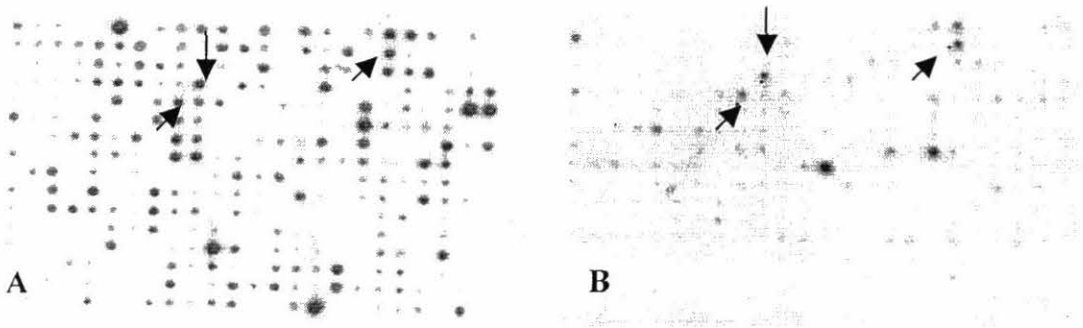

Figure 2 Array of 376 clones shown after hybridization of probes derived from a subtracted population of *B. decumbens* under Al stress (forward subtractive library). **A**. Dot blots hybridized with forward-subtracted cDNA **B**. Dot blots hybridized with non-subtracted cDNA. Dark spots in the autoradiography indicate clones that were expressed in high levels (arrows), or low abundance transcripts enriched during subtraction, which are candidates for differentially expressed genes.

32 clones were identified as differentially expressed genes and were sequenced. 20 of the 32 clones were found to have sequence homology in the GenBank and Gramene protein databases as shown in Figure 3 and Table 1.

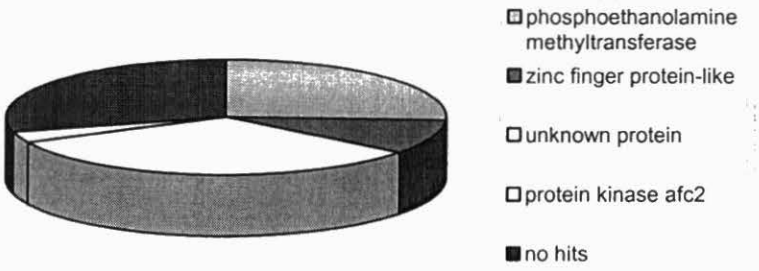

Figure 3 Functional categories found in candidate of differentially expressed genes as a response to Al toxicity in *B. decumbens*.

Among 32 clones, 8 clones of differentially expressed genes had the same sequence which had high sequence similarity with phosphoethanolamine methyltransferase (Table 1). This enzyme catalyzes sequential methylations to form phosphocholine, a key precursor in the synthesis of phosphatidylcholine and glycine betaine in plants. High levels of salinity and hyperosmotic stress induce rapid increases in phosphatidylcholine in *Arabidopsis*. (Pical et al 1999)

3 clones showed sequence similarity to a zinc finger-like protein from rice. This protein contains a domain that is related to the zinc finger (FYVE type) family of proteins. It functions in membrane recruitment of cytosolic proteins by binding to phosphatidylinositol 3-phosphate (PI3P), which is found mainly on endosomes. It was suggested that these proteins regulate multiple functions in cells. Al is capable of affecting all isoforms of phosphatidylinositol specifically in roots. (Jones & Kochian 1995). Other 2 clones showed similarity to protein kinase afc2 from Oryza sativa.

10 independent clones matched against unknown sequences or expressed proteins found in the Genbank database. 9 clones did not match significantly with any existing sequences. Thus, all ten appear to be unique sequences at the cDNA level. 18 clones contained a poly (A) signal, suggesting that part of the sequence consists of UTR 3′region

Clone category	Size (bp)	Blastx similarity	bits	E value
Contig ^{AA}	835	Phosphoethanolamine methyltransferase	162	2e-39
Contig	635	Protein kinase afc2	275	1e-72
Contig ^{AA}	426	Zinc finger protein-like	79.0	5e-14
contig	603	Unknown protein	219	5e-56
singleton	247	hypothetical protein LOC_Os12g3865	87.0	2e-16

Table 1. List of some candidates of differentially expressed genes as a response to Al toxicity in *B. decumbens* obtained after screening for differential expression. The top Blastx hit match is shown. Size refers to the number of nucleotide base pairs in the sequence. ^{AA} means poly A signals that were found in the cDNA clones.

Ongoing activities

Candidate genes listed above will be analyzed their expressions at different time points using Real Time PCR technology to confirm differential expression under Al stresses. To evaluate function of the candidate genes we plan to isolate the full-length cDNA to evaluate phenotype under Al stresses *in planta* with altered expression of the gene of interest.

The sequences of genes obtained will be used for gene mapping in Brachiaria and aid in the identification of molecular markers and QTL's associated with Aluminium tolerance in *Brachiaria*.

References

Jones & Kochian (1995) Aluminum Inhibition of the Inositol 1,4,5Trisphosphate Signal Transduction Pathway in Wheat Roots: A Role in Aluminum toxicity? The Plant Cell. 7: 1913-1922,

Pical et al (1999) Salinity and Hyperosmotic Stress Induce Rapid Increases in Phosphatidylinositol 4, 5-Bisphosphate, Diacylglycerol Pyrophosphate, and Phosphatidylcholine in *Arabidopsis thaliana* Cells The journal of biological chemestry Vol. 274, No. 53 pp. 38232–38240,

Wenzl P, Patiño GM, Chavez AL, Mayer JE, Rao IM (2001). The high level of aluminum resistance in signalgrass is not associated with known mechanism of external aluminum detoxification in root apices. *Plant physiology* 125: 1473-1484.

CONTENT

OUTPUT 3. Collaboration with public and private partnerts enhanced

Activity 3.1 New Collaborative Arrangements, Networks, Databases,Training and Workshops

3.1.1 Development of a web-based resource for molecular marker-assisted selection (MAS) in cassava

C. Buitrago, F. Rojas, M. Fregene
CIAT

Funding: CIAT

Important Outputs

Introduction

Molecular breeding is now a routine operation in private sector breeding companies but it remains a new phenomenon to public sector breeding programs and they have been slow to adopt the use of marker technologies. To facilitate the spread of molecular marker assisted selection (MAS) in a cassava breeding, CIAT is helping to set up simple marker laboratories in the NARS and a web-based resource to share information on protocols, markers, genetic stocks, etc available at CIAT and elsewhere for MAS work in cassava. We expect the cassava MAS data base (MASCAS) will facilitate a molecular breeding communities of practice that will enable participants learn from each other and develop a culture of sharing information, technologies, methodologies, and ways of addressing common problems. MASCAS builds upon another hugely successful web accessible database of the molecular diversity network of cassava (MOLCAS) that was set up to make the results of its SSR marker assessment of local cassava diversity in selected countries of Africa and Latin America widely available. The MOLCAS database is updated regularly with completed country studies and has become a very useful facility for the cassava research community, this year alone over 50,000 visits to the site were recorded. We describe below the MASCAS web-site and visits paid to it since its inception.

A web-based resource for MAS in cassava
MASCAS (**http://www.ciat.cgiar.org/mascas** and Fig 1) was conceived as a mechanism to document and make freely available to the cassava community information being generated by CIAT and other partners on MAS. It is an oracle-based web accessible database that is a repertoire of protocols, molecular marker tools, and genetic stocks available at CIAT as well as interesting links to other cassava biotechnology sites. The MASCAS web-based data base has since its launch last year received a total of 154 visits since its inception in January, 2005. Table 1 shows the summary of all visits and visits since January.

Figure 1. MASCAS home page on the web showing links to protocols for MAS, molecular tools, genetic stocks, partners, and other cassava links

Table 1. A record of visits to the MASCAS web-based data between January and October 2005

Web Page	Jan-Jun 2005	July-Oct 2005
/mascas/	17	23
/mascas/protocols.htm	7	15
/mascas/partners.htm	8	14
/mascas/tools.htm	8	12
/mascas/stock.htm	8	12
/mascas/introduction.htm	6	10
/mascas/links.htm	4	10
Total	58	96

3.1.2 An Advanced Cassava Breeding Training Course for Cassava Breeders from Africa and Brazil

Introduction

With funding from the Rockefeller Foundation and the Generation Challenge Program (GCP), an advanced breeding course was held at CIAT from April 10- May 8, 2005. The course brought together cassava breeders from Mozambique, Kenya, Tanzania, Ethiopia, Nigeria, Uganda, Ghana, and Brazil (Table 12.29 and Figure 12.42). The immediate objective of the course was to provide participants up to date information on field-based and molecular marker-assisted selection (MAS) methods in cassava breeding. Subjects covered include basic quantitative genetics, selection methods, crossing designs, field evaluations, molecular markers, QTL/gene mapping, association mapping, and linkage analysis. Others topics were genetic transformation, haploid technology, molecular breeding, tissue culture (in vitro propagation and post flask management), germplasm development, host plant resistance, integrated pest management, cassava production, and genetic diversity. Most of the subjects were covered in formal class room lecture but practical sessions were also included in the program from field tours, lab demonstrations of DNA isolation, gel electrophoresis, polymerase chain reactions, embryo rescue, in vitro propagation, two-node cutting multiplication, screen house screening of pests and disease, to computer sessions in gene mapping and diversity assessment analysis using Q-gene, Mapmaker/QTL, NTSYS, and GENESURVEY software packages.

Country presentations were also done to enhance exchange of experiences among participants and facilitators to explore new approaches to practical solutions for intractable breeding problems. On the last day of the class-room section of the course, participants were asked what they had learnt and would put to immediate use back home, top of the list was the use of a selection index in cassava breeding, second was the simple specific gravity method for measuring dry matter content, other lessons include increased replication to improve quality of field data, new methods of breeding that incorporates inbreeding, and integrating conventional breeding with new marker tools. The last week of the advanced breeding course was spent in Villaviciencio, in the Eastern Savannahs of Colombia, harvesting a cassava breeding trial. The cassava breeders were taken through the entire process of harvesting and evaluation of 2 cassava breeding trials, a clonal evaluation and preliminary yield trial. They were also shown simple equipments, for example the tripod for weighing roots and foilage, and simple methodologies, for example de-topping cassava plants before pulling up the roots that ease the drudgery of field-based cassava breeding.

A round-up session was spent discussing with the breeders the social context in which they work and they need to set-up clear achievable goals that takes into cognizance the producer/end-user and then stick doggedly to them. Two senior members of CIAT staff, Drs Pachico and Cock, who in the past led very successful commodity programs stressed to the participant that it takes 15-20 years to develop and disseminate improved cassava varieties over a significant acreage, therefore varietal development today needs to take into consideration the challenges, including globalization and the environment, opportunities, and needs of cassava in 2020AD and beyond. The advanced breeding course was undoubtedly an eye-opener of sorts for all participants and we intend to continue holding the course on an annual or biannual basis to provide support to practicing cassava breeders.

Table 1. List of participants from Ghana, Uganda, Nigeria and Brazil in the Advanced Cassava Breeding Course held at

No.	Participant	Institution and Acronym	Location	Nationality	Position	E-mail
1	Dr. Edward Eneah Kanju	International Institute of Tropical Agriculture, IITA	Tanzania	Tanzanian	Breeder	e.kanju@cgiar.org
2	Heneriko Philbert Kulembeka	Agricultural Research Institute Ukiriguru	Mwanza, Tanzania	Tanzanian	Breeder	kulembeka@yahoo.com
3	Esther Andrew Masumba	Sugarcane Research Institute, SRI	Kibaha, Tanzania	Tanzanian	Breeder	emasumba@yahoo.com
4	Anthony Pariyo*	National Agricultural Research Organization, NARO	Namulonge, Uganda	Ugandan	Breeder	apariyo@naro-ug.org ó apariyo@agric.mak.ac.ug
5	Robert Kawuki	Namulonge Agricultural & Animal Production Research Institute, NAARI	Namulonge, Uganda	Ugandan	Breeder	kawukisezi@naro-ug.org
6	Joseph Wainaina Kamau	Kenya Agricultural Research Institute, KARI	Machakos, Kenya	Kenyan	Breeder	jkamauw@yahoo.com
7	Theresia Luvuno Munga	Kenya Agricultural Research Institute, KARI	Machakos, Kenya	Kenyan	Breeder	tlmalamala@yahoo.com; karimtw@africaonline.co
8	Anabela Matangue Zacarias da Silva	INIA	Maputo, Mozambique	Mozambican	Breeder	azacarias@map.gov.mz ó a.zacarias@intra.co.mz
9	Chiedozie Ngozi Egesi	National Root Crops Research Institute, NRCRI	Umudike, Umuahia, Nigeria	Nigerian	Breeder	cegesi@yahoo.com
10	Elizabeth Okai	Crops Research Institute, CRI	Accra, Ghana	Ghanaian	Breeder	elizabeth_okai@yahoo.co
11	Emmanuel Okogbenin	CIAT -National Root Crops Research Institute (NRCRI) &GPC project	Umudike, Umuahia, Nigeria	Nigerian	Breeder	eokogbenin@yahoo.com
12	Alfredo Augusto Cunha Alves	EMBRAPA	Salvador, Bahía, Brazil	Brazilian	Physiologist	aalves@cnpmf.embrapa.br
13	Davi Theodoro Junghans	EMBRAPA	Salvador, Bahía, Brazil	Brazilian	Geneticist	davi@cnpmf.embrapa.br
14	Wondyifraw Tefera	Jimma Agric Research Station	Jimma, Ethiopia	Ethiopian	Breeder	wondyfraw@yahoo.com
15	Asmare Moges	Jimma Agric Research Station	Jimma, Ethiopia	Ethiopian	Breeder	asmare75@yahoo.com

CIAT, April 11 to May 7, 2005

Fig 1. 42 Participants from Mozambique, Kenya, Uganda, Tanzania, Nigeria, Ghana, and Brazil at the Advanced Cassava Breeding Course that took place at CIAT Headquarters, April 10-May 8

3.1.3 A workshop to train NARs partners in participatory plant breeding and molecular marker-assisted selection (MAS)

[1]Alois Kullaya, [2]Jane Ininda [3]Edward Kanju, [4]Morag Ferguson [5]Martin Fregene
1.ARI-Mikocheni 2. KARI-Nairobi/RF 3. IITA-Tanzania 4. IITA-Nairobi, 5. CIAT

Introduction

An important output of the Tanzanian MAS project is capacity building of NARS partners in MAS and participatory plant breeding (PPB) through graduate level training and workshop. A workshop was organized for scientists from several Tanzanian NARs from September 12 to 16, 2005, in Dar es Salaam. CIAT's participation in the workshop was a resource person on capacity building for molecular marker-assisted selection (MAS).

Report

This workshop was held from 12-16 September 2005 at Kunduchi Beach Hotel in Dar es Salaam, Tanzania. It was attended by eighteen (18) plant breeders from the following seven Agricultural Research Institute: Mikocheni, Kibaha, Maruku, Ukiriguru, Tengeru, Mlingano and Naliendele. Other participants came from IITA (5), WARDA (1) and CIAT (1). Dr. Jane Ininda from Kenya Agricultural Research Institute (KARI) (Nairobi) attended as resource person/instructor on PPB and while Mrs Mulemia Maina, Senior Program Assistant on Food Security, Rockefeller Foundation (RF). Nairobi Office represented Dr. D'vries.

The workshop began with an update on cassava improvement activities in Tanzania presented by Dr. Geoffrey Mkamilo, the national cassava coordinator, followed by the following presentations:
Progress in Breeding Cassava for Resistance to Brown Streak Disease in the Coastal Lowlands of Eastern and Southern Africa (Kanju, IITA scientist)
Strategic Cassava Brown Streak Disease Research for Development (Herron, IITA)
Value Addition for Income Generation in Cassava (Rweyendela, IITA)

Progress of the Cassava MAS project (Kullaya, MARI)
A Brief of Rockefeller Foundation's Improved Crop Varieties (M. Maina, RF)

The presentations were followed by two days of PPB by Jane Ininda, and a visit to Kibaha Agricultural Research Institute to see different cassava germplasm in the field. The fourth day was devoted for MAS (by Ferguson and Fregene) and a short visit to the MARI's laboratory.

One thing that became very apparent from the workshop is that all breeders who attended the workshop were, at some point, involving farmers and other stakeholders in the developed of their varieties, but this was mainly at the advanced variety evaluation stage. Participants saw the need to improve the participatory approach by involving the stakeholders at a much earlier stage of the breeding process, but of course within the context of the available financial, human and other resources.

With respect to MAS it became apparent that all participants had heard of MAS, but most of them did not understand what it is. The workshop therefore provided an opportunity to inform participants what MAS is, why and how MAS is applied, some examples of MAS applications and economics of MAS. The visit to the biotechnology laboratory was most rewarding because participants could see how some of what appeared to be very complicated during the theoretical session in the morning is done under local setting.

The fifth day of the workshop was used for group discussions:
One group deliberated on the Cassava MAS Project by reflecting on the on-going work, progress achieved, problems encountered and activity plan for Year 3 of the current phase. The group also discussed in detail plans for a follow-up phase (October 2006 – September 2009).
The second group deliberated on potential collaborative biotechnology projects that could have potential impact on the improvement of food security, income and livelihood.

At the end of it all participants were of the opinion that, though short, the workshop had been very useful because they felt more encouraged and armed to integrate both PPB approach and MAS as modern tools for improving the efficiency and effectiveness of their breeding programs.

3.1.4 Strengthening Farmer Seed Systems in Stress

L. Sperling
SB-2 Project

Introduction

Seed Aid

Seed aid is increasingly applied as an emergency response throughout Africa. The logic looks straightforward. Beyond free handouts, communities affected by emergency (for example, drought, flood, short-term conflict) should be given the means to sow (and harvest) their own food., i.e. via seed provision.

However the practice of seed aid, as increasingly analyzed via a research/development lens, is raising a series fundamental concerns. *Inter alia,* studies are showing that seed delivered in emergency is often is not needed at all (as systems have proven remarkably resilient); and the kind of aid given, and repeatedly, is promoting farmer dependencies in a range of countries. In terms of markets, free seed delivery has also been shown to undermine local seed/grain markets and to compromise the development of longer-term more commercial seed supply.

Seed aid is key for the CGIAR and CIAT as an issue, as a) it is most frequently delivered among the most vulnerable populations- and in the marginal areas (i.e. among our prime client groups); and b) such aid is the entry point for a set of wider agricultural responses to help vulnerable regions. Note that the phenomenon of crisis, or 'disaster' is vividly real in multiple countries in which CIAT operates, including many areas in East, Central, Southern and West Africa.

Germplasm Restoration

Restoration of local varieties was originally conceived as an extension of seed aid thinking, so as to help restore system sustainability. Germplasm restoration was given highlight in the mid 1990's when the Rwanda genocide and war exploded in a key secondary center of bean diversity. Plant genetic resource (PGR) practitioners worried about the possible loss of varieties not just for farmers, but also as genetic stocks for future generations (Seeds of Hope, Document 10, 1996).

While newer than seed aid and practiced in a narrower set of contexts, the field of germplasm restoration has been given visibility quickly due to its sanctioning in the Global Plan of Action. (FAO, 1996) There, under the activity *Assisting farmers in disaster situations to restore agricultural systems*, the logic of germplasm restoration, and its base assumptions, are clearly laid out:

"In the modern world and especially in developing countries, people are threatened with and vulnerably to natural disasters, civil strife and war. Such calamites pose huge challenges to the resilience of agricultural system. Often, adapted crop varieties are lost and cannot be recuperated locally."

"Indigenous landraces/farmers' varieties lost during calamites can frequently be found in *ex situ* collections outside the effected (sic) country. Properly multiplied, such stocks can be returned to reconstitute locally-adapted planting material, an essential component of sustainable agricultural systems."

So, in brief, like seed aid, germplasm restoration aims to 'correct a loss' and its primary response is an asset transfer: seed of PGR-specific varieties.

In the current sequence of disaster response, germplasm restoration is probably best described as a post-crisis, recovery activity. Goal 1 is to make sure people are fed, goal 2 to get them the means to sow, -- and then activity turns to addressing concerns of resilience. Figure 1 suggests the linear sequence of humanitarian response whereby the engine of food aid triggers a chain of support activities (seed aid, germplasm restoration...).

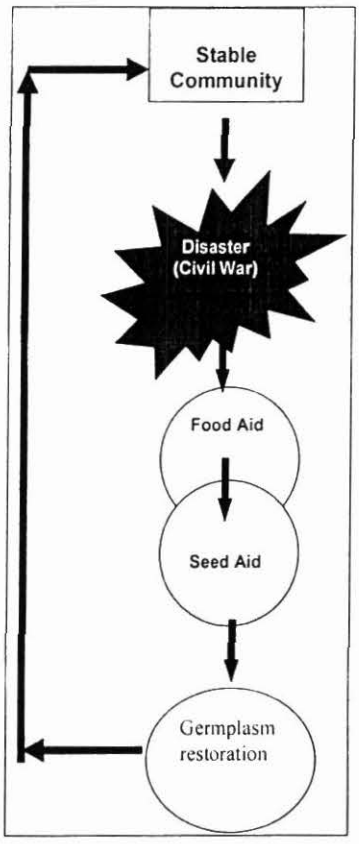

Fig 1: Schematic sequences of aid delivery following a 'disaster' (from Abamu, 2004)

Interestingly, while the foci of seed aid and germplasm restoration are closely related in rationale and substance, the two fields (conceptually as well as on the ground) have been virtually segregated: primarily because chief practitioners are drawn from different arenas. Seed aid has been spearheaded by emergency aid practitioners: the International Non-Governmental Organizations, UN agencies such as the Food and Agriculture Organization, and National Governments. Germplasm restoration, in contrast, has remained principally in the domain of scientific researchers, particularly plant genetic resource specialists, who have good access to the international germplasm banks.

CIAT and Partners' Roles

CIAT, in partnership with relief, developmental and donor agencies, facilitates a multi-activity initiative to improve responses which foster seed security (including PGR aspects), in acute and chronic stress contexts. The CIAT-led Seed Systems Under Stress Program, concentrates on:

- Helping to shape immediate emergency relief, particularly in terms of seed , germplasm and livelihood insight;
- 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.

Below, we briefly present milestones for 2005 and then describe recent progress across a series of specific activity areas.

Bulleted Activity Summary: 2005

- In-depth case studies analyzing the effects of short-term seed aid interventions on seed systems and diversity completed. This includes 8 case studies in 7 African countries. Published in English as "Addressing Seed Security in Disaster Response; Linking Relief with Development". Overview findings also published in French and Portuguese (http://www.ciat.cgiar.org/africa/index.htm)

- Germplasm Restoration overview, focusing on links of international genebanks to farmers in emergency situations, presented as draft report to donor (International Development Research Centre). Worldwide set of cases (N=8) examined.

- Long-term seed aid project launched, in core partnership with Ethiopian NARS. . Ethiopia has received continuous food aid since 1983 (22 years), and seed aid on and off throughout the same period..

- Seed Aid Practice Briefs (N=11) finalized in text form (that is, formatting and translation is still required.) They are aimed at donors, project managers, and humanitarian practitioners and provide insight into the design, implementation and evaluation of emergency seed security projects.

Activity 1: *Short-term Seed Aid* (Case studies of specific emergency interventions and their effects on seed systems and diversity

Collaborators

Editors:

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Tom Remington	Catholic Relief Services, Nairobi
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Paula Bramel	Consultant Care Norway and CRS
M. Alexander Phiri	Bunda College, Malawi

Methods

The case studies were undertaken to evaluate various forms of emergency seed aid and to couple these with analyses of the fuller seed and crop systems. The objectives were to understand if and how vulnerable farmers are being helped by the kind of assistance they receive—and how to move forward in improving practice.

The work was undertaken over a two-year period, in seven countries in Africa. In all cases, the seed aid practitioners were also engaged in the evaluations and reflections, so that 'lessons learned' could immediately influence the "next steps of practice." It is to the credit of the participating national agricultural research systems and nongovernmental organizations (NGOs) that they were willing to take a hard look at the effectiveness of their interventions. Equally, the donors, both USAID/OFDA and the Ministry of Foreign Affairs/Norway, are to be lauded for promoting substantive follow-up on emergency assistance: such follow-up is rare.

Table 1 gives the overview of the major features of the case studies: the countries in which they were undertaken, the stresses that originally triggered a decision to supply seed-related assistance, and the types of interventions that eventually unrolled.

Table 1. CIAT/CRS/CARE-Norway Project: Major Descriptors

Case study descriptors	Content
Countries	Burundi, Ethiopia, Kenya, Malawi, Mozambique, Uganda, Zimbabwe
Trigger Stresses	Drought, civil strife, flood, plant disease (and crop breakdown), distorted political economy
Interventions	▪ Direct seed distribution ▪ Seed vouchers and fairs ▪ Starter packs and targeted input distribution ▪ Community-based seed production ▪ Introduction of new varieties
Crop foci	Maize, beans, cassava, sorghum, rice, millet, cowpeas, bananas, sweet potatoes also: wheat, barley, vanilla, cocoa, moringa

Table 2 hones in on the salient (defining) questions of each field program. Five of the cases addressed key features of specific interventions (such as introductions of new varieties), while three presented overviews of the practice and evolution of seed aid on a country-wide basis.

Table 2. CIAT/CRS/CARE-Norway Project: Defining Questions

Specific site	Defining question
Analysis of Specific Interventions	
Eastern Kenya	Direct seed distribution and seed vouchers and fairs: what is their relative cost-effectiveness?
Northern Burundi	Seed vouchers and fairs and the role of traders: who benefits?
Western Uganda	Seed vouchers and fairs: real agro-biodiversity gains?
Western Kenya	Introductions of new (self-pollinated) varieties in period of crop breakdown: do informal farmer producer groups move quality seed, and quickly?
Northern Mozambique	Introductions of new varieties in a period of crop breakdown: are there special concerns with vegetatively propagated material?
Overview of Seed Relief and Evoution of Practice	
Malawi	Direct seed distributions Seed vouchers and fairs Starter packs/targeted input programs Community-based seed production
Ethiopia	Direct seed distributions/local procurement
Zimbabwe	Direct seed distributions

Select Results

Select Results are sketched below. The full volume (c. 180 pages) has been published in hard copy and is also available of the CIAT website: **(http://www.ciat.cgiar.org/africa/index.htm**

Relief organizations are generally using an 'acute' response- seed aid--' to treat what are more often 'chronic problems' (and poverty-based)

Emergency seed system assistance was delivered in six out of the eight cases examines in response to what was characterized as an acute stress. That is, acute seed insecurity was presumed to have been brought on *by distinct, short-duration events* that affected a significant portion of the population. However, more in-depth analysis, in all of the six cases, showed the problems to be of a more chronic, systemic nature: e.g., declining productivity, water-related stress, ongoing civil unrest, and/or misplaced political policies.

The other two cases, both of crop breakdowns (one in western Kenya with beans and the other in northern Mozambique with cassava), were the only ones in which prior assessments actually took place. These revealed that the 'acute manifestation' was also due to more systemic pressures: build-up of plant disease, lack of crop rotations, declining farm sizes.

The result of an 'acute' response in a more chronically stressed context is that the problem is not alleviated and that seed system assistance is then needed again—and again.

Chronic seed distribution is resulting in the emergence of a Relief Seed System

Seed aid distribution is taking place in an alarmingly large number of countries: one season, two seasons, three seasons, and beyond. The giving of seed aid is itself becoming a 'chronic' activity. Table 3 summarizes the number of years seed aid has been given in several countries. Figures have been amassed from actual government records, from NGO reports, and from the accounts of implementers working on the ground. There seem to be few checks for stopping such assistance (simply when funds dry up?) and deliberate exit strategies have not been planned.

Table 3. Chronic Seed Aid Distribution

Country	Seed Aid Distributions
Burundi	22 seasons since 1995
Eastern Kenya	1992/93, 1995/97, 2000/02, 2004
Ethiopia	Food aid 22 years since 1983/84 Seed aid on and off much of the time
Malawi	12 seasons or more
Zimbabwe	13 years (food aid, seed aid, or both)

The rise of a chronic seed aid system has been identified as a profitable business opportunity for the entrepreneurial, who specialize in quick delivery of a small range of crops. It has also led to the rise of a separate relief seed system (see the Ethiopia and Zimbabwe cases, in Sperling et al. 2004 volume). Relief seed systems are created to assist farm communities post-disaster and are based on the assumption that other seed channels (in both the formal and farmer seed systems) are simply nonfunctional. This assumption often does not hold.

No diagnosis and a (mis)-assumption of seed availability problems has been triggering seed-related disaster responses

The lack of any diagnosis related to the seed system has now become a commonplace observation within the disaster literature (Sperling and Cooper 2004). In practice, one of four strategies is employed for "assessing" seed security and none is sufficiently accurate or timely for assessing seed security among vulnerable farming populations:

- No assessment is done at all—and seed need is assumed.
- Food security assessments are effected—and seed need is assumed.
- A crop production fall (decline) is measured—and seed need is assumed.
- Lengthy surveys of farming and rural production systems are completed—and the results are analyzed and written up—after emergency seed has been delivered.

In the absence of seed-related needs assessment, the default option has been to assume that there is a lack of available seed. This has been done in a wide range of disaster contexts since the start of seed aid practice.

Two sources of concrete information, from very different perspectives, indicate how incorrect this automatic assessment of 'lack of availability' often is.

1. A growing number of studies have actually traced where farmers in "disaster" situations sourced the seed they planted—in areas where seed aid distribution had taken place. Table 4 indicates that in contexts where precise data were examined (and with larger sample sizes), relatively little of the seed sown came from emergency aid (with the importance of the assistance varying by crop and context). This means that, as farmers were lining up to become beneficiaries of free seed aid, they were simultaneously primarily sourcing other non-aid channels to access needed seed supplies.

Table 4. Importance of Relief Seed in Farmers' Overall Supply during Disaster Periods

Context	Crop	% of seed sourced via relief	Source
Zimbabwe/drought/political instability/2003	Pearl millet	12*	Bramel and Remington 2004
Rwanda/war/1995	Beans	28**	Sperling, 1997
Kenya/drought/1997	Maize	11	Sperling, 2002
Somalia/drought/2000	Sorghum	10-17*	Longley et al., 2001
Somalia/drought//2003	Maize	3	Longley et al., 2001

*This figure includes seed delivered by NGOs and the government during the stress period. During 'normal' times, farmers access 5% of their pearl millet seed from these channels.
** The figure of 28% came from the first seed distribution, two months after intensive fighting ceased. Relief seed was then distribution again, the next major planting, and in January 1996, and only 6% of the bean seed shown came via relief channels.

2. This project also set out to assess seed availability via the "experts" who may supply seed in crisis periods: that is, the local seed/grain traders. In Burundi, where seed aid has been given since 1995, 41 traders recounted their experience with seed sourcing over the last 10 years of drought and war. Seventy-eight percent indicated that there had never been a problem with availability. The other 22% nuanced their answers, with only one (item *a* below) suggesting an absolute lack at one point in time.

    a. only once—during the 1993/94 war—when everyone was fleeing (n=1);
    b. in 1993, when all seed had been bought up by the emergency NGOs;
    c. during the "events", seed was available in Rwanda (30 km away) but "my bicycle broke down";
    d. the problem was price.

Trader remarks highlight how relative the term "availability" is and how directly linked it is to a trader's means. Those who source seed using bicycles, and with slim price margins, have different parameters of availability than those with large trucks (and who also easily cross borders).

To date, only two type cases have been identified that show when availability of seed in a disaster context may be a fundamental constraint.. The first is where local seed on offer is no longer adapted to local growing contexts, often due to crop breakdown (e.g., cases: in eastern Kenya, due to bean root rots, and northern Mozambique, due to cassava brown streak, Sperling et al. 2004). Second involves contexts where there have been substantial production shortfalls and local markets have never sufficiently developed to deliver routine seed or planting supplies.

So, in brief, in terms of assessment, the field-grounded studies show that in multiple contexts (e.g., drought, civil strife, or both), farmers have been able to access the large majority of their seed from local channels.

Local seed/grain markets emerge as the core element for seed system stability

The more one looks at seed systems in detail, the more the role of local seed/grain markets appears as central in promoting seed security. Varied market-related findings are emerging from direct field analysis:

- Market-sourced seed (especially for self-pollinated varieties and cereals, with the exception of maize) provides a core for farmer seed security, especially among the more vulnerable, e.g., in this volume, Burundi, Zimbabwe, and western Kenya; see also Rwanda (Sperling, 1997) and eastern Kenya (Sperling, 2002).
- Local grain markets, from which seed is selected, have been shown to be more durable than expected in stress periods, with case analysis showing their functioning in periods of civil strife (e.g., Burundi) as well as in periods of drought and floods.
- The genetic quality of seed sourced in markets is most often acceptable to farmers, as it is generally grown in surrounding agroecological contexts.
- Surprisingly, the physiological and phyto-sanitary quality of seed purchased in local markets can also be partially regulated (through purchase from known contacts and rigorous farmer sorting). Laboratory analyses (for purity, health, and germination) show objectively positive health parameters for the market seed examined. Such data do not mean that all market seed is of high quality. They do, however, firmly show that the reverse is not universally true. Market seed, *a priori*, should not be equated with low-quality seed.
- For the non-hybrids, local seed/grain markets are proving an important channel for moving new varieties. In fact, for some crop types, local markets seem to move new varieties more effectively than formal diffusion channels.
- Markets have proven to be a useful source for re-accessing seed of desired types and quantities that has been lost or temporarily abandoned in stress periods.

Given their pivotal role in seed system stability—and resilience—one of the major conclusions of our case studies is that local grain/seed markets must be strategically supported, not undermined, in post-stress periods. They provide a central core of seed security, particularly for the vulnerable.

Seed systems during crisis are generally resilient—<u>except</u> in cases of crop/variety breakdown

Evidence shows that seed system resilience, of the local, farmer system, is the norm, rather than the exception during periods of stress. 'Resilience' in this context means that seed channels continue to provide varieties and seed that farmers find of acceptable quality, and which will grow when sown. Further, those analyses that focused on varietal diversity, generally find that major varieties are not lost—not during drought, war, nor even select cases of flood.

There are important exceptions to this observation on seed system resilience. In areas of crop breakdown, when existing varieties no longer perform due to formidable pressures (usually disease), the local systems may not have the capacity themselves to bring in new materials. Particularly in cases where vegetatively propagated crops (e.g., cassava, sweet potatoes) provide the base of food security, outside assistance may become key. The problem of cassava mosaic virus in East and Central Africa since the late 1980s demonstrates such need.

Misplaced seed-quality parameters in emergency response result in overemphasis on "health" to the detriment of genetic quality

Issues of seed quality very much shape the types of seed assistance (and asset transfers) that can unfold. In emergency seed procurement, quality issues most often focus on whether the seed is certified or not (as many donors require formal verification as a prerequisite for seed procurement.)

Quality stereotypes have equated certified and formal sector seed as being of high germination and good seed health, with poor assessments, stereotypically, applied to farmer seed (home-produced and procured from the market), which is deemed as generally poor. Case study analyses have shown that such labels can be deceptive. The quality of formal-sector seed may not be as advertised (western Kenya case) and emergency-grade seed overall is of highly variable health and genetic quality (eastern Kenya case). Farmer seed and market seed has also proven to be "objectively" of good quality, as assessed in laboratory analyses (western Kenya case).

The focus on the seed health parameter of "quality" has diverted attention away from what is probably the more important quality issue for seed: the seed on offer, at the very least, must be adapted to the stress conditions at hand. Puzzlingly, genetic quality, in practice, has been given second priority in emergency responses. Varieties emerging from formal research sectors or on offer from commercial companies are assumed 'good enough', whether or not they have been selected for use in the regions of stress or for growing under the management conditions practiced by beneficiary farmers.

Optimally, the genetic quality on offer should anticipate on-site stresses, e.g., they should be early maturing for those facing a hungry gap or resistant to specific disease pressures in areas with marked pathogen build-up.

Aside from these overview findings, the work also examined the effectiveness of specific types of emergency interventions, including, *inter alia,* seed vouchers and fairs and the introduction of new varieties.

Activity 2*: Seed Aid and Germplasm Restoration after Disaster* (Overview Analysis of Trends cross-continents)

Collaborators

Louise Sperling	International Center for Tropical Agriculture, Italy
Edward Zulu	SADC Seed Security Network, Bostwana
Tom Remington	Catholic Relief Services, Kenya
Frank Abamu	West Africa Rice Development Association-Ivory Coast
Rowland Chirwa	International Center for Tropical Agriculture-Malawi
William Fiebig	Save the Children Federation, United States
Massamba Gningue	Catholic Relief Services- Senegal
Patrick Kapukha	World Vision International—Sierra Leone
Christopher Kanema	SADC Plant Genetic Resources Center- Zambia
Wardie Leppan	International Development Research Centre- Canada
Godfrey Mwila	SADC Plant Genetic Resource Centre-Zambia
Edson Musopole	Actionaid/Malawi
Claude Nankam	World Vision International- US
Godfrey Nehanda	VeCo-Zimbabwe
Paul Omanga	Catholic Relief Services- Kenya
Jean-Claude Rubyogo	International Center for Tropical Agriculture-Malawi
Robbert van der Steeg	International Plant Genetic Resource Institute (IPGRI)
Leif Tore Tredal	Consultant (University of Norway)
Steve Walsh	Catholic Relief Services, Burundi
Eva Weltzien	International Center for Research in the Semi-Arid Tropics- Mali

Methods

The two 'aid themes' – seed aid and germplasm restoration--- were coupled in a single project review 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.

Scientists, development specialists and relief personnel prepared their own analysis of select seed aid and germplasm restoration interventions carried out by their respective organizations (effected through refereed journals, field reports and interview). The project cohort commented and debated each case for further revision. As a second thrust, 'global consensus conclusions' emerged via facilitated face-to-face workshops, with the final written draft actively signed off on by all. Hence the work combined description and reflections of organizations on their own intervention strategy and performance, as well as across-the board agreement (by diverse organizations) on trends and challenges in the aid and restoration fields.

Twenty-one seed aid and germplasm restoration were specifically analyzed within the project and key complementary volumes, reviewing another ten (distinct) projects, were drawn on to support conclusions about the way forward (see volumes, Sperling, Remington, Haugen and Nagoda, 2004; Sperling, Osborn and Cooper, 2004). The focus of the seed aid analysis was clearly on Africa as such aid is practiced more prolifically on this continent than elsewhere; germplasm restoration cases were drawn from experience worldwide—as so few cases have actually unfolded in practice. Potential restoration cases were reviewed for the Philippines, war-torn countries of West Africa, , Eritrea, Rwanda, Somali, the Andes; Cambodia, Sierra Leone and Mozambique..

Results

Overview

This review showed the degree to which both fields , seed aid and germplasm restoration, share common goals, are evolving comparable trends in the field and exhibit a similar set of current weaknesses. In terms of general findings, both sets of aid activities tend to assume a lack of seed or of PGR resources , and assume that needed materials are not available locally or regionally. These assumptions unfold in an environment in which diagnoses (either seed security or PGR-security assessments) routinely do not take place. Both fields generally are characterized by supply side interventions (i.e. seed dumping) and, oddly, both lack rigorous follow-up and evaluative capacity to gauge the effectiveness and to learn from what was implemented. The report makes a series of concrete, practical recommendations.

We focus below on the restoration cases, as, we believe, this is the first time, this body of work has been looked at as a whole.

Germplasm Restoration

The most salient point about the review of the group of germplasm restoration cases is that careful analysis shows that the germplasm restoration generally did not happen. The public communication around the case spread one kind of message, while the detailed field documents, scientific reports, and scientist interviews, describe other set of events.

This does not suggest a lack of positive activities carried out under a banner of germplasm restoration. Some germplasm-related actions were effected to farmers' great benefit--- but germplasm restoration *per se* was not usually among them.. To give profile to the reported cases, each is highlighted in the report (in case boxes), and the totality is summarized an annex. (country, dates of stress implementers, range of activities.) Directly below, we give glimpse of the key variations which did unfold.

- In one case, after a genocide and civil war, (Rwanda) an in-depth field assessment showed that local germplasm had not been lost at all. So while restoration activities had been prepared for a broad scale, implementers and scientists shifted concrete action to other realms – to respond to real farmer need (case 7).

- In two cases, after extreme stresses of prolonged war (West Africa) and a combination war and drought (Eritrea), the assistance activities focused on introducing new promising breeding materials to National Agricultural Research Institutes. The West Africa case saw in post war reconstruction an opportunity to move promising varieties known as 'NERICAs' (new rices for Africa). In the Eritrea case, priority was given to strengthening the NARI capacity itself, via introduction of new breeding materials, and training in evaluation procedures and seed production.(see cases 8 and 9).

- Another potential restoration case (in the Philippines) was spurred after a back-to-back sequence of drought and flood events. However, more refined analysis showed that the shifts away from 'traditional varieties, toward modern varieties was due more to farmers' aim to intensify production, coupled with their ongoing problems in managing the local materials (which were of long duration and had seed storage problems). Implementers did multiply and re-supply some new and old varieties to select villages, but follow- up several years later showed farmers did not maintain the seed (case 10).

- In Somalia, germplasm *expansion* activities (not restoration) were prepared in the area of the Middle Shebelle, with the goal being to broaden the diversity used on farm for the crops of maize and sorghum. Existing agriculture practice focused on a limited 12 varieties (for both crops) so researchers aimed to screen some 165 additional materials for possible on-farm use. A baseline activity documenting seed and variety was duly completed , the operational portion of the project was halted due to severe security constraints (case 11).

- The single clear restoration case documented is unusual in two respects: it was partly demand-driven (initiated at the request of communities) and responded to a slow-onset but escalating stress, that is, viral build-up in native potatoes which resulted in yield losses of up to 80%. This case of Andean potato, although not strictly one of emergency, was indeed one of crisis and the components of the response are interesting as they went beyond germplasm reintroduction and cleaning—to extensive community group organization work and technical capacity building. (case 12).

Practitioner suggestions for moving forward

In terms of considering how to forward on 'better practice in germplasm restoration', the projected started with two framing caveats: first, implementation practice has been limited; and second, in

most of the cases analyzed, it is not clear if restoration was needed (either because it clearly was *not* needed, or because an assessment to determine need was not made).

Below, the group focused in a first set of pivotal steps which should then lead to a better understanding of whether germplasm restoration—post acute crisis or disaster---is an issue which needs to be put on the international aid agenda.

The Goals of Germplasm Restoration Need to Be Better Defined.

The goals of germplasm restoration in a good number of cases seem overly simplified. Giving germplasm 'back to farmers' can have few sustainable results-- unless the stresses which caused germplasm loss are also addressed. Evidence suggests that acute events alone (war, flood, drought) do not cause germplasm loss. Therefore, emptying a genebank and multiplying materials in response to a humanitarian crisis may achieve little. Germplasm reintroduction always has to be combined with knowledge sharing and the building of skills to counteract losses. It is unlikely to be effective as a one-off transfer.

In-depth germplasm and seed system analysis increasingly also paints a picture of system dynamism: there is (elective) varietal turnover; farming systems may physically evolve (or deteriorate); or farmers' goals may change (e.g. towards more commercial enterprise). 'Getting varieties back"--- may be exactly what farmers don't want.

Thinking of restoration, as a restoration of processes, however, may be more on the mark. This might include, ensuring farmers renewed and continuing access to diversity (new and old), building farmer skills to better control their germplasm (such as in seed quality), and creating a farmer-accessible information framework--- that indicates what kind of germplasm stocks might be available and where--- for possible re-access or new use.

Assessment methodology has to start to 'come of age' and move out of a PGR box.

The PGR assessment methodology—for use as an implementation tool to strengthen farming systems either post crisis or in contexts of chronic stress—is still in the early developmental stages. Such assessments are warranted in relation to crises when done within a livelihood, farming systems and seed systems framework---, taking account of ecological shifts, economic factors, social, and even political shifts. Practitioners felt strongly that PGR assessments embrace, but go far beyond morphological or molecular analysis and PGR expertise alone. Practitioners emphasized that PGR analysis has to be grounded in an understanding and analysis of the factors that are shaping diversity plus assessing evolving trends.

In terms of methodological specifics, certain shifts or additions to existing assessment repertoire might be warranted to respond to stress situations:

1) more creativity or options need to be pursued for reconstructing baselines—(what was there before);

2) there might best be a move away from a focus on assessing germplasm per se (whether at the varietal, molecular or morphological level) and towards assessments of the *processes* which shape germplasm use (have they been disrupted or not –and how).

Perhaps also, contrary to popular view, PGR assessments might be best seen not only as scientific assessments, but as combined scientific, developmental and humanitarian assessments. By implication, PGR assessments require a multidisciplinary approach and multi-partner approach (research, development and community-based),

As two basic tenets of PGR analysis, the project practitioners suggested that analysts:

- have to distinguish between cases of varietal or genetic erosion and evolution (and whose perception guides the interpretation
- have to clearly underscore what farmers real demands are (for restoration or otherwise...).

In terms of 'do's and don'ts—for lay public consumption—project participants agreed on the following recommendations:

"Better Practice"
Germplasm Restoration—associated with crisis

To be discouraged
• Be cautious about assuming germplasm loss—after a crisis (The evidence for this across crises is very slim)
▪ Avoid claiming 'germplasm restoration' has taken place—when nothing has been returned to farmers fields.
• Avoid solely supply-side interventions (i.e. genebank empyting and multiplication)
▪ Do not assume post-disaster that farmer needs are the same as before. (Work to understand dynamic situation: PGR profiles change),
To be encouraged
❖ Bring in rounded agricultural/livelihood expertise. PGR is not just the domain of technical PGR specialists; it is rooted in developmental and livelihood concerns.
❖ Assess PGR Security—to determine *if* problems and, if so, *which?*
(Here, focus on germplasm-shaping processes--- rather than germplasm per se Processes can signal if systems can recover or evolve without intervention—and also signal dynamic trends)-
❖ Develop an explicit *Information Strategy* in PGR stress-related activities: *Farmers need information on access (where, whom what conditions), they require choices (new, old...), they need in be able to make informed decisions about possible planting materials.*
❖ Promote only demand-driven restoration, introduction or diversification scenarios.

Activity 3: Long-term seed aid and the most Vulnerable: Focus on 'Classic Case' – Ethiopia

Collaborators
(Team leaders)

A. Deressa, T. Assefa	Ethiopian Agricultural Research Organization (EARO)
L. Sperling, J.C. Rubyogo	International Center for Tropical Agriculture (CIAT)
A. Asfaw, A, Tenaye	Southern Agricultural Research Organization (SARI- Ethiopia)
Wondafrash Mulugeta	Amhara Agricultural Research Institute: Debre Birhan
S. McGuire	Overseas Development Group (ODG) (member from University of East Anglia (UEA)

Background

The foci of this IDRC-supported project are a) the analysis of the effects of chronic aid , and b) the identification of positive developmental ways to move chronically- stressed populations forward. These thrusts have strong support from actual practitioners—and are particularly important themes in the country of interest: Ethiopia.

Within Ethiopia, two diverse emphases suggest the urgent need for such a chronic stress analysis, and it is fortunate that the Ethiopian Agricultural Research Organization (EARO) is engaged as a co-leader of this work.

First, government policy is advocating strong intensification of production, including among small holder farmers and in areas not previously reached by research products. For beans, for instance, the Program Coordinator has managed to multiply 80T of seed already-and received word in October 2004 to put another 15 ha under irrigation immediately. For the project, this push means that NARS collaborators want to look forward on how to boost chronically stressed communities, as well as back, that is, on the effects of seed aid.

Second, the emergency specialists launched another appeal for international assistance in both December 2004 and December 2005. These crises are not new or unexpected in Ethiopia. Between 1970 and 1998, the country experienced more than 56 large-scale disasters, including one as recently as 1999-2000, when drought was exacerbated by conflict and more chronic issues of low agricultural productivity, few employment opportunities, and depressed marketing outlets for Ethiopian coffee and livestock exports. An emerging issue for the Ethiopian government and donors alike is how to respond to these repeated emergencies effectively, without damaging the resilience of agricultural systems and basic -livelihoods more generally.

Objectives/Methods

The project was launched in June 2005 and has three basic objectives:

Objective 1: The Effects of Longer-term Seed Aid on farming families understood so as to promote effective assistance particularly for the vulnerable.

This will be pursued through survey, key informant interview and focus groups at the farmer level in four regions. We aim to give insight into how beneficiaries in crisis periods are aided (or not) by standard and repetitive humanitarian practice. Analysis will differentiate among the needs of those in diverse stressed farming systems. It will also investigate the root causes (chronic as well as acute) for food and seed insecurity

Objective 2: Governmental lessons and evolution of seed aid policy documented so as encourage policy support for 'better practice' initiatives

Via review of official and gray government documents spanning 25 years, as well as extensive interview, we aim: a) to document evolving seed aid policy at the national level so as to stimulate reflection on policies shape emergency response, b) to highlight emerging lessons across several regions in Ethiopia so as to inform current seed emergency program design. A key feature here should be identification of enabling policies' which encourage 'better seed aid practice' to emerge.

Objective 3: Institutional links between Emergency Practice decision-makers and Agricultural Development Decision-makers catalyzed.

This will be encouraged in the both field studies (design and follow-up), and during the policy analysis, which will be conducted jointly with policymakers in both the development and emergency sectors.

Activity 4: Seed Aid Briefs: Outreach to Donors and Humanitarian Relief Practitioners

Collaborators

Key inputs across briefs by:

Louise Sperling	International Center for Tropical Agriculture
Tom Remington	Catholic Relief Services
Jon Magnar Haugen	Consultant for Care Norway

Office of Foreign Disaster Assistance (Laura Powers and Julie March)

Inputs on select briefs from:

Jean Claude Rubyogo	International Center for Tropical Agriculture
Robbert van der Steeg	International Instite for Plant Genetic Resources
Paula Bramel	International Institute for Tropical Agriculture
Tom Osborn	Food and Agriculture Organization of the United Nations (FAO)
Dennis Lattimer	FAO
Geoff Heinrich	Catholic Relief Services

Background

This activity aims to provide rigorous 'better practice' guidance for both donors and managers shaping emergency seed security projects, as well as field staff involved in on-the-ground humanitarian practice.

The contents of the briefs have been shaped from extensive field experience, across different types of disasters and seed security constraints and opportunities. Aside from the writers (drawn from different institutions and disciplines), the briefs are being reviewed by the key donor: The Office of Foreign Disaster Assistance/USAID.

Results

Eleven practice briefs are in near final form. They will be published in French and English.

#	**Seed Aid Practice Briefs**
1	Seed Systems in Stress Who we are and what we do
2	Understanding Seed Security (acute versus chronic)
3	Introduction to Case Studies
4	Agrobiodiversity and Seed Relief
5	Using Seed Aid to Facilitate Farmer Access to New Varieties
6	Seed Quality and Seed Relief
7	Assessing Seed System Security
8	Understanding Seed Systems Used by Small Farmers in Africa: Focus on Markets
9	Understanding of Relief & Recovery Seed Approaches
10	Evaluation and Seed Relief
11	Seed Assistance Proposal Development: A Rapid Review Checklist for Practitioners

Future Plans

The program has focused till now on documenting what actually happens to farmer systems in emergency; and in evaluating the effectiveness of varied responses. Both seed aid and germplasm restoration have been foci of analysis.

For the next two years, the program will be moving research emphasis to the chronic stress areas, those regions where seed aid is delivered on a repeated basis and which generally embrace poorer farmers in marginal areas.

Substantial effort will also be placed of finalizing Seed System Security Assessment (SSSA) tools to assist donors, managers and implementers in addressing issues of seed security

practically. Tool development, capacity building in field assessments and awareness raising in centers of seed aid policy form the three-pronged agenda.

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Activity 3.2 Cassava Biotechnology Network's activities

3.2.1 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. The fund aims at achieving the following over the 5-year span:

To provide opportunities and support to female and male master's students from the developing countries of the world to undertake thesis research addressing key elements of the sustainable use and conservation of agricultural biodiversity, in particular:
a) intellectual property rights and access to agricultural genetic resources
b) molecular characterization of agrobiodiversity
c) community-based conservation of genetic agrobiodiversity.

To promote the bridging of the research/ development divide, by encouraging researchers and their home universities to develop linkages with research for development projects, and to undertake applied research which informs development processes.

To explore opportunities for further expansion of this initiative in order to involve other stakeholders.

To encourage and support the exchange of information, knowledge and technology between the stakeholders in agricultural biodiversity conservation in these countries.

A total of 22 proposals were evaluated and 7 fellowships were approved and started in October/2003 until 2005 (**see Table1**).

A total of 10 proposals were evaluated and 2 were approved started in March 2005 until 2008.(**see Table 2**).

A total of 11 proprosals were received to MSC – Africa. Dr. Ronnie Vernooy will review and will send his result on November,05

Table1. – BUDGET Projects Approved supported by the "Ginés-Mera Memorial Fellowship Fund for Postgraduate Studies in Biodiversity". 2003-2005

Student	Project Title	University	Award (US$)
Adriana Mercedes Alzate	Identification of cassava (*manihot esculenta crantz*) genetic diversity from small farmers using molecular markers and evaluation of adoption's level of ciat breeding lines in the atlantic colombian coast.	Nacional de Colombia. Palmira Colombia	12000
Astrid Johanna Arango Ulloa*	Totumo: (*Crescentia cujete L*): Diversity and Uses of this multipurpose tree in Colombia.	Georg-August-Universitat-Goettingen, Germany	8000
Constanza Maria Quintero Valencia	Genetic Mapping and use of single nucleotide Polymorphisms (SNPs) for the characterization of common bean (*Phaseolus vulgaris* L.) germplasm	Nacional de Colombia. Palmira Colombia	12000
Javier Llacsa Tacuri*	Determination of socioeconomic, cultural, agronomic and environmental factors that they carry weight in the Conservation of the variability of local potato varieties in small farming Communities of Amaru, Chahuaytire and Viacha – Pisac district – Calca province – Cusco Departament – Perú.	Nacional San Antonio Abad del Cusco University. Cusco Peru,	12000
Juliana Chacón Pinilla	Phylogenetic patterns In the Genus *Manihot* (*Euphorbiaceae*): Biogeography and Comparative Ecology of Mesoamerican and Southamerican species.	Andes University, Bogotá Colombia	14000
Nelson Arturo Royero Moya	Molecular Characterization Of The Genetic Variability In Guanábanos (*Annona Muricata* L.) And Anonáceas Species Related To Hortícola Importance	Nacional de Colombia , Palmira, Colombia	8000
Roosevelt Escobar Pérez	Establishment of low cost *in-vitro* technologies for the cleanliness and conservation of local cassava varieties and use in farmer's safety food programs.	Nacional de Colombia, Palmira Colombia	8000
TOTAL			**74.000**

Table 2. BUDGET Projects Approved supported by the "Ginés-Mera Memorial Fellowship Fund for Postgraduate Studies in Biodiversity". 2005-2008

Student	Proyect Tittle	University	Award (US$)
Bohorquez Adriana	Isolation of Expressed sequences during the defense response of Mecu-72 to white fly attack using subtractive libraries and microarrays expression of cDNA	Nacional de Colombia , Palmira Colombia	20.000
Ocampo John	Diversity within the genus passiflora (passifloraceae) based on ecogeographic, morphological and molecular evidence	Montpellier University, France	20.000
TOTAL			**40.000**

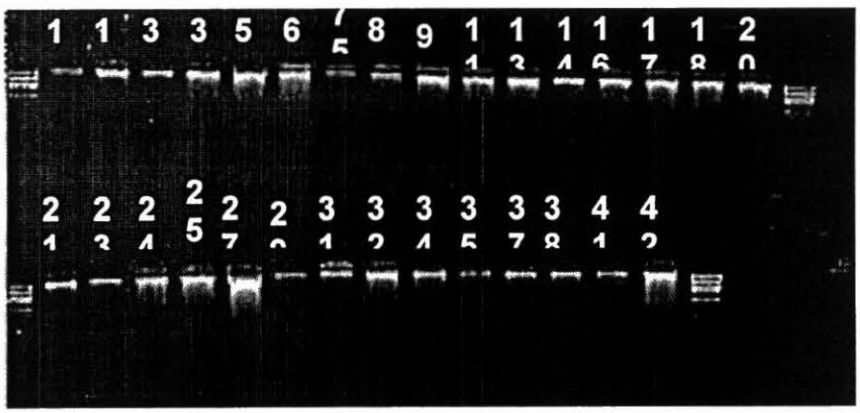
Thesis title:

IDENTIFICATION OF CASSAVA (*MANIHOT ESCULENTA CRANTZ*) GENETIC DIVERSITY FROM SMALL FARMERS USING MOLECULAR MARKERS AND EVALUATION OF ADOPTION'S LEVEL OF CIAT BREEDING LINES IN THE ATLANTIC COLOMBIAN COAST.

Adriana Alzate, Universidad Nacional, Palmira - Colombia
a.m.alzate@cgiar.org

Abstract

The collection of cassava varieties planted in the Atlantic Colombian coast by small farmers was done in the departments of Cordoba, Sucre, Atlantico, and magdalena.
An stratified sampling with statistically significant size of sample was done to collect the cassava samples. The sampling method incidated the number of municipalities to visit in each department and 10 surveys were perfomed in everyone. In Atlantic, Magdalena, Cordoba and Sucre 10, 9,11 and 10 municipalities were visited respectively. 100, 90, 110 and 100 surveys were done in each department as well.
The type of varieties planted by each farmer showed a rank between 1 to 9. A sample or seed from each variety was requested to the farmers. In this way, 1048 samples were collected, transferred to CIAT and planted in the greenhouse. After some months, young leaves were collected from the plants and DNA was extracted using the modified Dellaporta Method (Dellaporta et al, 1993).



GEL DE CALIDAD DE 30 MUESTRAS ESCOGIDAS AL AZAR

The genetic diversity evaluation is being done using Microsatellite markers (SSR, Simple Sequence Repeats). Iniciallly, a preliminary study was done evaluating 30 samples random selected out of the 1048 set with the 36 SSR markers previously used to estimate genetic

diversity and differentiation in Cassava (Mba et. al, 2001). The results from this evaluation were analyzed with CERVUS and 9 out of the 36 SSR markers were selected because of the high polymorphic information content (PIC). In addition, the linkage of these 9 markers was considered to have a representative marker from different linkage groups of the cassava genetic map. The 9 selected SSR makers will be used to evaluate the 1048 samples collected in the Atlantic Colombian coast. DNA dilutions from all the samples are being done for further PCR amplification and evaluation in polyacrilamyde gels that will be used to score the alleles per locus corresponding to each cassava varietiy.
The data analysis will include a Principal Coordinate Analysis and a Cluster Analysis using the genetic distances and the proportion of alleles shared.

Finally, the adoption's level of CIAT breeding lines in the Atlantic Colombian coast will be done processing and analyzing the surveys.

Thesis Title:

TOTUMO: (*CRESCENTIA CUJETE L*): DIVERSITY AND USES OF THIS MULTIPURPOSE TREE IN COLOMBIA.

Astrid Johanna Arango Ulloa
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Tesis para obtener el Titulo de Maestría en Agricultura Tropical e Internacional
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Abstract
Germplasm of the calabash tree (*Crescentia cujete* L.) was collected in five regions of Colombia, i.e. Andean, Caribbean, Amazonian, Orinoquean and Pacific regions. Collecting this multipurpose tree was guided by the indigenous knowledge of farmers in each region, which served as well for compiling ethnobotanic information. Large variation in fruit shapes and sizes was found, of which some forms were typical for certain regions. Overall 58 accessions were collected and roughly classified into 22 types by eight fruit shapes and sizes, each, integrating regional common names. Molecular markers (AFLPs) were applied to leaf tip tissue originating from vegetatively propagated plants in order to assess the diversity available in the germplasm collected as well as to detect patterns of geographical or morphological similarity. One accession each of *C. alata* H.B.&K. and *C. amazonica* Ducke were used as outgroups. On average, 86.8% of polymorphism was detected with each fingerprint of AFLPs, indicating a high marker index. The analysis accounted for 95% of the total variation. Overall, genetic similarity was extremely low (0.43) varying from 0.22 to 0.82, which suggests high genetic diversity among the accessions of this collection. Eight groups were identified by Multiple Correspondence Analysis (MCA) and 3 cluster analysis applying UPGMA and based on Nei-Li genetic similarity. No patterns could be established between either geographical provenance or fruit morphology and genetic diversity. Concerning the outgroups, the *C. amazonica* accession appeared to be a distinct species. The *C. alata* accession, however, was not sufficiently distinct from *C. cujete* to merit species status. The latter material might as well be a hybrid or serve to further challenge the validity of interspecific organization of the genus *Crescentia*. This study is the first approach with molecular markers of genetic diversity of *C. cujete* and provides information on biodiversity that can be used in the conservation and use of germplasm of the species.

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Thesis Title:

GENETIC MAPPING AND USE OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) FOR THE CHARACTERIZATION OF COMMON BEAN (*Phaseolus vulgaris* L.) GERMPLASM.

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Abstract

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 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. The purpose of this project is to add to the CIAT's principal mapping population, *P. vulgaris* SNP markers developed at CIAT (Gaitán and Tohme, 2002) together with soybean SNPs (developed at BARC-USDA); study their potential for characterizing genetic diversity of wild *P. vulgaris* core collection, and to explore the association either of single SNPs or haplotypes blocks in cultivated varieties with well known response to some of the most important biotic production problems of the crop, such as bean golden mosaic virus (BGYMV), anthracnose (ANT), common bacterial blight (CBB) and angular leaf spot (ALS). SNP detection was carried out using the single base extension (SBE) methodology described by Chen *et.al.* (2000) standardized by Cregan (USDA Beltsville Agricultural Research Center) and implemented at CIAT by Gaitán-Solís *et.al.* (2004), with a Luminex-100 flow cytometer as platform. Allele calling was done using Masterplex GT software (MiraiBio, INC). Plant material included a population of F_9 plants from 87 recombinant inbred lines (RILs) developed at CIAT from the cross between G19833 and DOR364; 108 wild and weedy *P.vulgaris* accessions representing the geographic distribution of the species and 165 Andean and Mesoamerican varieties belonging to the known bean market classes. Data for resistance to ANT, CBB, and ALS were kindly provided by S. Beebe (Mesoamerican Bean Genetics, IP-1 Project, CIAT). MAPMAKER/EXP (version 3.0) (Lander *et.al.*, 1987) was used to assign all the markers to linkage groups at a minimum LOD score of 4.0. A total of 58 SNP markers (39 from soybean designed by Quigley, P. Cregan's Lab., BARC-USDA; and 19 from *P. vulgaris* designed by Gaitán-Solís, *et.al.*, 2004) were added to the linkage map previously developed at CIAT (Beebe *et.al.*, 1998). Five soybean SNPs remained unlinked. The total cumulative length was 2000cM (Kosambi units) and the average chromosome length was 181.7cM. Each of the eleven *P. vulgaris* linkage groups had at least two SNP markers attached. The average number of SNPs per linkage group was five with B03 and B07 having the highest number of SNPs placed on them (nine) while B05 had only two. The distribution of the SNPs along linkage groups was variable and the average distance between them in this map was 13.1cM. Single marker analysis for QTL detection was carried out using WinQTLCartographer (version 2.5) software (Wang et. al., 2005) and showed QTLs for ALS in chromosome B10, for ANT in chromosome B03 and B11, and presumably for BGYMV in chromosome B03. Some SNP markers seem to be in or surrounding these QTL regions, but more accurate analysis such as interval mapping is in process to finally declare QTLs and associated

SNPs. Although more SNP analysis needs to be done, genome-wide scan of *P. vulgaris* germplasm has been initiated. All the 108 wild and weedy accessions and the 165 cultivated varieties have been genotyped with 21 *P. vulgaris* SNPs discovered by Gaitán and Tohme (2002). These 21 SNPs clearly separated the accessions in the two major *P. vulgaris* genepools, Andean and Mesoamerican. All *P.vulgaris* SNPs were polymorphic in wild germplasm. In cultivated varieties, only CIATSNP-13TC was found to be monomorphic. Codominant nature of SNPs allow to distinguish between homozygotes and hetezygotes individuals for almost all loci. No heterozygote individuals were identified in cultivated varieties with CIATSNP-22TA, and in wild germplasm with CIATSNP-30CT and CIATSNP-13TC. The SNPs reported here are all biallelic, thus a PIC value of 0.50 is the maximum achievable. The range of values of PIC founded in cultivated varieties was between 0.04 (CIATSNP-7GC) to 0.36 (CIATSNP-17TC), with 8/21 (38%) of the primer pairs having PIC values >0.30. In wild germplasm, PIC values ranged from 0.11 (CIATSNP-17TC) to 0.37 (CIATSNP-22TA) with 9/21 (43%) of the SNPs having PIC values >0.30 (Table 1). These data are comparable to those reported in wheat by Sommers *et.al.* (2003) who suggest that SNPs can be very useful in genetic diversity studies, given their high abundance, their amenability to high throughput detection platforms, and the reasonable PIC values observed in a large proportion of the SNPs assayed. Since the information about the order of SNP markers along the genome is essential for haplotype prediction, block partition (Halperin and Eskin, 2004) and for association of SNP haplotypes and phenotypic traits (Zaykin *et.al.*, 2002), the validation of a second batch of 84 soybean SNPs (also developed at BARC-USDA) in eight Andean and Mesoamerican bean genotypes has been initiated. Once mapping analysis is completed and representative SNPs selected, genotyping *P. vulgaris* germplasm will continue in order to achieve haplotype-trait association. On-going activities are: to place in the linkage map of *P.vulgaris* (DOR364 x G19833 population) 84 soybean SNP markers, continue the characterization of genetic diversity in *P. vulgaris* with representative single nucleotide polymorphisms and study the association of SNP markers with ALS, ANT, CBB, BGYMV.

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Thesis Title:

DETERMINATION OF SOCIOECONOMIC, CULTURAL, AGRONOMIC AND ENVIRONMENTAL FACTORS THAT THEY CARRY WEIGHT IN THE CONSERVATION OF THE VARIABILITY OF LOCAL POTATO VARIETIES IN SMALL FARMING COMMUNITIES OF AMARU, CHAHUAYTIRE AND VIACHA – PISAC DISTRICT – CALCA PROVINCE – CUSCO DEPARTAMENT – PERÚ.

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Abstract

The present study intends to come near interpret the factors that have influence in the decision of the peasant families to preserve native varieties of potatos in Amaru, Chahuaytire and Viacha communities – Pisac district - Calca province, Cusco department - Peru.
The objective is to contribute to the analysis of factors of conservation of native potatos, with the purpose to name a bigger analysis and consideration in the processes of intervention for rural development, so that these resources have better alternatives of use, doing possible that genetic resources may be preserved to the equal to improve the standards of living of the farmers.

The principal hypothesis in the decision of the peasant families to grow native potatos, as a consequence to preserve, she is given and conditioned for various factors being these of cultural, environmental nature and of agronomic traditional practices of the native potatos, getting constituted like favorable to the conservation. The socioeconomic phenomena, the external pressure, the focuses of rural development no in agreement to the Andean reality and the globalization, they get constituted like a threat to the conservation of native cultivations, which as they are out of the andean families control.

Methodology responds to study of cases a kind of explicative and descriptive fellow's investigation, coming true, longitudinal in the time, checking vivencial, interviews structured, direct observation and workshops or meetings with groups of families. In the three communities focal, groups constituted have each one join for ten themselves families. The ten families are the result of selection among those that they preserve many varieties of native potatos and those that they preserve few or very few varieties. An each one a diagnosis of his socioeconomic situation, of grade of identity with the Andean culture of agronomic practices of one's own of his relation, and with the environment is done to him, contrasting each one join of theses aspect with the number of to than, grow and most of all with the grade of knowledge and uses that he gives him to each join of his varieties. All it will permit to bring us near to interpret the rationality peasant of the conservation of cultivations native.

Thesis Title:

PHYLOGENETIC PATTERNS IN THE GENUS *MANIHOT* MILL. (EUPHORBIACEAE): BIOGEOGRAPHY AND COMPARATIVE ECOLOGY OF MESOAMERICAN AND SOUTHAMERICAN SPECIES.

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Abstract

Evolutionary relationships among wild *Manihot* species are still uncertain. Current studies have emphasized on the origin of cassava (*Manihot esculenta* subsp. *esculenta*), one of the most important crops in tropical countries, without regarding the rest of species. In order to quantify inter-specific genetic variability among wild *Manihot* species, and to establish a molecular phylogeny of the genus, three plastid (*accD-psaI* spacer, *trn*L-F spacer, and *trn*L intron), and three nuclear DNA regions (*G3pdh*, *CAM1*, and *CAM2*) were sequenced. *Cnidoscolus* was included as outgroup. Tree topology and geographical distribution of species were used to infer a biogeographic hypothesis of the genus. The age of the different nodes was estimated by means of a molecular clock calibration. Ecological data obtained from the last monograph of *Manihot*, was also used to infer the adaptation process of the species to their current habitats. Nuclear *G3pdh* was chosen to infer the evolutionary relationships of the species, due to the lack of variation of the chloroplast genome, the possibility of gene duplications in the *CAM1* region, and the positive effect of natural selection on the *CAM2* region. The phylogeny shows a Central American clade, sister to the South American species. The diversification of the last clade began in Brazil during the Pleistocene, followed by migration of species towards other parts of the South American continent. Glacial and interglacial periods could play an important role, modulating the adaptation to dry habitats. The predominance of shrub forms could occur early in the evolution of *Manihot* species, although a better sampling of species is needed.

Distribución geográfica del género *Manihot* y centros de diversidad propuestos por Rogers y Appan (1973).

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Thesis Title:

MOLECULAR CHARACTERIZATION OF THE GENETIC VARIABILITY IN GUANÁBANOS (*ANNONA MURICATA* L.) AND ANONÁCEAS SPECIES RELATED TO HORTÍCOLA IMPORTANCE

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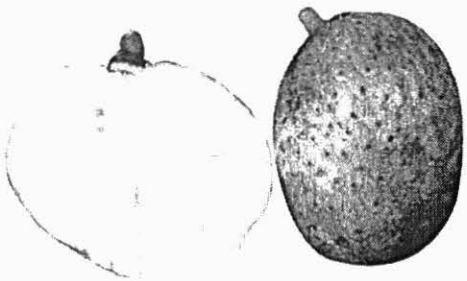

Abstract

The genetic variability of horticulturally important Annonaceae species was characterized at the intra- and interspecific level using AFLP molecular markers. These species have a neotropical origin, produce edible syncarpous fruits and have been classified in the genera *Annona* and *Rollinia*. The accessions were divided in two sets for the study. Set 1 comprised 39 accessions of at least 10 different species. A total of 496 AFLP bands, obtained with two combinations of primers, were analized applying similarity, multiple correspondence, diversity and phylogenetic analysis. In the similarity analysis, using the Nei-Li coefficient, most of the species showed similarity below 40% among them, which might be related to low success in their, man-mediated, interspecific hybridization. Phylogenetic analysis showed *Rollinia* accessions as a group inside *Annona*, being closer to *Annona* species with subglobose flowers than to *Annona* species with elongate flowers, thus suggesting a taxonomic and systematic revision of both genera. Two hundred forty one bands were analysed for the characterization of the second set of accessions, which consisted of 36 accessions of soursop. Applying similarity, correspondence and diversity analysis, these accessions showed a genetic variability that ranged between 0.75 and 0.95 of similarity. This level of variability might reflect germplasm exchange between farmers. It may also reflect the fact that the accessions were selected by agronomic traits, which may not mirror the genetic variation found in natural populations of the species. In addition, an inventory of herbarium collections of *Annona* and *Rollinia* species was made. A total of 196 samples of 39 different species were found from all the biogeographic regions of Colombia. Although there is important genetic variability, useful for breeding of cultivated Annonaceae, it is only a fraction of the variability available in Colombia and tropical America. The AFLP analysis indicate that there is a need to increase the number of species, and the number of accessions per conserved species, of Annonaceae through new collections of germplasm in the neotropics.

Thesis Title:

ESTABLISHMENT OF LOW COST *IN-VITRO* TECHNOLOGIES FOR THE CLEANLINESS AND CONSERVATION OF LOCAL CASSAVA VARIETIES AND USE IN FARMER'S SAFETY FOOD PROGRAMS.

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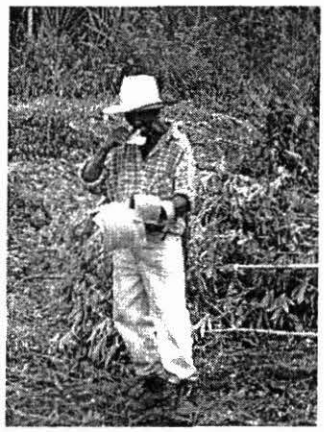
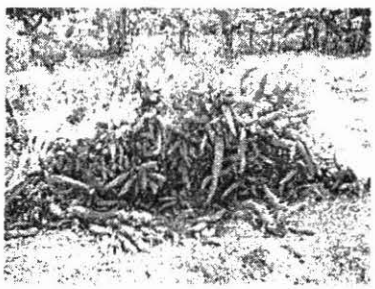
Abstract

The Agrobiodiversity and Biotechnology Project (SB-2 Project) promote the efficient conservation and use of agro-biodiversity. Farmers in the Cauca department of Colombia have traditionally cultivated cassava as a food and cash crop. This staple, however, has been increasingly affected in the past decade by a major pest, the whitefly (*Aleurotrachelus socialis* Bondar) and a chronic phytosanitary problem frogskin disease (FSD). These production constrains have negatively affected the income derived from cassava and, thus the well being of many resources-poor farming communities in the department of Cauca.

The SB-2 Project initiated a series of activities in three localities: Alegrias, Pescador, and Santa Ana of the Cauca Department, with a view to reactivating cassava production and improving the well-being of farmers in this regions. As part of this work different activities were developed under this award:

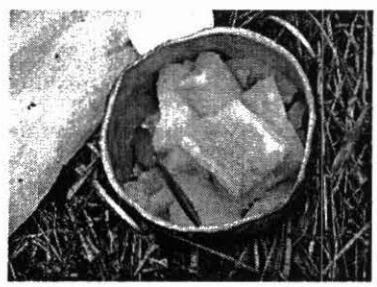


Yield of in vitro plant under farmer conditions.



Yield of in vitro plant under farmer conditions

A new clon (CM 2772-3) tested with farmers. Its has a yellow pulp



A. Production of planting material of the whitefly-resistant cassava cultivar Nataima-31:

The Tropical Whitefly IPM Project, with the financing of the New Zealand Agency for International Development identified resistance to the whitefly (*A. Socialis)* in cassava clones from Ecuador and Peru. This resistance was used to bread a high-yield cassava cultivar possessing high levels of resistance to whitefly damage: The cultivar "Natiama-31".
In June 2002, the Colombian Corporation for Agricultural Research (Corpoica), the Colombian Ministry of Agriculture and Rural Development (MADR), and CIAT released the new cultivar in Colombia "Nataima-31" being a good option because it yields well average 33 tons of fresh roots/Hectare and does not need pesticide application for whitefly control, which maximizes household income for cassava farmers. However, there is not enough planting material of "Natima-31" to satisfy its demand. This project aims to produce two seed lots of "Nataima-31", consisted of 5000 plants for a cassava development project (Polo de Desarrollo) managed by CIAT-MADR, and 500 plants for small farmers' associations (AMUC) to produce good quality

planting material, free of diseases such as FSD. These part of our project allowed establishment of local seed's plot that permit AMUC distributed cutting among its partners.

B. Production of cassava planting material possessing tolerance to Frog Skin disease (FSD)

Frog Skin Disease (FSD) is a serious phytosanitary problem that affects cassava production in Colombia, and particularly in the department of Cauca. The causes of FSD are still uncertain and diseased cassava plants do not produce marketable toots.

Research conducted by the Virology Research Unit of CIAT has shown that some cassava clones are tolerant to FSD, resulting in acceptable yields despite being infected. The SB-2 project using tissue culture techniques aims to produce a seed plot with 500 plants of the FSD-tolerant genotypes: HMC-1, MPer 183, CM 7951-15, and SM 653-14 and Nataima-31.

C. Production of small-scale horticultural crops for food security and income generation

Discussion with different communities allowed identification of crops of interest for management in a small plot of 200 m^2. Farmers from Alegrias, Cauca, were not interested in working with these kinds of crops because they considered they required a lot of management. Four groups from Pescador and five from Santa Ana decided to participate in this part of the project.

We chose materials produced by the Horticultural Research Group of the National University of Colombia, Palmira, because they were developed with a low input-technology. The implementation of this activity has different purposes: (a) opening a space for women to discuss their expectations and interactions with men in agriculture activities–gender and participatory thematic were developed with a sociologist's support, (b) production activity as home food supply, (c) income generation possibility, and (d) open discussion about CBN.
Crops considered by participating farmers were: common beans, maize, summer squash, carrot, tomato, pepper, and coriander.

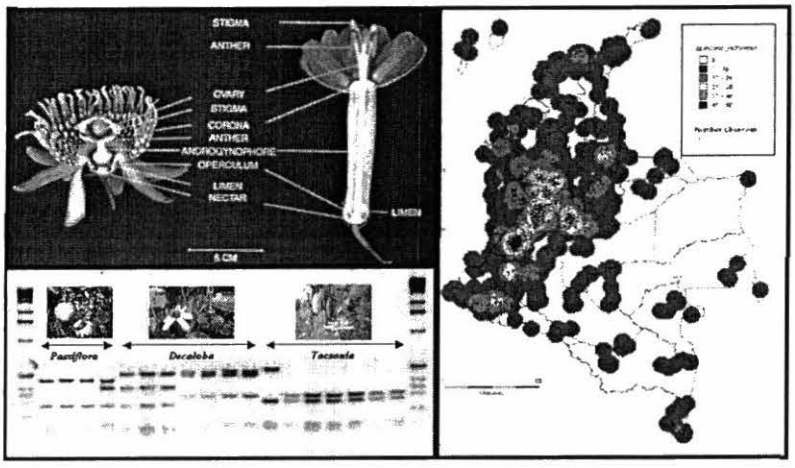
Ginés Mera Fellowship - Abstracts
Project Technical Report 2005- 2008
(Current activities)

Project Title:

DIVERSITY WITHIN THE GENUS PASSIFLORA (*PASSIFLORACEAE*) BASED ON ECOGEOGRAPHIC, MORPHOLOGICAL AND MOLECULAR EVIDENCE

John Ocampo, Montpellier University, France
John.ocampo@spymac.com

Colombia is the country that has the highest diversity of *Passifloraceae* species in cultivated and wild forms, both at intra- and inter-specific level. Among the cultivated species, grown because of their eatable fruits, the most important ones are: *P. tripartita* var. *mollissima* (curuba de castilla), *P. tarminiana* (curuba india), *P. ligularis* (granadilla), *P. maliformis* (granadilla de piedra), *P. quadrangularis* (badea), *P. popenovii* (grandilla de Quijos), and the two varieties P. *edulis*, *flavicarpa* (yellow maracuja) and *edulis* (purple maracuja). For these reasons, passion fruits could be used as biodiversity indicator in environmental studies, because anthropological and natural components involved. The distribution and the diversity of this family were studied using ecogeographical, morphological and molecular tools (CAPS-mt/cpDNA). Passport data of 162 Colombia species were gathered from 22 national and three international herbaria. Based on this inventory distribution maps were generated using DIVA-GIS. These maps were used to define zones showing a potential interest for collection activities. In 16 departments field collections where done, the collected material was included in a reference herbarium and into two *ex situ*. The distribution maps show a high concentration of passiflora species the Andean zone, mainly between 1000 and 2000 m., the area that also includes the highest anthropological activity in Colombia. Using a list of 125 morphological descriptors, species were divided into subgenera; descriptors related to the flower resulted to be the most discriminating ones. From the cytoplasmatic DNA analysis an apparent biparental inheritance of some species could be observed. Besides this, some taxonomic inconsistencies with respect to the present taxonomic classification were found. The obtained results and their extrapolation will allow the identification of factors causing genetic erosion and the possible risk areas. This new information will be used to define conservation practices and strategies, to promote fruit diversification and the *in situ* conservation.



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Project Title:

ISOLATION OF EXPRESSED SEQUENCES DURING THE DEFENSE RESPONSE OF MECU-72 TO WHITE FLY (*Aleurotrachelus socialis* Bondar) ATTACK USING SUBTRACTIVE LIBRARIES AND MICROARRAYS EXPRESSION OF cDNA

Adriana Bohorquez Chaux, Universidad Nacional de Colombia
a.bohorquez@cgiar.org

Abstract

I am on a Plant Breeding PhD program at the National University of Colombia (Palmira, Valle del Cauca), since January 2005. In going for this degree, I have taken on the added challenge of utilizing cutting edge scientific tools to contribute to the understanding of the mechanisms underpinning resistance to one of cassava's major production constraints.
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. 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. 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. The using subtractive libraries and microarrays profiles provides an immediate and productive method of gene discovery.

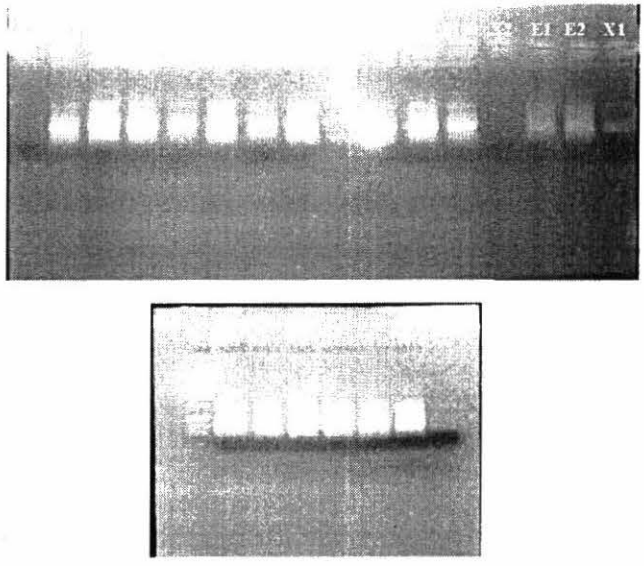
Project Objectives

To identify and access exotic or novel genes and gene combinations which can contribute to germplasm enhancement for whitefly resistance in cassava.

Isolation of expressed sequences during the defense response MEcu-72 to whitefly attack, using subtractive libraries and microarrays of cDNA.

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 CMD, 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. This endeavour and by so doing make definite contributions to advancing the global community's understanding of cassava's resistance to pests and diseases.

Amplicons of M: Mcol-2246 and E: Ecu-72 with Primers Bam I and Bam II for the subtraction (X1 and X2 negative controls)



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3.2.2 Current CBN – LAC Small Grants Scheme

	Name	Proyect Title	Country	Institution
1	Batista de Souza Claudia Regina	Aislamiento de secuencias promotoras de genes posiblemente involucrados en la formación7 de la raíz de reserva de yuca	Brazil	Universidad Federal do Pará – UFPA, Brazil
2	Francisco A.P. Campos	Development of transgenic varieties of cassava resistanse to infection by *Xanthomonas axonopodis* pv *manihotis*"	Brazil	Federal University of Ceara
3	Ricardo Medina	Análisis de proteinas, enziams y genes relacionados con la tuberización y biosíntesis de almidón de raíces tuberoas de mandioca (*Manihot esculenta* Crantz)	Argentina	IBONE
4	José Restrepo	Mejoramiento de la competitividad y productividad de los cultivadores de yuca de la región Andina de Colombia a través de la oferta tecnológica relacionada con la diversificación de variedades y uso de semilla certificada empleando propagación *in vitro*	Colombia	CIAT- Grupos locales del Norte del Cauca y Risaralda
5	Teresa Losada Valle	Production of high quality cassava planting material	Brazil	Laboratorio de Fitovirologia e Fisiopatologia Instituto Biológico Sao Paola
6	Luis Joaquim Castelo Branco	Cloning and characterization of the gene coding for branching enzyme in sugary and comercial cassava (*Manihot esculenta* Crantz)	Brazil	EMBRAPA /CENARGEN
7	Danilo Moreta	Identificación de genes que controlan la acumulación de azúcar en variedades de yuca de Ecuador	Ecuador	CIAT- INIAP
8	Sergio Rodriguez	Impacto de los Programas de Fitomejoramiento en la Producción de yuca, mediante biotecnologia participativa"	Cuba	INIVIT
9	James Montoya Lerma/A.M.Caicedo	Biochemical and Molecular Characterization of anti-imnmune compounds that suppress Cyrtomenus bergi Froeschner (Hemipter: Cydnidae) immune response	Colombia	UNI VALLE
10	Aldo Villar Trindade	Improving cassava a drought tolerance through enhanced mycorryzal simbiosis	Brazil	EMBRAPA
11	Yoel Beovides	Desarrollo de marcadores moleculares ligados a porcentaje de materia seca en yuca (Manihot esculenta Crantz) mediante técnicas de mejoramiento genético asistido por marcadores	Cuba	INIVIT
13	Margarita Hdez	Implementación de unidades de micropropagación en zonas rurales del occidente cubano para la obtención d material de plantación de alta calidad en cultivos de reproducción agámica	Cuba	INCA

Activity 3.3 DataBases and Libraries

3.3.1 Paddy Gene Book: A phenotypic database for a rice T-DNA insertion lines

M. Lorieux – J. Lozano – M. Bouniol – S. Acosta – 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

Fifteen 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, called *Paddy Genes Book*, 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 27,000 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

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Figure 1. Screenshots of the Paddy Genes Book phenotypic database
Figure 1a. Main menu

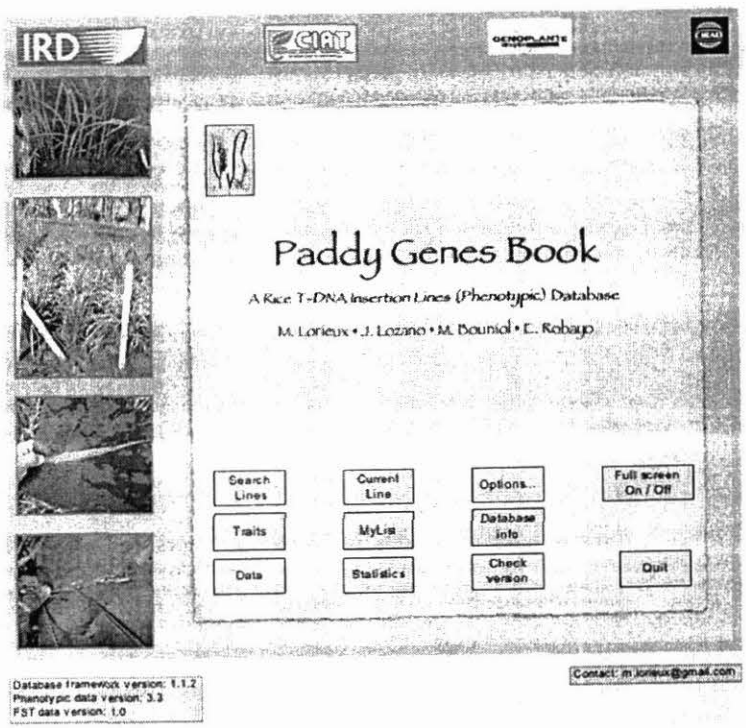


Figure 1b. Trait browsing

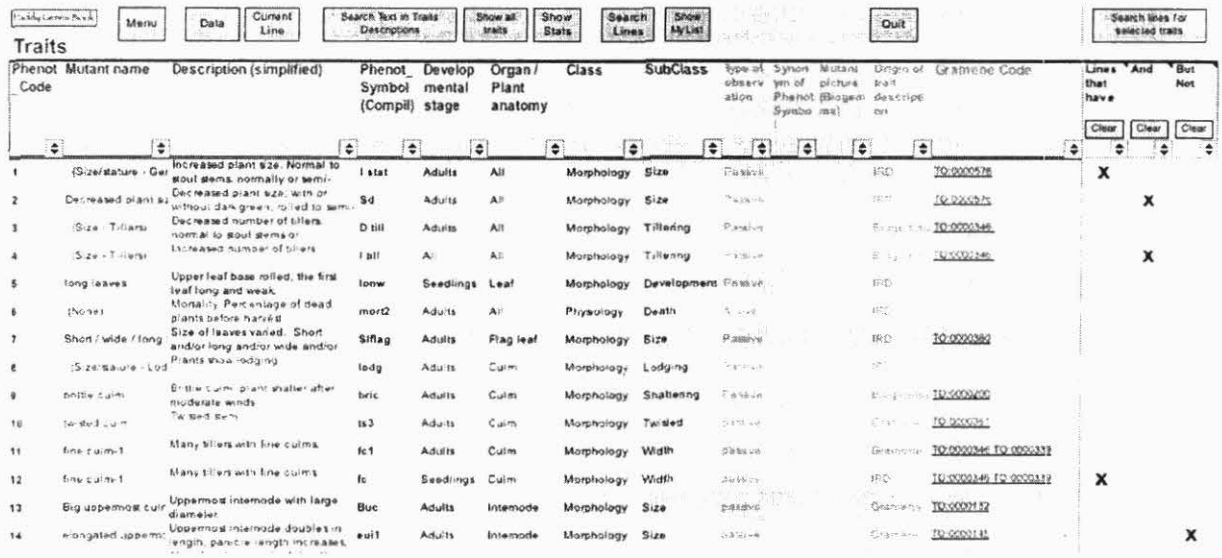


Figure 1c. Search for lines by trait code or full text

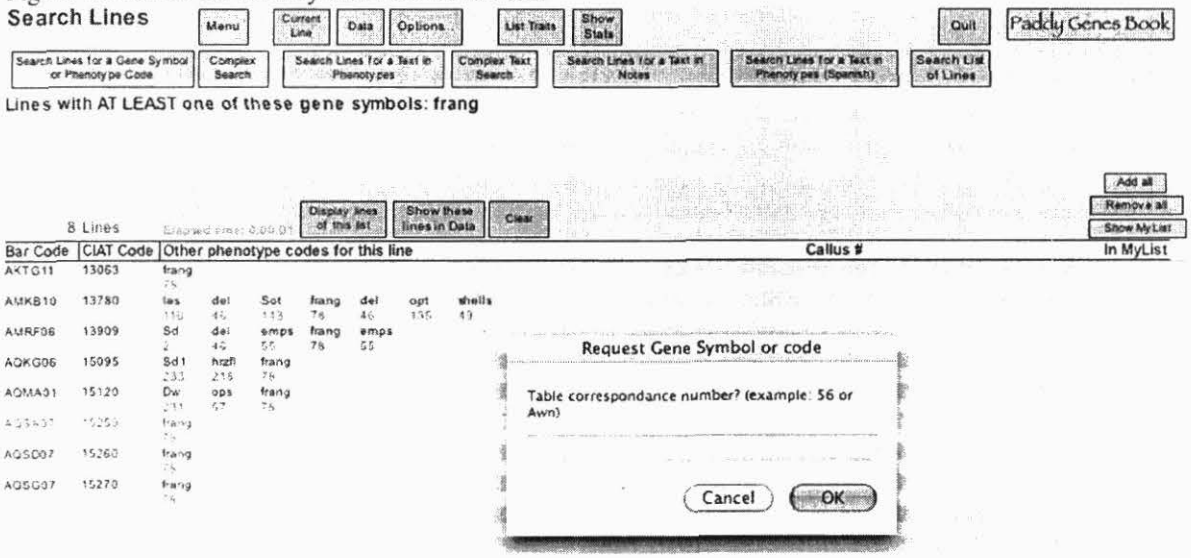


Figure 1d. Statistics on traits and lines

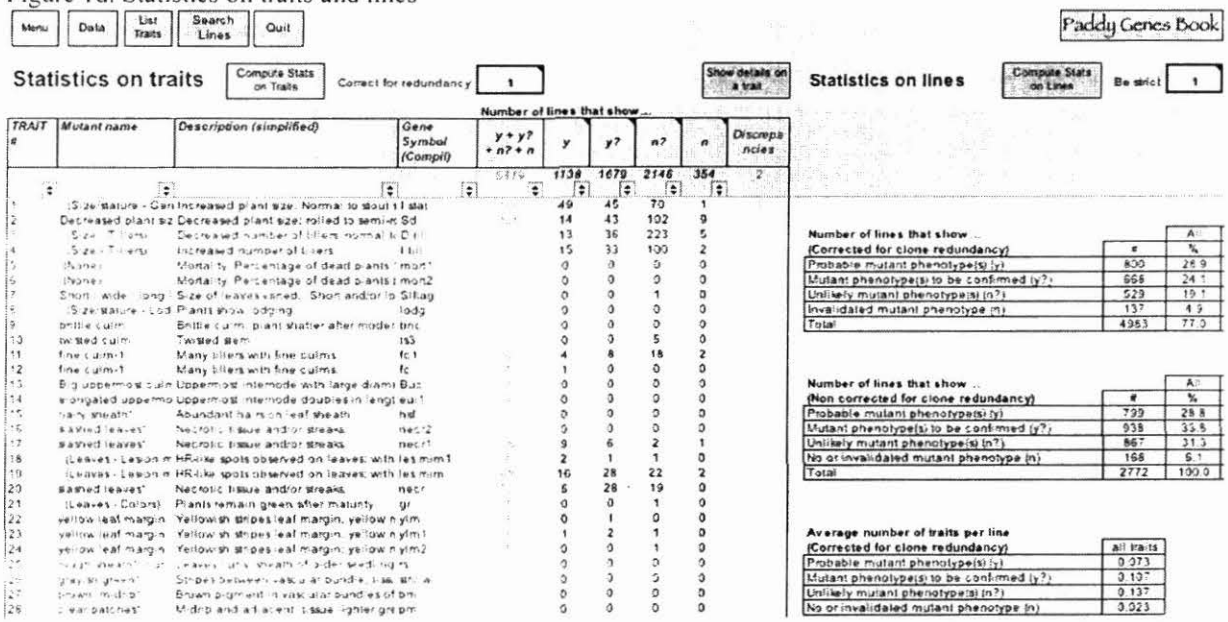


Figure 1e. Display of phenotype, codes, statistics, heading date graphics and photographs for each line

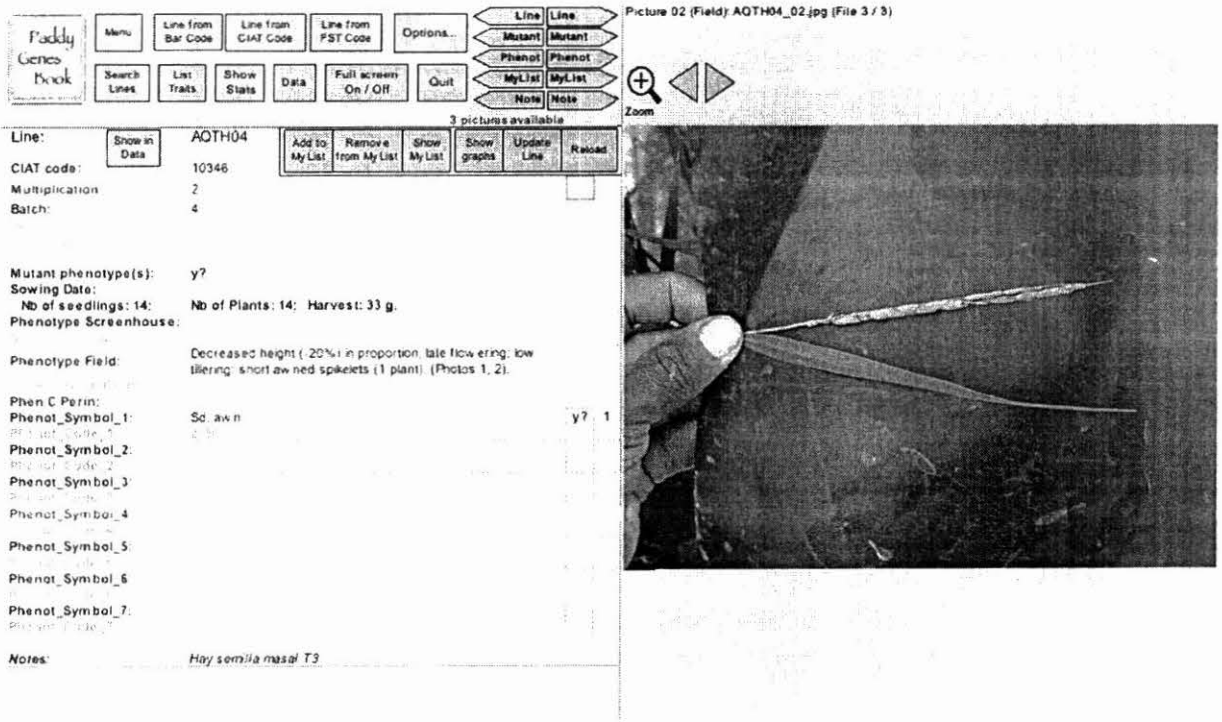
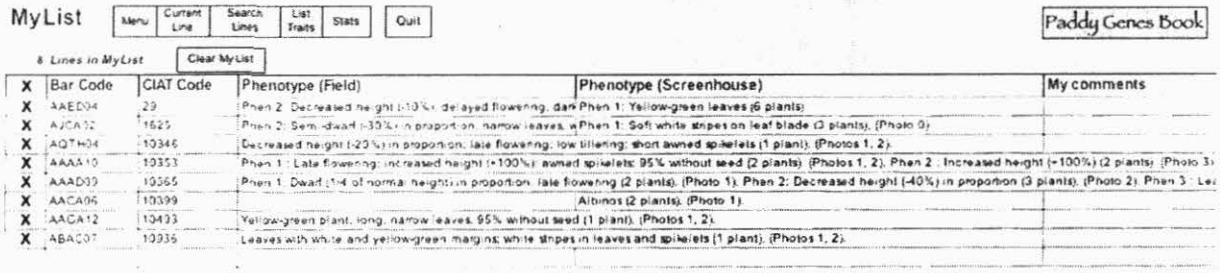


Figure 1f. Customized search list


MyList | Menu | Current Line | Search Lines | List Traits | Stats | Quit | Paddy Genes Book

8 Lines in MyList | Clear MyList

	Bar Code	CIAT Code	Phenotype (Field)	Phenotype (Screenhouse)	My comments
X	AAED04	29	Phen 2: Decreased height (-10%); delayed flowering; dark	Phen 1: Yellow-green leaves (6 plants)	
X	AJCA02	1625	Phen 2: Semi-dwarf (-30%) in proportion; narrow leaves; w	Phen 1: Soft white stripes on leaf blade (3 plants). (Photo 0)	
X	AQTH04	10346	Decreased height (-20%) in proportion; late flowering; low tillering; short awned spikelets (1 plant). (Photos 1, 2).		
X	AAAA10	10353	Phen 1 : Late flowering; increased height (+100%); awned spikelets; 95% without seed (2 plants). (Photos 1, 2). Phen 2 : Increased height (+100%) (2 plants). (Photo 3)		
X	AAAD09	10365	Phen 1: Dwarf (1/4 of normal height) in proportion; late flowering (2 plants). (Photo 1). Phen 2: Decreased height (-40%) in proportion (3 plants). (Photo 2). Phen 3 : Le		
X	AACA06	10399		Albinos (2 plants). (Photo 1).	
X	AACA12	10403	Yellow-green plant; long, narrow leaves; 95% without seed (1 plant). (Photos 1, 2).		
X	ABAC07	10936	Leaves with white and yellow-green margins; white stripes in leaves and spikelets (1 plant). (Photos 1, 2).		

3.3.2 Bio Informatics Support at CIAT 2005

Fernando Rojas ;Joe Tohme; Mathias Lorieux; GCP Participant Institutes

This Report brings information about the main activities involved in the Bio Informatics Support at CIAT in 2005 and will be focused in the 2 main topics:

Activities for Developing and installing Software Tools for BioInformatics for this year
The participation of CIAT in the Generation Challenge Program(GCP) – SP4 Subprogram.

Introduction

The CIAT Agrobiodiversity and Biotechnology project has been developing bioinformatics capacity in the following domains:

- development of annotated databases (e.g. cassava and bean ESTs – rice mutant phenotypes),

- implementation and development of bioinformatics tools (sequence analysis pipelines; retrieving, data mining and storage tools),

- software development (e.g. LIMS) in collaboration between CIAT researchers and specialists in software & algorithms development (ParqueSoft system engineers).

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.

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

PIPELINE CLUSTERING running and installed in our server for proceed to build the CONTIG DATABASE; USING GNU SOFTWARE (It's Based Mainly in the Perl Script of Perpignan University mailto:piegu@univ-perp.fr and the StackPack Tool; The stackPACK clustering system allows rapid clustering, alignment and analysis of EST's as well as full-length sequences by providing sophisticated gene indexing capabilities that go well beyond other clustering and assembly tools.

EST's for Cassava (Manihot Sculenta) Database and site Ready and in use : Actually we have established an EST's Database for Manihot Sculenta available at: http://gene3.ciat.cgiar.org/meest/html/ and the Contigs search tool at: http://gene3.ciat.cgiar.org/meest/html/bycn/cn1/

Support for the installation of the New version of LIMS: Demo at :
http://198.93.225.51:9000/axlims/
Login: guest
Pwd: anonymous

Maintenance and update of the CIAT Bio Informatics site (http://gene3.ciat.cgiar.org/blast/inicio.htm)

ICIS Website for Phaseolus (IPHIS) based in the ICIS 5 version in collaboration with IRRI (http://gene3.ciat.cgiar.org:8080/ICIS5) currently in implementation.
Molecular Map of Cassava being published at NCBI : Using the Map Viewer of NCBI it allows the representation of mapped objects (in the same, or different coordinate systems) to be displayed simultaneously. They have several genomes represented and Cassava will be the next; after we can start with Phaseolus Data too.
Bio Case Web Services adopted and installed in our server: http://gene3.ciat.cgiar.org/biocase/:
Under the GCP-Central Registry task we are suppose to make the data available to all GCP partners. In order to achieve this target, as a first layer, we propose to use BIOCASE wrapper through which our data can be linked to the central registry portal using templates created under the separate GCP task. At first we will start with the GCP passport schema and with time more schemas for other data sets will be added, as they get ready.

Pipeline for SNP's (Starting to work with ICRISAT for design and implement this Pipeline; including Phraph, Phred, Consed and Polybayes Packages.

Markers Database: Have designed a Database running under MySql for storage of information about distinct markers(SSR's RFLP's and others) for distinct crops(Cassava, Bean, Rice); currently loading data.
GCP (Generation Challenge Programme http://www.generationcp.org/):
Actually SP4 (BioInformatics Subprogram) have 3 big "PROJECTS":

4.1 Establishment of the GCP Information Platform
4.2. Improvement of the GCP Information Platform components
4.3. Creation of software in support of GCP activities

CIAT is involved and budgeted in:

Task 7: Improvement of Quality of Existing Databases
Task 8-15: Creation of Institutional BioInformatics Capacity

And is participating in (Not budget):

Task 2: Implementation of Web Services Technology in GCP Consortium
Task 3: Creation and maintenance of GCP repository

And could participate in:

Task 19: Development of Crop gene expression database and data minig tools
Task 20: Development of Decision support tools for MAS and MAB

Conclusion

Implementation of the Clustering Pipeline

Implement of Web-Services Technology Based in BioCase

Research and Install New Software tools as:

G-pipe: Graphical Pipeline Generator; PISE: Web Interfaces for G-Pipe

CIAT Expose SINGER(cassava, Bean, Forrages) data-banks with WEB-SERVICES technology

Continue working in the SP4 GCP.

Future Plans

Implement of Pipelines

Implement of Web-Services Technology Based in BioMoby

Continue working in the GCP SP4 tasks

AxLims Working
Grid Computing Projects

3.3.3 Contribution made towards protected areas in Latin America: databases about distribution of wild relatives of crops

We have continued with the establishment of databases about the geographic distribution of wild relatives for the so-called CIAT 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: BR, CR, CICY, ENCB, K, INB, M and USJ. 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, 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.

Contributor: D.G. Debouck

3.4 Training and Workshop Agrobiodiversity and Biotechnology Principal Staff

3.4.1 National and International Collaboration

Martinez, C. Attend and present a poster at Plant & Animal Genome Meeting. San Diego, California, USA. January 15-20/2005

Blair Mathew. Rockefeller Foundation Research meeting. Presented results of marker assisted selecion in bush and climbing beans. January 23 – 29, 2005

Tohme. Joe. Visit Universidad de Michigan and Iowa, USA, January 5-6, 2005

Tohme, Joe. Meeting Humboldt – CIAT. January 17 –18, 2005

Fregene, Martin. Attendance of the Rockefeller Foundation Second General Meeting on Breeding, Biotechnology, and Seed system of African crops. Harvest of the CIAT introductions in Tanzania. Trip to Kenya and Tanzania. January, 2005

Tohme, Joe. Retreatment Harvest, USA. January 23- February 2, 2005

Sperling, L. EARO/Melkassa, Etiopía. Impact-oriented bean Dissemination: collaborative survey results. February, 2005

Sperling, Louise. Ethiopian Bean Dissemination Analysis and innovation history workshop. Nairobi, Kenya and Kampala, Uganda. January 30-February 10, 2005

Martinez, C. Santa Cruz, Bolivia. Visit to Ciat-Bolivia and Aspar to coordinate Bolivia participation in the CIDA funded rice biofortification project. February 2-5.2005

Tohme, Joe. Meeting on Project: Conservation and sustainable use of wild relatives of Neotropical, IICA, Costa Rica. February 18-19, 2005

Martínez, C. Brazil.Visit to CNPAF-Goiania and Londrina-EMBRAPA to coordinate activities related to GCP and drought screening. February 21-25.2005

Tohme, Joe. Thesis Submitted: Fausto Rodriguez. Andes University , Bogotá-Colombia. March 2, 2005

Sperling, Louise. Reaching end User HarvestPlus Consultation. March 3-4, 2005.

Lentini, Z. Danforth Center. San Louis, Missouri, USA. Lentini Z. to represent CIAT in Preparatory meeting of The Public Research and Regulation Initiative (PRR) for Conference of the Parties MOP2 serving as the Meeting of the Parties to the Cartagena Protocol in Montreal, Canada, 2005. Elected member of steering committee. March 13-17, 2005.

Sperling, Louise. Support to MSc Students, Norweigian University of Life Science. OS, Norway. March 14-15, 2005

Debouck, D.G. Master's Degree programme in Plant Genetic Resources. Use of molecular markers for germplasm characterization. Universidad Nacional Agraria La Molina. Lima, Peru. March 2005.

Fregene Martin. Project planning meeting for Brazilian activities, held in Brazil, in: Embrapa/CNPMF, Cruz das Almas, BA (14-18 March 2005) and *Embrapa Headquarters and Embrapa/Cenargen,* Brasília, DF (17-18 March 2005). Brazil March 2005.

Tohme, Joe. Meeting COLCIENCIAS, Bogotá – Colombia. March 8, 2005

Tohme, Joe. Collaborative CIAT-USAID Projects. Visit bean Program in Malawi. March 11-26, 2005

Martinez, César and Manabu Ishitani. Visit with Dr. Fusaka Uga to Santa Rosa, Villavicencio headquarters, March 11-12, 2005

Debouck, D.G. Lima, Peru, 29 March 2005, invited conference in the symposium to honour D^{r} Miguel Holle: "La importancia de lo no-planificado en la planificación de trabajos en recursos fitogenéticos: layos del zorro y pichupa numías para los tiempos de filogeografía y genómica".

Lentini, Z. Development of Cassava Haploid Technology within the Course on Cassava Breeding for Africa. CIAT, Cali. April 2005

Tohme, Joe. Genomica Workshop. CEISA, Bogotá. April 5-6, 2005

Debouck, D.G. Mérida, Mexico, 12 April 2005, invited seminar at the Centro de Investigaciones Científicas de Yucatán: "El fríjol peregrino: sus viajes desde el Pliocénico tardío".

Ishitani, M. Workshop & Project meeting on "Development of Integrated Rice Cultivation System under Water-Saving Conditions" at IRRI, April 2005

Sperling, Louise. Participatory Plan Breeding Monitoring Tour. Nazret and Awasa, Ethiopian; Arusha Tanzania; and Kabale Uganda. April 24-May 7, 2005.

Tohme, Joe. Biosafety Projects, Bogotá. April 26-27, 2005

Martinez C. Panama. Attend and present a paper at LI Reunión Anual PCCMCA on activities related to High iron and zinc rice lines. May 2-6,2005.

González, R.I., Palmira, Colombia, 21 May 2005. Presentation during Workshop on Genebanks operations for Botanical Gardens. "Estudio y caracterización de la diversidad genética". CIAT.

Tohme, Joe. Meeting with Dr. Elizabeth Bray, Chicago University. HarvestPlus Meeting, Washington and Visit North Caroline University, USA. April 29 – May 5, 2005

González, R.I.,Ciudad de Panama, Panama, 5 May 2005. Presentation realized by Rodolfo Araya during Workshop LI of PCCMCA. "Flujo genético en frijol común: consideraciones para el manejo de cultivos genéticamente modificados".

Fregene Martin. Attendance of the CIAT board meeting in Uganda, Visit to GCP project at NAARI, Namulonge, Visit to South African collaborators with Dr Joachim Voss, Visit to the GCP projects at NRCRI, Umudike, Nigeria, and CRI, Kumasi, Trip to Ghana. Uganda, Tanzania, South Africa, Nigeria, Ghana. May17- July 2, 2005.

Sperling L. NARO/Uganda: Recent Advances in Participatory Plant Breeding: Focus on Methods. May, 2005

Louise, Sperling. CIAT Africa Staff Meeting and Reaching End User HarvestPlus Meeting. Kampala, Uganda. May 30-June 6, 2005

Martínez, C. Caracas, Venezuela. Participate in "Workshop Herramientas Moleculares para el Mejorameinto de la Tolerancia al Stress Abiótico en Plantas", organized jointly by IVIC, INIA, FONACIT, CIET, MCT,and CIAT funded by UNESCO. July 4-8, 2005.

Fregene Martin. Attendance of a biotech conference on novel approaches for designing disease resistance in crops using genomics; Visit to collaborators at the IBRC to discuss a new genomics project on cassava in collaboration with South Africa to be submitted to JSPS. Trip to Japan. July 22-August 1, 2005.

Tohme, Joe. Meeting HarvestPlus, CIMMYT, México. May 18 – June 1, 2005

Martínez, C. La Habana, Cuba. Present a paper on the CIDA –funded Rice Biofortification Project at the III Encuentro Internacional del Arroz and carry out a Workshop on Rice Biofortification with national rice programs participating in this project. June 6-10,2005.

Tohme, Joe. Meeting with Drs. Maria H.Bonilla, Edgar Echeverry – Genoma del café, Bogotá, Colombia. June 17, 2005

Tohme, Joe. Meeting with Planeación Nacional on Biotechnology for Vision 2019 Document, Bogotá - Colombia. June 28, 2005

Lentini Z and Ishitani, M. Molecular tools for the improvement of plant tolerance to drought stress. IVIC (*Instituto Venezolano para Investigaciones Científicas*). Transgenic crops and drought tolerance: The case of rice. Environmental Biosafety. July 4-8, 2005. Caracas, Venezuela.

Fregene, M. Trip to Japan. Attendance of a biotech conference on novel approaches for designing disease resistance *in crops using genomics.* Visit to collaborators at the IBRC to discuss a new genomics project on cassava in collaboration with South Africa to be submitted to JSPS.

Tohme, Joe. Thesis Submitted: Carolina Rosero – Andes University. July 27, 2005

Martínez, César. Evaluation and selection rice lines. Villavicencio, August 01, 2005

Tohme, Joe. Meeting with Dr. Francisco Arias Invimar, Bogotá – Colombia. August 07,2005

Debouck, D.G. San José, Costa Rica, 10 August 2005, invited conference: "Gallo pinto en horas de la mondialización: cómo añadir valor a la agrobiodiversidad?".

Mejía Alvaro, Biotechnology Fruit Consortium Meeting. Bogotá, August *11-13, 05*

M. Blair. Attended subprogram 1 genotyping workshop of Generation Challenge Program as Bean Crop Leader – Chennai, India. Present results of genotyping experiments. August 22-26, 2005

Fregene Martin. Attendance of the 2nd general meeting of the African Molecular marker network (AMMANET) in Nairobi and the GCP Drought planning meeting at IITA-ILTAB in Nairobi. Attendance of the RF funded PPB and MAS workshop to train NARs partners. Attendance of the GCP annual meeting in Rome. Attendance of a meeting on mapping wild QTLs and project planning for new projects involving wild *Manihot* species at SLU, Uppsala, Sweden. Trip to Kenya, Tanzania, Italy, and Sweden August 28 – October 5 2005

Sperling, L. EARO/Addis - Ethiopia HQ: Seed Relief and Long-Term Seed Aid in Etiopía. September, 2005

Sperling, L. UN/FAO :Addis Invited Expert : Agricultural Emergency Task Force, Etiopía. Seed System Security Assessment Methodology. September, 2005

Debouck, D.G. Bogotá, Colombia, 2 September 2005, invited conference in the 1st Regional Workshop about Biopiracy in Latin America: "Observaciones sobre un frijol amarillo, o cuando un sistema de DPI extraviado llega a ser contraproduciente".

Sperling Louise. Launch of Long-term Seed Aid Special Project and Reaching EndUser Consultation. 5 sites in Ethiopian and Kampala, Uganda. September 12 – October 2, 2005.

Martínez, C. Brazil. Visit to CTAA-Embrapa in Rio de Janeiro, CNPAF-Goiania ,and Universdidade Rio Grande do Soul, Porto Alegre to coordinate activities related to CIDA-funded Rice Biofortification project. September 4-10,2005.

Blair Mathew. Bean/Cowpea CRSP all researchers meetings. Participated as part of the Bean/Cowpea CRSP technical committee and to give a presentation on future priorities of bean research. Senegal Sept 11 – 17, 2005

Lentini, Z.. Venezuela. To initiate germplasm collection of wild/ weedy rice in natural environments and rice fields. Status of Guarico, Cojedes and Portuguesa with National partners as collaborators of the BMZ funded gene flow project. September 19-23, 2005

Lentini, Z.. Porto Alegre, September 26 - 29, 2005. To represent CIAT in the IV Brazilian Congress on Biosafety and IV Symposium on Transgenic Products and have a preliminary meeting with National partners from Brazil, Costa Rica, Colombia, Mexico and Peru to prepare workshop for development of Regional Proposal on Biosafety funded by GEF/ World Bank. September 26 - 29, 2005

González, R.I., Cali, Colombia, 13 October 2005. Presentation during the XL National Congress of Biological Sciences. "Determinación de flujo de genes entre especies de frijol (*Phaseolus ssp.*) en Colombia y Costa Rica mediante marcadores microsatélites".

Lentini, Z. Biosafety in Centers of Biodiversity: Building Technical Capacity in Latin America for Safe Deployment of Transgenic Crops (A multi country approach for Brazil, Colombia, Costa Rica, Mexico and Peru). GEF/ World Bank. Cali. October 24-26, 2005.

Ishitani, Manabu Generation CP annual meeting, Rome, October 2005

Ishitani, Manabu. DREB meeting, Rome, October 2005

Ishitani, Manabu. Trip to Japan, MAFF, MOFA, JIRCAS and RIKEN, October 2005

Ishitani, Manabu. Starch Update, Bangkok, November 2005

Blair Mathew. Generation Challenge Program annual meeting – Rome, Italy – present results of TILLING experiments. September 28-30, 2005
Martínez, C. Visit to Nicaragua and Dominican Republic to discuss and supervise activities related to CIDA-funded Rice Biofortification Project. October 17-22, 2005.

Sperling Louise. Resource Person, CIMMYT Seed Systems Learning Workshop. Fregene, Italy. October 26, 2005

Fregene Martin. Trip to Japan and Thailand. Visit to scientific collaborators at University of Tokyo, JIRCAS, RIKKEN, and the Japanese ministry of foreign affairs (MOFA) and Ministry of Agriculture and Fisheries (MAFF). October 23-29, 2005, October 23-29, 05

Lentini, Z. Biosafety in Centers of Biodiversity: Building Technical Capacity in Latin America for Safe Deployment of Transgenic Crops (A multi country approach for Brazil, Colombia, Costa Rica, Mexico and Peru). GEF/ World Bank. Cali. October 24-26, 2005.

Blair Mathew. Attend Bean improvement Cooperative meetings (Newark, Delaware). Present results of Biofortification. Oct 31 – Nov 3, 2005

Sperling, L. CIMMYT/Rome Seed System Learning Workshop: Resource Person. Emergency Relief and Seed Systems: Experiences from Africa. October, 2005

Lentini, Z. Biosafety Course (Corpoica) within the Workshop Biotechnology Tools, genomics and proteomics for plant breeding. *October* 24-26,2005 CIAT, Cali.

Louise Sperling. Participatory Variety Selection Support tour for Souther Africa. Malawi, Mozambique, Zambia and South Africa. November 6-20, 2005.

Debouck, D.G. Popayán, Colombia, 8 November 2004, invited opening conference at the 3rd National Botanical Congress of Colombia: "Trenta mil años de tolerancia hacia ciertas plantas malezas en el Neotrópico".

Fregene Martin. Attendance of a conference on starch in Bangkok and a workshop on CIAT-Thailand cassava biotechnology collaborations; visit to cassava biotech projects at the Mahidol and Kasesart Universities and the Department of Agriculture biotechnology center. October 30 November 5, 2005

Martínez, C. Philippines. Attend and present a paper at the 5 th International Rice Genetics Symposium and participate in the *HP-Rice Crop Meeting*. November 16-26, 2005.

Fregene Martin. Trip to South Africa and Nigeria. Attendance of the 4th USAID-African partnership conference on biotechnology, November 14-18, 2005.

Debouck, D.G. Chapingo, Mexico, 17 November 2005, invited conference at the Universidad Autónoma de Chapingo in an international workshop organized by SOMEFI: "Las plantas viajeras: sus sueños, sus oportunidades, y sus limites".

Fregene Martin. Visit to NRCRI, Umudike, Nigeria, to discuss status of the cassava proposal submitted to the Government of Nigeria, November 20 – 25, 2005

Debouck, D.G. Montevideo, Uruguay, 24 November 2005, invited conference in the V Simposio de Recursos Genéticos de América Latina y Caribe: "Cuantas veces vino el poroto a nuestra Terra Australis? Reflexiones sobre la formación de los Recursos Fitogenéticos".

Martínez, C. New Haven, CT. Visit to Steve Dellaporta'Lab at Yale University to review status of the CIAT-Yale collaborative Project and discuss future plans. November 28-30,2005.

Blair Mathew. Phaseomics – Salta, Argentina - attended as a member of the organizing committee and to coordinate with collaborators from the University of Geneva for TILLING project. Nov. 29 – Dec 3, 2005

Lentini, Z. Tracing tansgenes in crop-wild-weedy populations. GEF/World Bank Colombia Biosafety Project. Ministry of Agriculture. December 5-6, 2005. CIAT, Colombia

Lentini, Z. Maracay. Venezuela. Lentini Z. To represent CIAT in the Tri-Annual meeting of the RedBio-Venezuelan Chapter of the Society on Plant Biotechnology with lectures in two symposia (rice and biosafety). December 5-9, 2005.

Tohme, Joe. Attendance to HarvestPlus Rice Crop Meeting on 24-25 November at IRRI. November- December 6, 05

Ishitani, M. CGIAR annual meeting, Morocco, December 2005

Louise Sperling. 2nd Meeting Long-term seed aid Project. Addis Ababa, Ethiopia. December 12-18,2005

Lentini, Z. Kansas City, Missouri. To attend symposium on Crop Gene Flow and The Occurrence and Consequences of Gene Introgression between Crops and Their Sexually Compatible Relatives, establish contact with Hector Quemada (WMSU) to be contracted as a consultant for developing Regional Proposal on Biosafety funded by GEF/ World Bank, and establish contact with Barbara Shall population geneticist (member of USA NAS) for collaboration in rice research and development of rice project .December 13-14, 2005.

3.4.2. Trainning, Visiting, Workshops, International and National Conferences for Agrobiodiversity and Biotechnology Personnel

Amsalu Berhanu. EARO /Melkasssa

Asfaw Asrat. Southern Agricultural Research Institute Awasa, Ethiopia.

Assefa Teshale. Ethiopian Agricultural Research Organization. Nazareth Ethiopia.

Bekele Adam. EARO/Melkassa

Dagne Belete. EARO/Melkassa

Kanyenga Lubugo Antoine. INERA Kipopo

Mbombesa, Festo. SARI. Arusha, Tanzania

MuiMui Kennedy. Zambia

Muthalmia John. KARI. Embu

Namayanja Annet. National Agricultural Research Organization Uganda.

Nsanzabera Felicite. Institut des Sciences Agronomique du Rwanda.

Nkonko Mbikayi. INERA Mulungo DRC.

Okwiri, P. Ojwang. KARI. Katumani. Kenya

Bernal Diana, Soto Mauricio. Min Agricultura, Thematic Genomic workshop – MADR. November, 2004

Caicedo, Elizabeth, CBN Activities. Santander de Quilichao and Pescador, Colombia. November, 2004

Caicedo, Elizabeth. Workshop CIAT with AMUC community. Integral strengthening of the cassava cultivation in the Cauca, Colombia, November 2004

Rojas Juan Jairo, Plantas evaluation. Pescador Cali. January 18, 2005

Yuko Ogo, Tokyo University, Japan-CGIAR Fellowship Program, 8 weeks, January 2005

Chavarriaga, Paul. Thesis submitted Felipe Sarmiento. January 28-29, 2005,

Romero Catalina. Interview with Harvard University. PhD. Program. January 28 –February 02, 2005.

Rodriguez Fausto. Molecular Biology and Bioinformatics. CIAT February 4-5, 2005

Giraldo Olga Ximena. Thesis Submitted Jose Leonardo Bocanegra. Tolima University, Tolima Colombia. February 3-4, 2005

Soto Mauricio. Work on Molecular Biology in Rice and Yuca. IRD, France. February 5- March 24, 2005

Université de Perpignan (Perpignan, France). February 05-2005 to april-16-2005. Working with Microarray analysis and Gene expression analysis using Real-Time PCR technology.

Rojas, Fernando, ICARDA – SYRIA, February 2005. Participation in the Singer Workshop

Rojas Fernando. Numhems – Holland , Feb 2005 Participation in the ICIS Workshop

Galindo Leonardo Miguel. Conference Javeriana University , Cali –Colombia. February 15, 2005

Romero Catalina. Interview with Stanford University. PhD. Program. February 27 – March 6, 2005.

Chavarriaga Paul. Biosafety Meeting. Danforth, USA. March 2-7, 2005

Mafla G., Curso Internacional sobre sistemas modernos de producción, procesamiento y utilización de yuca': Conservación del germoplasma *in vitro* de la yuca. 18 – 27 April, 2005, CIAT, Cali, Colombia.

Salcedo, J.M. 21 April 2005. Invited presentation at 'Curso: Biodiversidad: conocer, conservar y utilizar'. "Bancos de semillas como una alternativa de conservación *ex situ*". Universidad Nacional, sede Palmira, Colombia.

Duque Myriam Cristina. Mixed models course. CATIE. Costa Rica. March 13-19, 2005

Caicedo Elizabeth. CBN Activities, Cauca. March 8 –10, 2005

Escobar Roosevelt. Visited Industrias del Maíz. Barranquilla, Colombia. April 5-6, 2005

Escobar Roosevelt. Technological transfer of cocoa and avocado Project. Results. April 18, 2005

Mafla Bohórquez, G. 18 April 2005. Invited presentation at 'Curso Internacional sobre sistemas modernos de producción, procesamiento y utilización de yuca': Conservación del germoplasma *in vitro* de la yuca. Palmira, Colombia.

Romero Catalina. Visited Harvard University. April 2-6, 2005

Escobar Roosevelt. Visited CENICAÑA. Florida, Valle, Colombia. April 27, 2005

Gallego Gerardo. COLCIENCIAS Meeting. Colciencias, Bogotá May 6, 2005

Chacón Juliana. Thesis Submitted corresponding Ginés Mera fellowship. Andes University, Bogotá Colombia. May 17, 2005

Ocampo, C.H. May 21, 2005. Presentation during the meeting of the Botanic Gardens Network in Colombia: Marcadores bioquímicos y moleculares para la caracterización genética del germoplasma vegetal. CIAT.

Rojas Fernando. IPGRI - Rome , June 2005. Participation in the Generation Challenge Program (GCP) -Web- Services Workshop

SB-2 Personnel attended Latin-American day of bioinformatica. Cartagena. June 20-24, 2005

Soto, Mauricio. Cartagena, Colombia. June 21-25. Bioinformatics meeting. Bioinformatics Latin American Network (RIB).

Soto, Mauricio. University of Wisconsin, Madison. June 27-28, 2005. USA. Connecting Underrepresented High School Students with Genomics Workshop.

Gallego Gerardo. Thesis submitted Carolina Rosero. Javeriana University. Bogotá. July 18, 2005

Chavarriaga Paul. Curso Análisis estadistico de datos e interpretación. Tecnológica de Pereira. Julio 29 2005.

Chavez Lucia. Carotens Analysis. Brazil Sao Paulo. EMBRAPA CNPMF, Salvador, Brazil. August 20, 2005

Rojas Fernando IPGRI - Rome ,September 2005. Participation in the GCP 2005 Annual Research meeting

Rojas Fernando. Participate In the Generation Challenge Programme Annual Research Meeeting at Rome. October, 04, 2005

Mafla G, Master's Degree programme in Plant Genetic Resources, Universidad Nacional de Colombia. October 2005.

Villafañe Fausto. Advanced Bioinformatics Course. Cold Spring Harbor, USA. October 11 – November 04, 2005

Gallego Gerardo. To attend meeting with Colombian Asociacion of Biological Science. Universidad Autónoma. October 11-14, 2005

Kyoko Miwa, Tokyo University, Japan-CGIAR Fellowship Program, 8 weeks, November 2005

Staff Sb-2 participated in: Two worshops were organized and conducted in collaboration with national research institutions. The first one dealt with "Rice biofortification" with Instituto de Investigaciones del Arroz en La Habana, Cuba, and the second one "Herramientas moleculares para el mejoramiento de la tolerancia al stress abiótico en plantas" coordinated by IVIC, Venezuela. Around 30-35 participants from Colombia, Venezuela, Cuba, Nicaragua, Dominican Republic, Bolivia, Chile, Brazil and Argentina were trained in each workshop.

Staff SB-2 participated in the II National Course on Hybridization and analysis of DNA Microarrays. December 5-7, 2005.

3.4.3 Current Students

Acherman Jessica. B.S.c. GIS – studying climbing bean adaptation with A. Jarvis and M. Blair.

Adeyemo Sarah. 2005-2006. PhD. Flowers, Fruits and Roots: Modification of flowering to improve traits of agricultural importance
University of Cologne, Germany.

Bengtsson Frida. 2005- MSc. The wider context of seed wouchers and fairs. Norway University of Life Science.

Bohorquez, Adriana. 2005- 2008. MSc. Isolation of expressed sequences during the defense response of Mecu-72 to white fly attack using substractive libraries and microarrays expression of cDNA. Universidad Nacional, Palmira, Colombia. Ginés Mera Fellowship.

Castro Ana Maria. M.Sc. Universidad de Valle, Cali, Colombia (expected finish date October 2006)

Chavarro Carolina. B.S.c. Univ. Javeriana – Bogotá (since June 2005) – studying heat tolerance in Andean common beans with M. Blair

Cichy Karen. PhD program, Dept of Agronomy, Michigan State University (visiting student – Fullbright Fellowship) studied low P adaptation in Andean beans – joint with M. Blair and S. Snapp.

Cruz Murillo Anna (Colombia) PhD. Universidad Nacional de Colombia (expected finish date October 2007)

Díaz Juan Manuel. M.S.c. Universidad Nacional – Palmira, Colombia (since Jan 2004) - evaluation of genetic diversity in Andean accessions of the common bean core collection with M. Blair.

Diaz Lucy. M.S.c. Universidad Nacional – Palmira, Colombia (since Jan 2004) – evaluation of genetic diversity in Mesoamerican accessions of the common bean core collection with M. Blair

Garavito Andrea. PhD. Búsqueda de genes de respuesta a la inoculación del arroz por Bradyrhizobium. CIAT-Montpellier University. November 1, 2005 – October 31, 2008

Garzón Luz Nayibe. – M.S.c.– Universidad Nacional – Bogotá, Colombia (Sept – Dec 2004) – development of molecular markers for anthracnose resistance in common bean and their application with M. Blair and G. Ligareto.

López Obando Mauricio. 2005-2006. BSc. Producción y purificación de la proteína recombinante fusionada NusA-cry1Ab, a partir de la expresión del gene en una cepa de E. coli con t-rna extras para codones poco frecuentes en bacterias. UNIVALLE, Cali, Colombia.

López Danilo. 2005-2006. MSc. Evaluación Agronómica de transgénicos de yuca en el campo. Universidad Nacional de Colombia, Palmira, Colombia

Lozano Henry. B.S.c. Universidad Nacional – Palmira, Colombia (July 2003 – Dec 2004) Advanced backcross – studying micronutrient content in wild common bean crosses with M. Blair.

Macia Eliana. (Colombia) Universidad de Valle, Cali, Colombia (expected finish date October 2006)

Manrique Carpintero Norma C. 2005-2006. MSc. Evaluación de nuevas condiciones de crioconservación de ápices de yuca "*Manihot esculenta* Crantz" para genotipos de respuesta baja. Universidad Internacional de Andalucia, Sede Iberoamericana Santa Maria de la Rábida. España.

Marín Diana Ximena/ Ana Lucía Henao –B.S.c. Univ. Javeriana – Cali, Colombia (Sept 2004 to Sept 2005) carrying out economic analysis of climbing beans in five department of Colombia with N. Jonson/C. Gonzalez and M. Blair.

Montoya Carmenza. 2005-2006. MSc. Marker assisted selection in snapbean backcrosses for "arrugamiento foliar" virus. Plant Breeding. Universidad Nacional de Colombia, Palmira, Colombia.

Ochoa Iván. PhD. Pennsylvania State University, USA – (Jan 2004) genetic mapping of low phosphorus tolerance in common bean. Jointly with M. Blair and J. Lynch.

Okai Elizabeth (Ghana) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date September 2007)

Oviedo Tatiana, Intern (Colombia) Universidad de Tolima, Ibague, Colombia (expected finish date December 2006)

Ojulong Henry (Uganda) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date September 2006)

Olalekan Akinbo (Nigeria) Ph.D. student University of the Free State, Bloemfontein, South Africa (expected finish date December 2007)

Pachón Jorge. PhD. Universidad Nacional de Palmira, Colombia. 2005-2007

Pantoja Wilfredo. M.S.c. Universidad Nacional – Palmira, Colombia (since Jan 2004) – evaluation of genetic diversity in Tepary bean accessions with M. Blair

Quintero, Carmenza. 2004-2006. MSc. Genetic Mapping and use of single nucleotide polymorphism (SNPs) for the characterization of common bean (*Phaseolus vulgaris* L.) germplasm. Plant Genetic Resources. Universidad Nacional de Colombia, Palmira, Colombia.

Roda, Federico. 2005-206. BSc. Aislamiento de la secuencia del gen homólogo al gen mir, candidato a mediador molecular en la resistencia de Brachiaria al salivazo. Universidad Nacional, Bogotá, Colombia

Rodriguez Nohra. M.S.c. program, Universidad Nacional – Bogotá, Colombia (visiting student) studying marker assisted selection with M. Blair

Sanabria Yamid. 2005-2006. BSc. Determinación Morfológica, citogenética y molecular de una especie tetraploide del género Oryza y evaluación de introgresiones en progenies F1, BC2 y BC3 originadas de cruces con O.sativa. Universidad del Tolima, Colombia

Sandoval Tito. – B.S.c. Univ. Del Valle – Cali, Colombia (since January 2005) studying phytate accumulation in common bean with M. Blair

Santana Gloria. PhD program, Univ. Nacional – Palmira, Colombia – studying resistance to bean common mosaic virus with M. Blair, F. Morales and G. Ligareto.

Tsegaye Bayush. 2005. PhD. Incentives for on-farm conservation in a centre of diversity in a centre of diversity: a case study of durum what (*triticum turgidum* L.) landraces from East Shewa, central Ethiopia.

Velez Leon Dario. PhD. Universidad Nacional – Bogotá, Colombia (July – Dec 2004) – studying the inheritance of intercropping ability between common bean and maize – joint with M. Blair and G. Ligareto.

West Jennifer Joy. 2005. MSc. The wider context of seed vouchers and fairs. Norway University of Life Science. (Joint Thesis).

Zhang Xiaoyan– PhD program, CAAS – Beijing, China (visiting student) – evaluation of genetic diversity in Chinese accessions of common bean with M. Blair.

3.4.4 Visiting Researchers

Angulo Luis Rafael, Universidad Central de Venezuela. November 21- December, 17 2005

Agudelo Ospina Edilson de Jesus. Colegio Educativa Santo Tomás. August 26 – September, 2005

Alzate Adriana Mercedes. Colombia. October 1, 2003 – September, 2005

Amsalu Berhanu. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Angle Fernando. Research Assistant Cenicaña. Genetic transformation mediated by *Agrobacterium*, Colombia. 2005

Angulo Luis. Fundacite. Venezuela. November 21 – December 16, 2005

Arbelaez Juan David. Proyecto Genoplante-IRD –France. June 17, 2005 – June 10, 2006

Avila Teresa– Centro Fitogenético Pairumani / Univ. San Simon – Cochabamba, Bolivia (October-November 2005). – evaluation of genetic diversity in Bolivian accessions of common bean with M. Blair.

Assefa Teshale. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Asfaw, Asrat. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Barry Gerard. IRRI. Philippines. August 16, 2005

Bekele Adam. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Beovides Yoel. Instituto Nacional de Viandas Tropicales. Cuba. September 2005 – 2006

Bohorquez Chaux Adriana. Colombia. March 1 2005 – February 28, 2008

Bonilla Martha Liliana. Centro Nacional de Investigacion de Caña de Azucar, Colombia. February 7 2005 – March 11, 2005

Bouniol Mathieu. France. June 1, 2005 – May 9, 2006

Caicedo Ana Milena. Colombia. June 25, 2005 – September 30, 2006

Carvajal B.Sc. Mónica Carvajal. Training in molecular markers of *Phaseolus vulgaris* L. 21 July-21 Sept-2005.

Chiedozie Egesi (Nigeria), NRCRI, Umudike, Nigeria (start date December 12, 2005).

Crouch Jonathan. CIMMYT, México. August 16, 2005

Dagne Belete. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Dagnew Asmaré. Etiopia. March 14 - April 13, 2005

David Lisa. USA . May 11 – September 30 , 2005

De la Cruz Ramiro – INIA – Lara, Venezuela (November 2005)- training in nutrition breeding and biofortification program.

Diaz Carlos Arturo. UNIVALLE. Colombia. October 1, 2005 – February 28, 2006

Espinoza Rosa Melania from INIA, Venezuela was trained in seed and *in vitro* conservation at the Genetic Resources Unit, 5-14 September 2005.

Fujita Kayo. MAFF Research Council Secretariat of Japan. March 8-13, 2005

Gerena Jershon. Colombia. December 15, 2004 – February 15, 2005

Ghneim Herrera Thaura. IVIC. Venezuela. November 1-14, 2005

González Carolina. University of Perpignan. November 8, 2004 – January 28, 2005

González Alonso. DBIRD, Australia. February 21-23, 2005

Holmes Cristina. Canada. August 12, 2005 March 31, 2006

Hurtado Karen Andrea. Educativa Santo Tomas, School. August 26 – September 26, 2005

Iglesias Carlos. Hybrid Research Director Weaver Popcorn Company, USA. February 21-23, 2005

Jaimes Hugo Arley. Colombia. June 23 2005 – June 22, 2007

Jean José Mauricio, José Luis Rodríguez and Reynaldo Cajina. MAGFOR/Nicaragua, were trained in seed conservation at the Genetic Resources Unit, 22 –25 February, 2005.

Kanyenga Antoine. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Katsuyama Tatsuro. MAFF Japan. March 8-9,2005

Londoño Angela Maria. República de Israel, School. August 26-September, 2005

Lopez Jershon. Kansas State University. December 11, 2004 – February 15, 2005

Male Alan – CIAT-Uganda – pathology assistant (July 2005) – training in marker assisted selection with M. Blair, G. Mahuku and J. Tohme

Martinez Carlos Andrés. Universidad del Tolima. Training in biochemical and molecular markers of *Phaseolus vulgaris*. Sept 2005-January 2006.

Mbombesa Festo. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Miwa Kyoko, JIRCAS. Japan. November 4 – Diciembre 23, 2005

Miranda Sandra. Training in SDS-PAGE technique for phaseolin. Instituto Nacional de Ciencias Agrícolas de Cuba. 19-30 september, 2005.

Moges Asmare Dagnew. Ethiopian Agric. Research Organization. Ethiopia. March 14, April 22, 2005

Montenegro Maria Fernanda. Universidad Nacional de Colombia. Colombia. June 8 2004 – January 7, 2005

Montes Luis Adolfo. Guatemala, Universidad de Guatemala, Guatemala city

Morales Mabel. BSc. Javeriana University. Bogotá Colombia. Training on molecular tools for genetic molecular characterization of plants and to assess gene flow in rice and other species. August-December, 2005

Moreno Adriana Carolina. Educativa La Merced, School. August 26-September 26, 2005

Moreta Danilo. Instituto Nal. De Investigaciones Agropecuarias. Ecuador. October 24, 2005 – September 30 , 2006

MuiMui Kennedy. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Muthalmia John. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

National University - Palmira, Colombia

National University - Medellin, Colombia

National University of Education and Technology - Duitama, Colombia

Nacional University - Bogotá, Colombia

Namayanja Annet. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Nkonko, Mbikayi. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Nsanzabrera Felicite. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Ocampo John Albeiro. Colombia. November 1 2003 – December 31, 2006

Ogo Yugo, Jircas, Japón. February 2 – March 7, 2005

Okamoto Hiroya. Embassy of Japan in Bogotá. March 8-13, 2005

Okwiri P. Ojwang. Participatory Plant Breeding Monitoring tour. 15 days conducted for ECABREN and SABRN Networks, of behalf of PABRA. April – May 7, 2005. (L. Sperling)

Ospina Jose Omar. Fedearroz – Saldaña Colombia. 2005

Pachón García Jorge. Colombia. February 23, 2005 – February 21, 2006

Palacio Juan Diego. Instituto von Humboldt. Colombia. June 28 Agosto 31, 2005

Perez Iris. INIA. Venezuela. April 11 – 23, 2005. Use of molecular techniques as tools in rice breeding.

Perez Miguel – INIA – Maracay, Venezuela (November 2005) – training in marker assisted selection with M. Blair.

PRONACA - Ecuador

Professors from the Quito University (Ecuador)

Rohan Mc Donald, Conditioning and establishment of micropropagated plants Ministry of Agriculture St. Vincent and The Granadines, 28-30 September 2005.

Rodríguez, Fátima. Training in conservation and management of *in vitro* cassava germplasm INTA, Nicaragua, 17 November, 2005.

Quesada Lina Maria. Andes University. Colombia. June 13 –26, 2005

Quintero Marcela. Fedearroz – Villavicencio, Colombia, 2005

Radulovich Ricardo. Costa Rica University. February 21-24, 2005

Rauscher Jason. USA. March 19 – May 15, 2005

Reyes Ximena – Centro Fitogenético Pairumani / Univ. San Simon – Cochabamba, Bolivia (July-December 2005). – evaluation of genetic diversity in Bolivian accessions of common bean with M. Blair.

Royero Nelson. Universidad Nacional. Colombia. October 1, 2003- June 30, 2005

Research Assistants. Fedearroz, Villavicencio, Colombia, 2005

Rice Growers Committee – Saldaña, Tolima, Colombia

Saito Masayoshi. JIRCAS, Japan March 8-9, 2005

Sanchez Inés. CORPOICA. Colombia. February 22 1995 – December 31, 1005

SENA - Buga, Colombia

Senators of the Republic of Colombia

Suarez Luz Stella. Universidad Nacional de Colombia. Colombia. September 13 - 16, 2005

Tefera Wondyfraw. Ethiopian Agricul. Research Organization. Etiopia. March 14 – April 13, 2005

Torre Do Vale Carla Manuela. Instituto Nal. De Investigacion Agronómica. Mozambique. November 25 – December 19, 2005

Torrealba Gelis. INIA. Venezuela. August 1 – 31, 2005 . Use of molecular techniques as tools in rice breeding.

Torres Salvador Andres. BSc. *Universidad* San Francisco de Quito. Ecuador. Training on Molecular tools for genetic molecular characterization of plants and to assess gene flow in rice and other species. June 20 – August 17, 2005

Uga Yusaku, National Institute of Agrobiological Sciences - NIAS. Japan. March 8-12, 2005

Velasquez Nubia. UNALMED-CIB. Colombia. August 8-19, 2005

Verdier Valerie. IRD. Francia. November 8 2004 – January 27, 2005

The Professional Staff of the Genetic Resource Unit attended the visit of 223 people from 60 different government bodies, institutions, companies, etc. A total of 240 students from 12 different universities of Colombia visited the Genetic Resource Unit, on May and October 2005 through the Open House day coordinated by CIAT's Training Office.

3.5 Publicaciones

3.5.1 Refereed Journals, Books

Balyejusa Elizabeth Kizito, Anton Bua, Martin Fregene, Thomas Egwang, Urban Gullberg and Anna Westerbergh (2005). The effect of cassava mosaic disease on the genetic diversity of cassava in Uganda. Euphytica 146:45-54

Balyejusa Elizabeth, Kizito1,2, Linley Chiwona-Karltun1, Thomas Egwang2, Martin Fregene3 and Anna Westerbergh1 (2005). Genetic diversity and variety composition of cassava on small-scale farms in Uganda: An interdisciplinary study using genetic markers and farmer interviews (Accepted November 2005, in press).

Beebe SE, Rojas M, Yan X, Blair MW, Pedraza F, Muñoz F, Tohme J, Lynch JP (2005). Quantitative trait loci for root architecture traits correlated with phosphorus acquisition in Common Bean. Crop Science (in press)

Blair MW*, Muñoz C, Garza R, Cardona C (2005) Molecular mapping of genes for resistance to the bean pod weevil (Apion godmani Wagner) in common bean. Theor Appl Genet (*in press*).

Blair MW, Astudillo C, Restrepo J, Bravo LC, Villada D, Beebe SE (2005) Análisis multi-locacional de líneas de fríjol arbustivo con alto contenido de hierro en el departamento de Nariño. Fitotecnia Colombiana (in press)

Blair MW, Nin JC, Prophete E, Singh SP, Beaver JS (2005) Registration of Two Bean Golden Yellow Mosaic Virus Resistant, Large Red-Mottled Common Bean Germplasm. Crop Science (in press)

Castelblanco W. and Fregene M. (2005) Identification of Naturally Occuring Mutant Alleles of the Granule Bound Starch Synthase (GBSS) Gene in Wild Relatives of Cassava. Fields Crop Research (Submitted October 2005).

Castelblanco W. and Fregene M. (2005). SSCP-SNPs based Conserved Ortholog Set (COS) Markers for Comparative Genomics in Cassava (*Manihot esculenta* Crantz) (submitted November 2005).

Chacón M.I., Pickersgill B. & D.G. Debouck. 2005. Domestication patterns in common bean (*Phaseolus vulgaris* L.) and the origin of the Mesoamerican and Andean cultivated races. Theor. Appl. Genet 110 (3): 432-444.

Díaz LM, Díaz JM, Blair MW (2005) Diversidad genética de fríjol común (*Phaseolus vulgaris* L.) en Colombia. Fitotecnia Colombiana (in press)

Durán LA, Blair MW, Giraldo MC, Machiavelli R, Prophete E, Nin JC, Beaver JS (2005) Morphological and molecular characterization of common bean (*Phaseolus vulgaris* L) landraces from the Caribbean. Crop Science 45: 13320-1328.

Dwivedi SL, Blair MW, Upadhyaya HD, Serraj R, Balaji J, Buhariwalla HK, Ortiz R, Crouch, JH. (2005) Using Genomics to exploit grain legume biodiversity in plant breeding. In Jules Janick (ed) Plant Breeding Reviews, Volume 26 (December 2005) ISBN: 0-471-73215-X, 394 pp

Florez-Ramos, C.P.; Z. Lentini, M.E. Buitrago, and J. Cock. 2005. Somatic Embryogenesis and Plantlet Regeneration of Mango (*Mangifera indica* L.). Acta Horticulturae (In Press)

Fregene M. and Mba C. (2005). Molecular Marker-Assisted selection (MAS) in cassava genetic improvement. In: Genetic Improvement of Cassava Hershey C. (ed) FAO, Via Caravalle, Rome, Italy. (In press).

Fregene M. and Ceballos H. (2005). Advantages and Disadvantages of Molecular Marker-Assisted selection (MAS) in cassava breeding. In: Guimares E. (Editor). FAO, Via Caravalle, Rome, Italy. (Submitted Nov 2005)

Frei A, Blair MW, Cardona C, Beebe SE, Gu H, Dorn S (2005) Identification of Quantitative Trait Loci for resistance to *Thrips palmi* Karny in common bean (Phaseolus vulgaris L.). Crop Science 45: 379-387.13.

Kizito, Elizabeth Balyejusa , Anton Bua, Martin Fregene,
Thaura Ghneim, Alejandro Pieters, Iris Pérez Almeida, Gelis Torrealba, César P. Martínez, Mathias Lorieux and J.Tohme. 2005. Venezuela joins the global efforts for breeding water-saving and drought tolerant rice. Plant Breeding News, Cornell Univ. Clair Heresy(ed).

Lentini Z and A.M. Espinoza. 2005. Coexistence of Weedy Rice and Rice in Tropical America: Gene Flow and Genetic Diversity. Chapter 19. p: 303-319. *In:* J. Gressel (Ed.). "Crop Ferality and Volunteerism: A Threat to Food Security in the Transgenic Era?". CRC Press. Boca Raton, FL

López C., Soto-Suárez M., Restrepo S., Piegu B., Cooke R., Delseny M., Tohme J. and Verdier V. 2005. Global transcriptome analysis of cassava responses to *Xanthomonas axonopodis* pv. *manihotis* infection using a cassava cDNA microarray. Plant Molecular Biology. 57, pp. 393–410.

Marin J., Ospina C., Morante N., Sanchez T., Ceballos H., Fregene M.Genetic Mapping of Beta-Carotene Content in cassava (*Manihot esculenta* Cranz) (2005). Theor and Appl Genet (submitted November 2005).

Martinez C.P., SJCarabali, JBorrero, MCDuque and J.Silva.2005. Genetic progress towards grain quality in rice(O.sativa) through recurrent selection. In: Population improvement: Away of exploiting the genetic resources of Latin America. Elcio P. Guimaraes(ed). FAO, Rome.2005:277-297 p.

Miklas PN, Kelly JD, Beebe SE, Blair MW (2005) Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. Euphytica: Invited review paper for special issue on 'Legume resistance breeding' (in press)

Ocampo, C. H., Martín, J. P., Sánchez-Yélamo, M. D., Ortiz, J. M. & O. Toro. 2005. Tracing the origin of Spanish common bean (*Phaseolus vulgaris* L.) cultivars using biochemical and molecular markers. Genet. Resources & Crop Evol. 52: 33-40.

Okogbenin, Emmanuel, Jaime Alberto Marin, and Marting Fregene (2005). A SSR marker based Genetic Map of Cassava Euphytica (Accepted September 2005, in press).

Pedrosa-Harand A, de Souza Almeida CC, Mosiolek M, Blair MW, Schweizer D, Guerra M (2005) Extensive Ribosomal DNA Amplification during Andean Common Bean (*Phaseolus vulgaris* L.) Theor Appl Genet (in press)

Ramírez M, Graham MA, Blanco-López L, Silvente S, Blair MW, Hernández G, Vance CP, Lara M (2005) A foundation for functional genomics of Phaseolus vulgaris: sequencing and analysis of root nodule, P-stressed root, pod, and leaf ESTs. Plant Physiology 137: 1211-1227.

Ruiz, J.; V. Segovia, Z. Lentini, M. Buitrago, C. Flórez, and J. Cock. 2005. *In vitro* Propagation and Regeneration of *Solanum quitoense* (Lulo) Plants and their Use as Elite Clones by Resource Farmers. Somatic Embryogenesis and Plantlet Regeneration of Mango (*Mangifera indica* L.). Acta Horticulturae (In Press)

Soto-Suárez M. 2005. DNA Microarrays: Gene function using one miniature matrix. Journal *Innovación y Ciencia*, ISSN 0121-5140, Bogotá. Colombia. 12, 3, pp. 18-21.

Soto-Suárez, M. Restrepo, Silvia. Mosquera, Gloria. Verdier, Valérie y Tohme, Joe. 2005. Análisis de Expresión Génica Durante la Respuesta de Defensa de la Yuca a la Bacteriosis Vascular (Añublo Bacteriano). Revista Colombiana de Biotecnología. Vol. VII No. 2

Thomas Egwang, Urban Gullberg and Anna Westerbergh (2005). The effect of cassava mosaic disease on the genetic diversity of cassava in Uganda Euphytica 146:45-54.

Xia L., Peng K., Yang S., Wenzl P., de Vicente M.C., Fregene M., and Kilian A. (2005) DArT for high-throughput genotyping of cassava (*Manihot esculenta*) and its wild relatives Theoretical and Applied Genetics 110(6):1092-8.

Sperling, L., T. Remington, J. M Haugen, et S. Nagoda (editors). 2004. Addressing Seed Security in Disaster Response: Linking Relief with Development, Overview. Cali, Colombia: CIAT -- Centre international de l'agriculture tropicale.
URL : http://www.ciat.cgiar.org/africa/seeds.htm

Volume overview findings also translated and posted in: .

French : L'aide humanitaire d'urgence et la sécurité semencière :Lier le secours au développement : Une vue d'ensemble de la problématique
Portuguese. DESCRIÇÃO DA SEGURANÇA EM SEMENTES COMO RESPOSTA AOS DESASTRES LIGANDO À ASSISTÊNCIA COM O DESENVOLVIMENTO (translated with help of ICRISAT)

Sperling, L. and A. Christinck. In A. Christinck, E. Weltzien and V. Hoffman, eds. Designing Strategies for Seed Production and Distribution (for PPB) 2005. Setting Breeding Objectives and Developing Seed Systems with Farmers: A Handbook to Practical Use in Participatory Plant Breeding Projects. Margraf Publishers, Scientific Books.

Torres González A.M. & C.M. Morton. 2005. Molecular and morphological phylogenetic analysis of *Brachiaria* and *Urochloa* (Poaceae). Molec. Phylogenet. Evol. 37: 36-44.

3.5.2 Proceedings, Abstracts, Posters and Others

Almeida, A.; Olga X.Giraldo, C.P.Martínez, J.Borrero, MC Duque, and J.Tohme. Identification of QTLs for Yield and Yield Components from Wild Species of Oryza. Abstracts 5th International Rice Genetics Symposium.IRRI, Manila, Philippines,19-23 November 2005. p42.

Almeida, A.; Olga X.Giraldo, César P. Martínez, Jaime Borrero, Myriam C.Duque, Jerson López and J.Tohme. 2005. Identification of QTLs for Yield and Yield Components in a Backcross Population between *O.sativa* and Lemont. Plant & Animal Genome XIII. January 15-19, 2005. San Diego, Ca USA.P294(p146).

Aranzales, E., Mafla, G., Escobar, R. & D.G. Debouck. 2005. Establecimiento de protocolos para la conservación in vitro de especies de la familia Palmae: modelo de la palma de chontaduro (*Bactris gasipaes* Kunth.). V SIRGEALC –Simposio de Recursos Genéticos para América Latina y el Caribe. November 23-25, Montevideo, Uruguay.

Arcia K., J.C.Flórez, L. Fory, Eliana González, R. Pineda, T. Agrono, C. Ordóñez, M.C Duque y Zaida Lentini.2005.XL Congreso Nacional de Ciencias Biológicas. Evaluación del flujo de genes desde arroz transgénico y no transgénico hacia el arroz maleza bajo condiciones de campo.11 al 14 de Octubre. Cali, Colombia.

Balcázar, M.S., Rivera, Á. L. & Pineda L., B. 2005. Actividad antagónica de bacterias aisladas de semillas de *Brachiaria y* asociadas con hongos de *Brachiaria.* In: Memoria XLV Congreso annual de la Sociedad Americana de Fitopatologia Division Caribe, VI Congreso nacional de Fitopatolgia y I Congreso Nacional de Fitoprotección. San José, Costa Rica, @7 Junio-1 De Julio de 2005. P. 89.

Balcázar, M.S., Rivera, Á. L. & Pineda L., B. 2005. Actividad antagónica de bacterias aisladas de semillas de *Brachiaria y* asociadas con hongos de *Brachiaria.* XLV Congreso annual de la Sociedad Americana de Fitopatologia Division Caribe, VI Congreso nacional de Fitopatolgia y I Congreso Nacional de Fitoprotección.7 June-1 July 2005. San José, Costa Rica.

Blair MW, Astudillo C, Beebe S. (in press) Analysis of nutritional quality traits in an andean recombinant inbred line population. Annual Report of the Bean Improvement Cooperative 483.

Blair MW, Pantoja W, Beaver JS, Nin JC, Prophete E. (in press) Genetic diversity assessment of Caribbean common bean germplasm. Annual Report of the Bean Improvement Cooperative 484.

Carabali, S.J.; CPMartínez, J.Borrero, A.Gutiérrez, MC Duque, J.Silva. 2005. Evaluación del Progreso Genético para Calidad del Grano(centro blanco, longitud y temperatura de gelatinización) del Arroz (*O.sativa*) mediante selección recurrente. 10 Congreso Nal.Fitomejoramiento. Palmira, Valle. Mayo 10-14,2005.

González C., Soto-Suárez M., Piégu B. and Verdier V. 2005. Identification of differentially expressed genes induced in rice by *Xanthomonas oryzae* pv. *oryzae* by suppression subtractive hybridization and microarray analysis. 6ème Congrès de la Société Française de Phytopathologie. Toulouse, France, 23-25 February. Poster.

González C., Soto-Suárez M., Piégu B. and Verdier V. 2005. Identification of differentially expressed genes induced in rice by *Xanthomonas oryzae* pv. *oryzae* by suppression subtractive hybridization and microarray analysis. Asociación Colombiana de Ciencias Biológicas (ACCB). Cali, Colombia. 10-15 October. Lecturer.

González-Torres, R.I., O. Toro, M. Carvajal & D.G. Debouck. 2005. Presencia de complejos "Silvestre –Malez-Cultivo" de *Phaseolus vulgaris* L. en Costa Rica y Bolivia utilizando marcadores de AND nuclear y cloroplástico. V SIRGEALC –Simposio de Recursos Genéticos para América Latina y el Caribe. November 23-25, Montevideo, Uruguay.

González-Torres, R.I., Araya-Villalobos R. & D.G. Debouck. 2005. Gene flow and its effect on biodiversity: common bean as model for future considerations of biosafety. Deutscher Tropentag 2005: The Global Food & Product Chain-Dynamics, Innovations, Conflicts, Strategies. October 11 – 13, 2005, University of Hohenheim, Stuttgart, Germany.

Ishitani, M. Proceeding on "Root exudates of *Brachiaria humidicola* inhibit nitrification characterization and quantification of this unique biological phenomenon. *Plant nutrition for food security, human health and environmental protection*, 444-445, 2005

Kimani PM, Beebe S, Blair M. (in press) Effect of Rhizobia inoculation on Seed Iron and Zinc. Annual Report of the Bean Improvement Cooperative 485.

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 31: 959-970.16.

Salcedo, J.M & D.G. Debouck. 2005 Comparison of stigma areas among wild and cultivated forms (landrace and modern cultivars) of *Phaseolus vulgaris* L. Annu. Rept. Bean Improvement Coop. (USA) 48: 34-35.

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 4: 44-54.

Salcedo, J.M & D.G. Debouck. 2004. Comparación de areas en la region del estigma entre las formas silvestres y cultivadas (tradicionales y modernas) de *Phaseolus vulgaris* L. III Congreso Colombiano de Botánica. November 2004, Popayán, Colombia.

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 265: 17-29.

Blair Mw, Astudillo C, Caldas GV, Rengifo J, Beebe SE, Graham R, Grusak M. (2005) Progress and potential for improving micronutrient content in common beans. Bean Improvement Cooperative – Oct 31 – Nov 3, 2005.

Blair MW, Giraldo MC, Buendia HF, Tovar E, Pantoja W, Diaz L, Diaz JM, Duque MC, Beebe SE, Borba-Olivieras T, Brondani R, Peloso MJ, Zhang X, Wang S, Miranda-Lorigados S, Reyes X, Davila A, Avila T. (2005) Microsatellite marker diversity in common bean (*Phaseolus vulgaris* L.). Phaseomics IV. Nov 30-Dec 2, 2005.

Beebe SE, Blair MW, Tohme J (2005) Progreso y Estado Actual en Mejoramiento del Frijol para Mayores Niveles de Hierro y Zinc - PCCMCA (Regional agronomy meetings), Panamá - May 6, 2005

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"Biofortificacion: Mejoramiento nutricional" - Joe Tohme, Matthew Blair, Steve Beebe, Hernán Ceballos - XIII Congreso de Fitomejoramiento y .Cultivos – Palmira – *Presentation.* May 11, 2005

"*Phaseolus* / common bean genotyping: SP1 - Generation Challenge Program" – Matthew Blair - Chennai, India – *Presentation.* August 22, 2005

"Priority constraints to increasing productivity of beans in developing countries and strategies to overcome these: a CIAT perspective" - Matthew Blair, Steve Beebe, Rowland Chirwa, Paul Kimani - CRSP All researchers meeting – Dakar Senegal – *Presentation.* September 11-16, 2005.

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"Progress and potential for improving micronutrient content in common beans" – M.W. Blair, C. Astudillo, G.V. Caldas, J. Rengifo, S.E. Beebe, R. Graham, M. Grusak – Meeting of the Bean Improvement Cooperative – Oct 31 – Nov 3, 2005. *Presentation.*

Phenotypic variation, mapping and QTL analysis of adventitious rooting under low phosphorus stress in common bean (*Phaseolus vulgaris* L.) Ochoa I, Blair MW, Brown KM, Lynch JP – poster at Agronomy Society of America. *Poster.*

Analisis multi-locacional de lineas de frijol arbustivo con alto contenido de hierro en el departamento de Nariño. Blair MW, Astudillo C, Restrepo J, Ojeda P, Villada D, Beebe S - poster at XIII Congreso de Fitomejoramiento y Cultivos. *Poster.*

Adaptación y uso de un marcador SCAR para resistencia a BCMV para el gen bc-3 en un programa de mejoramiento de fríjol andino. HF Buendia, MW Blair, A Hoyos, M Castaño, F Morales - poster at XIII Congreso de Fitomejoramiento y Cultivos. *Posters.*

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Diversidad genética de razas andinas de fríjol comun *Phaseolus vulgaris* L. Díaz JM, Duque MC, Blair MW - poster at XIII Congreso de Fitomejoramiento y Cultivos. *Poster*

Diversidad Genética de Fríjol (*Phaseolus vulgaris*) en Colombia. LM Diaz, JM. Diaz, MC Duque, MW Blair - poster at XIII Congreso de Fitomejoramiento y Cultivos. *Poster*

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3.5.3. Thesis

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Buitrago Aya Carolina, 2005. BSc Biologia, "Evaluación de la patogenicidad y virulencia de *Bacillus popilliae* Dutky sobre larvas de tercer instar de Phyllophaga menetriesi Blanchard (Coleoptera:Melolonthidae)" Universidad del Valle, Cali, Colombia.

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Chaparro Catalina Astrid. 2005. MSc. Transformación genética mediante biobalística y evaluación de nuevos protocolos embriogénesis somática en aguacate (Persea americana Mill). Universidad Internacional de Andalucía y Universidad de Málaga , España.

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Guevara Edward. 2005. B.S.c. GIS – studying climbing bean adaptation with A. Jarvis and M. Blair.

Gutiérrez Andres Gonzalo 2005. BSc. Construcción y evaluación de líneas de introgresion interespecíficas de Arroz (Oryza sativa L.) Utilizando microsatélites. Universidad del Tolima, Colombia

Hernández, Erick Giovanni. B.Sc. Tesis: Caracterización genotípica y fenotípica de accesiones de arroz rojo *(Oryza sativa f. spontanea*) procedentes de los Departamentos de Tolima y Valle del Cauca. Universidad Francisco de Paula Santander Facultad de Ciencias Agrarias y del Ambiente Plan de Estudios de Ingeniería Producción Biotecnológica Cúcuta 2005. Tesis en Curso.

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Nuitjen Edwin. 2005. PhD. Farmer management of gene-flow: The impact of genetic diversity and crop improvement in the Gambia. Wageningen University. Defense November 30, 05 (L. Sperling).

Pineda Rosana, M.Sc. Thesis. Flujo de genes desde arroz transgénico al arroz maleza. Universidad Nacional. Sede Medellín Abril 2005. Tesis Meritoria.

Rodriguez Fausto. 2005. BSc. Aislamiento de candidatos a genes de resistencia contra la mancha angular de la hoja en el frijol común.
Universidad de los Andes, Bogotá Colombia.

Rodriguez Lina Maria. B.S.c. Universidad de Los Andes – (July 2004 – Sept 2005) studied geminivirus resistance genes with M. Blair.

Rosero Carolina. 2005 BSc. QTLs Mapping of Aluminum Resistance in *Brachiaria* sp. Universidad Javeriana, Bogotá

Royero Nelson Arturo. 2005. MSc. Caracterización molecular de accesiones de guanábana (Annona Muricata L.) y otras especies de importancia hortícola de la colección Colombiana de Anonáceas. Universidad Nacional de Colombia, Sede Palmira.

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Sarmiento Felipe, 2005. BSc. Búsqueda y Caracterización de Secuencias de Expresión Específica en la Raíz de Yuca (*Manihot Esculenta,* Crantz). Univ de los Andes, Bogotá, Colombia.

3.6 Projects

3.6.1 Project approved or on going

BMZ-Germany - "Bean genomics for improved drought tolerance in Latin America",

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" Universidad Nacional with CIAT

Generation Challenge Program - "TILLING mutagenesis and drought gene analysis"

Harvest Plus Challenge Program – "Biofortified crops for human nutrition" Harvest Plust challenge program, various donors 300,000 USD/yr (2003-2008).

USAID – "Breeding staple crops for improved micronutrient value", 400,000 USD (2002-2004)

EcoFondo – "Manejo del germoplasma local y aumento de la agrobidodiversidad de frijol y maiz con variedades biofortificadas para mejorar la nutricion en comunidades rurales del departamento de Nariño" – FIDAR with CIAT –

Fontagro – "Mejoramiento de la nutricion humana en comunidades pobres de America Latina utilizando maiz (QPM) y frijol comun biofortificados con micronutrientes"

BioCassava Plus, a project to develop new cassava cultivars with improved nutritional status for 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.

Development of Molecular Markers Associated with Genes for Useful and Unique Traits in Wild Progenitors of Cassava for Marker-Aided Introgression into Elite Cassava Cultivars, SIDA-FORMAS, SEK494,000 for 2 years.

Identification of Genes Controlling Starch Accumulation and Quality in Cassava (*Manihot esculenta* Crantz) Towards the Development of Varieties with Novel Quality and High Content of Starch (High-Value Cassava), SIDA-FORMAS, SEK495,000 for 2 years

Graduate Level (M.Sc) Training in Plant Breeding for NARs Scientists from Mozambique, Rockefeller Foundation, US$144,000 for 2 years

A One-Month Intensive Course in Cassava Breeding for NARs Cassava Breeders from Uganda, Kenya, Tanzania and Mozambique, Rockefeller Foundation, US$44,000 for 1 year

Improvement of the nutritional value of cassava: high storage protein content and zero cyanide cassava., DANIDA, US$120,000 for 3 years

Proof-of-Concept "Molecular Breeding Communities of Practice", GCP commissioned grants, US$30,000 for 2 years

A dataset on allele diversity at orthologous candidate genes in GCP crops (ADOC), GCP Commissioned grants, US$30,000 for 2 years

A Public-Private Partnership to Commercialize High Protein and Beta-Carotene Rich Cassava Varieties for the Animal Feed, Flour, and Cassava Chip Markets, The Government of Nigeria, US$14million for 4 years

CIDA, Canada. Agrosalud, High iron and zinc rice lines. US$235,000

GCP-Unlocking genetic diversity SP1 and SP2. US&4,500

GCP- Evaluation of T-DNA mutants to drought stress. US$ 3,000

GCP-Exploring natural genetic variation: developing genomic resources and introgression lines for four AA genome rice relatives. US$ 4,500

CIAT-Yale Univ. Consortium: Screenhouse and field evaluation of Ac/Ds mutants. USDA. US$ 4,000.

HP- Rice crop. Identification and expression analysis of genes important for iron translocation to the rice grain.US$200,000 Two years project starting in 2006. PI. Dr. Janette Palma Frett. Universidade Rio Grande do Soul. Porto Alegre. Brazil.

Assisting Disaster affected and chronically-stressed communities in East and Central Africa. USAID/OFDA. 8 case studies 7 countries. Full volume published. March 2002-March 2005. Total Amount: USD305,650

Seed Aid & Germplasm restoration in disaster situations. IDRC.Shows that CGIAR not involved in Germ. Restoration in acute stress. June 2003-March 2005 (ext to August). Total Amount: 198,000 CAD (126,000 USD).

Long-term seed Aid in Ethiopia. IDRC. Addresses chronic stress areas and extreme poverty. June 2005-May 2007. Total Amount: 232,000 USD.

Getting high-yielding and adapted bean varieties into the hand and fields of seed-stressed farmers. USAID. High impact: 73% of farmers reached had never been reached with new bean varieties prior. October 2003-February 2005. Total Amount: 150.000 USD.

Systematic evaluation of rice mutant collections for conditional phenotypes with emphasis on stress tolerance. Generation CP 40,000 US$/2005

Crop gene expression profiles and stress-gene arrays. Generation CP 23,000 US$/2005

Evaluation and deployment of transgenic drought tolerant varieties. Generation CP 33,479 US$/ 2005-2006

Bean genomics for improved drought tolerance in Central America, BMZ 16,000 US$/2005

3.6.2 Projects funded and their Donors (Oct 2004 – Sept 2005)

BioCassava Plus, a project to develop new cassava cultivars with improved nutritional status for 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.

Development of Molecular Markers Associated with Genes for Useful and Unique Traits in Wild Progenitors of Cassava for Marker-Aided Introgression into Elite Cassava Cultivars, SIDA-FORMAS, SEK494,000 for 2 years
Identification of Genes Controlling Starch Accumulation and Quality in Cassava (*Manihot esculenta* Crantz) Towards the Development of Varieties with Novel Quality and High Content of Starch (High-Value Cassava), SIDA-FORMAS, SEK495,000 for 2 years

Graduate Level (M.Sc) Training in Plant Breeding for NARs Scientists from Mozambique, Rockefeller Foundation, US$144,000 for 2 years

A One-Month Intensive Course in Cassava Breeding for NARs Cassava Breeders from Uganda, Kenya, Tanzania and Mozambique, Rockefeller Foundation, US$44,000 for 1 year

Improvement of the nutritional value of cassava: high storage protein content and zero cyanide cassava., DANIDA, US$120,000 for 3 years
Proof-of-Concept "Molecular Breeding Communities of Practice", GCP commissioned grants, US$30,000 for 2 years
A dataset on allele diversity at orthologous candidate genes in GCP crops (ADOC), GCP Commissioned grants, US$30,000 for 2 years
A Public-Private Partnership to Commercialize High Protein and Beta-Carotene Rich Cassava Varieties for the Animal Feed, Flour, and Cassava Chip Markets, The Government of Nigeria, US$14million for 4 years.

Delivery of Transgenic Rice Cultivars to Seed Producers and Farmers in Tropical America. US$ 300,075. (2001-2005). Donor: The Rockefeller Foundation

Development and use of inbred lines in cassav

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 (2004-2007). 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 (2004-2008).

Gene Flow Analysis for Environmental safety in the Tropics. CIAT – University of Costa Rica – Hannover University and BBA, Germany. Donor: EURO 450,000 (2005-2006).

Development and evaluation of drought-tolerant rice transgenic plants. GCP SB3 USD 70,000 (2005-2006)

Lulo (*Solanum quitoense*, naranjilla) with added value: New alternatives for the small farmer. CIAT-CORPOICA. Col $ 499 million (2006-2008)

Biosafety in centers of biodiversity: Building technical capacity in Latin America for safe deployment of transgenic crops. GEF-World Bank. USD 5 million. PDF-B (pre-proposal) approved August 2005. USD 260,000 (November 2005-July 31, 2006).

Development and evaluation of drought-tolerant rice transgenic plants. GCP SB3 USD 70,000 (submitted March 2005)

Lulo (*Solanum quitoense*, naranjilla) with added value: New alternatives for the small farmer. CIAT-CORPOICA. Col $ 499 million (submitted July 2005)

CIDA, Canada. Agrosalud, High iron and zinc rice lines. US$235,000

GCP-Unlocking genetic diversity SP1 and SP2. US&4,500

GCP- Evaluation of T-DNA mutants to drought stress. US$ 3,000

GCP-Exploring natural genetic variation: developing genomic resources and introgression lines for four AA genome rice relatives. US$ 4,500

CIAT-Yale Univ. Consortium: Screenhouse and field evaluation of Ac/Ds mutants. USDA. US$ 4,000.

HP- Rice crop. Identification and expression analysis of genes important for iron translocation to the rice grain.US$200,000 Two years project starting in 2006. PI. Dr. Janette Palma Frett. Universidade Rio Grande do Soul. Porto Alegre. Brazil.

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)

High 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

3.6.3 List of Partners/Donors

CIBIOGEM, UNAM, Mexico

University of Costa Rica, Costa Rica

ICA, Corpoica, Institute von Humboldt, Colombia

UCV, IVIC, INIAP, Venezuela

CONAM, Peru

EMBRAPA, Brazil

University of Hanover, BBA, University of Braunschweig, Germany

ETH, Switzerland

PRI, University of Wageningen, The Netherlands

JIRCAS, Japan

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.

CIAT Core Budget.

Ministerio de Agricultura y Desarrollo Rural of Colombia.

Ministerio de Agricultura y Desarrollo Rural of Colombia (special project: Development of cryopreservation techniques for palm species in view of developing cost effective genetic banks).

World Bank (special project: Rehabilitation of International Public Goods; CGIAR Genebanks Upgrading Project).

Bundesministerium fuer Wirtschaftliche Zusammenarbeit und Entwicklung (BMZ) of Germany (special project: studies of gene flow in the bean model).

Chinese Academy of Agricultural Sciences – S. Wang

Clemson University Genomics Institute – J. Tomkins

Centro Fitoecogenetico Pariumani – Bolivia – T. Avila

Cornell University – T. Fulton, S. Kresovich

CORPOICA – G. Santana. M. Lobo

EMBRAPA-Centro Nacional de Pequisa Arroz e Feijao – M.J. Peloso, R. Brondani

INIA – Lara – Venezuela – ME Morros

INIA – Peru – A. Valladolid, M. Gamarra

INIAP – Ecuador – E. Peralta

FIDAR – J. Restrepo

INIFAP-Mexico – R. Garza

Universidad Autonoma de México – G. Hernandez

Universidad Nacional de Colombia – G. Ligareto

University of Geneva – B. Broughton, CE Pankhurst

Universidad del Valle - A. Pradilla

UAGRM – BOLIVIA- J. Ortubé

Chinese Academy of Agricultural Sciences – S. Wang

Clemson University Genomics Institute – J. Tomkins

Centro Fitoecogenetico Pariumani – Bolivia – T. Avila

Cornell University – T. Fulton, S. Kresovich

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INIAP – Ecuador – E. Peralta

FIDAR – J. Restrepo

3.7 Current SB-2 Investigators: Discipline, position and time fraction

Name	Discipline	Time dedication%
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
Pfeiffer, Wolfgang	Coordinator HarvestPlus-LA	100
Sperling Louise	Seed Systems	20
Tohme Joe	Genomics, Project manager	100

Tissue Culture/Cryopreservation/Plant Transformation

Bolaños, Eugenio	Technician
Brito Marco	Technician
Buitrago Maria Eugenia	Research Assistant
Criollo Arturo	Technician
Dorado, Carlos	Technician
Escobar, Roosevelt	Research Assistant
Echeverry, Morgan	Research Assistant
Fory, Luisa	Rice Biotechnology Research Coordinator
Galindo, Leonardo F.	Research Assistant
González, Eliana	Research Assistant
Herrera, Pablo	Technician
Ladino, Janeth Julieta	Research Assistant
Manrique, Norma	Research Assistant
Muñoz, Liliana	Research Assistant
López, Danilo	Research Assistant
Rios, Auradela	Technician
Ríos, Alexander	Technician
Ruiz Juan Jairo	Research Assistant
Tabares, Eddie	Research Assistant
Tigreros, Humberto	Technician
Villamizar Johnna Patricia	Research Assistant

Genome Diversity

Barrera, Edgar.	Research Assistant
Bernal Diana	Research Assistant
Bohorquez, Adriana.	Research Assistant
Galindo, Lonardo M.	Research Assistant
Gallego,Gerardo.	Asociate – Laboratory Coordinator
Giraldo,Olga X.	Research Assistant
Gutiérrez, Janeth P.	Research Assistant
Londoño, Claudia	Technician
Posso Duina	Research Assistant
Quimbaya Mauricio	Research Assistant
Quintero, Constanza.	Research Assistant
Roda Federico	Thesis Student
Romero Catalina	Research Assistant
Vargas, Jaime	Research Assistant

Plant-Stress interactions

Arango Jacobo	Thesis Student
Chaves Alba L.	Research Associate
Deguchi Michihito	Posdoctoral Visiting Research
Soto Mauricio.	Research Assistant
Galindo Leonardo Miguel	Research Assistant
Salcedo Andrés Felipe	Research Assistant
Recio Maria Eugenia	Technician
Montenegro Maria Fernanda	Visiting Research
Sarmiento Felipe	Research Assistant
Jacobo Arango	Thesis Student
Andres Bolaños	Technician

Administrative

Cruz, Olga L.	Bilingual Secretary
Duque, Myriam C.	Statistical Consultant
Quiceno, Jorge	HarvestPlus – Administrative Assistant
Rojas Fernando	System Analist
Zuñiga, Claudia S.	Bilingual Secretary

Institute A. von Humboldt

Camacho Lindamar	Thesis Student
Cardoso Carolina	Thesis Student
Diaz Carlos	Visiting Research
Escobar Selene	Visiting Research
Palacio, Juan D.	Visiting Research
Silva Bernardo	Thesis Student
Villafañe Carolina	Visiting Research

Bioinformática

Garcia Alexander	Postdoctoral Fellow
Plata German	Research Assistant
Rodriguez Fausto	Research Assistant
Rojas Fernando	System Analist