ANNUAL REPORT 2002

PROJECT SB-2

ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

CIAT

For internal use only

October, 2002

Dedication

This report is dedicated to the memories of our two fallen colleagues, ***Maria de Jesús (Chusa) Ginés*** *and* ***Verónica Mera****, of late Coordinator and Social Scientist, respectively of the Cassava Biotechnology Network for Latin America and the Caribbean (CBN-LAC).*

The promising lives of these two highly respected and beloved scientists, wives and mothers were tragically cut short in the morning of Monday, 28 January 2002 when the aircraft they were aboard crashed into the Cumbal volcano in the Colombia – Ecuador border. They were on an official trip from their base in Quito, Ecuador, to the CIAT headquarters in Cali, Colombia. Chusa and Vero believed in and tirelessly championed the causes of the resource-poor farmers of the world. It is remarkable that they ultimately paid the supreme price while in active pursuit of the goal of ensuring that the voices of these farmers are heard in priority setting for cassava biotechnology research. By dedicating the SB-02 Annual Report for 2002 to the memories of these two wonderful people we are affirming our resolve to continue to positively impact on the lives of the poor farmers of the tropics by the strategic use of novel biotechnological tools to increase agricultural productivity through the exploitation of the rich agrobiodiversity of our mandate crops.

CONTENT

Project SB-2: Assessing and Utilizing Agrobiodiversity through Biotechnology

PROJECT OVERVIEW

Project Description

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

Outputs:

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

Milestones:

2002 Cassava cryopreservation implemented. Gene transfer used to broaden the genetic base and enhance germplasm of rice, cassava, and the forage grass *Brachiaria*. Screening with microarray technology initiated. Marker-assisted selection implemented for rice, beans, and *Brachiaria*. ESTs generated for cassava starch and CBB. A LIMS developed. Procedures developed for conservation of wild species and landraces, based on studies of seed biology and physiology. Safe-duplication and restoration continued.

2003 Efficient transformation system devolved for beans. Transgenic cassava tested for resistance to stemborer. Bioreactor technology implemented for cassava and rice. Markers developed for iron and zinc in beans. Collaboration with public and private partners strengthened. Advanced backcross populations of rice characterized. Protocols for cryoconservation of seeds and tissue germplasm established. Germplasm collections regenerated. Safe-duplication and restoration continued.

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

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

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

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

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

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

Work Breakdown Structure

Project SB-2: Assessing and Utilizing Agrobiodiversity through Biotechnology

PROJECT GOAL

To contribute to the sustainable increase of productivity and quality of mandated and other priority crops, and the conservation of agrobiodiversity in tropical countries.

PROJECT PURPOSE

To ensure that characterized agrobiodiversity, improved crop genetic stocks, and modern molecular and cellular methods and tools, are used by CIAT and NARS scientists for improving using and conserving crop genetic resources.

OUTPUT 1. Genomes characterized: Genomes of wild and cultivated species of mandated and non-mandated crops, and associated organisms characterized.

- Molecular characterization of genetic diversity
- Identification and mapping of useful genes and gene combinations
- Development of molecular-genetic techniques for assessing genetic diversity.

OUTPUT 2. Genes modified: Genes and gene combinations utilized for broadening the genetic bases of mandated and non-mandated crops.

- Transfer of novel genes and gene combinations by means of cellular and molecular gene transfer techniques.
- Identification of points for genetic intervention in plant/stress interactions.
- Development of cellular and molecular techniques for genome modification.

OUTPUT3. Collaboration with public and private sector partners enhanced.

- Organization of conferences, networks, workshops and training courses.
- Assembling of data bases, genetic stocks, maps and probes, and related information.
- Publications, project proposal development and contribution to IPR and biosafety management.

CIAT: SB-2 Project Log Frame (2003-2005)

Project: **Assessing and Utilizing Agrobiodiversity through Biotechnology**
Project Manager: Joe Tohme

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

Output 3 Collaboration with public- and private-sector partners enhanced.	CIAT partners in LDCs using information and genetic stocks. New partnerships with private sector.	Publications. Training courses and workshops. Project proposals.	Government and industry support national biotech initiatives.
Output 4 Mandated crops conserved and multiplied as per international standards.	Germination rates for long-stored materials. Cost per accession/year, compared with other gene banks.	Visits to GRU substations and conservation facilities.	Absence of uncontrolled diseases. Quarantine greenhouse space available at different altitudes.
Output 5 Germplasm available, restored, and safely duplicated.	Number of germplasm requests received and satisfied annually. Users received germplasm and data. Users asked for novel germplasm and data.	Visits to multiplication plots. Reports on requests and delivery. Number of core collections multiplied and shipped.	Agreement with CIAT holds.
Output 6 Designated Collections made socially relevant.	Landrace diversity restored to farmers. Farmers use new varieties. Breeders use novel genes.	Germplasm catalogs. Plant variety registration logs. National catalogs.	International collecting possible. Quarantine matters cleared.
Output 7 Strengthen NARS for conservation and use of Neotropical plant genetic resources.	NARS germplasm collections conserved. Number of trainees trained at CIAT. Number of universities and NARS using training materials.	Country questionnaires. Courses registered. Distribution and sales of training materials.	NARS and networks willing to cooperate.
Output 8 Conservation of Designated Collections linked with on-farm conservation efforts and protected areas.	Number of case studies and pilot *in situ* conservation projects.	Project documentation.	NARS interested in conservation efforts. Farmers interested in conservation efforts.

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumption
OUTPUT 1: Genomes characterized			
Activity 1.1.Molecular characterization of genetic diversity	• Characterization of core collections • Identification of sources of resistance to disease • Genetic structure of wild and cultivated beans and cassava available Phylogenic trees based on ITS sequences. • Characterization of genetic diversity of endangered palms and soursop in Colombia	Report, articles, databases of molecular fingerprinting	Availability of structure collections Material supplied by GRU
Activity 1.2 Identification and mapping, of useful gene and genes combinations	• Marker assisted scheme established for bean rice, and *Brachiaria* • Linkage detected between markers and important agronomical traits • QTL analysis for quality traits,disease resistance, and agronomic performance in bean, cassava and rice.	Draft articles, Annual Report, publications	Availability of mapping populations and phenotypic characterization
Activity 1.3 Development of molecular techniques for assessing genetic diversity and mapping useful genes	• Bean, Cassava and *Brachiaria* microsatellites developed • New technologies - SAGE, cDNA AFLP , and microarray implemented. • Resistance genes analogues identified, characterized and mapped in bean and *Brachiaria.* • Mapping resistance genes in *Brachiaria* rice.	Sequences available Report, draft articles	Access to facilities in advanced labs
OUTPUT 2. Genomes modified	•		
Activity 2.1 Transfer of novel genes an gene combinations by cellular/molecular techniques	• Expression of insecticidal protein • Isolation of lignin biosynthetic genes from *Brachiaria* • Generation of transgenic *Brachiaria*, cassava, rice, tomato, sugarcane. • Field test of transgenic rice with virus resistance • Backcross conversion from transgenic rice.	Transgenic plants in biosafety field Report, draft articles	Biosafety regulation approved Biosafety greenhouse space available Collaboration with NARS
Activity 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base	• Rapid propagation rates of cassava cultivars improved by bioreactors. • Low cost cassava in vitro propagation method transferred to farmers association. • Cost analysis of propagation and conservation of cassava germplasm by different methods. • Use of bioreactors for rice anther culture • Adaptation and use of selection system for genetic transformation non dependent on antibiotic resistance	Farmer reports, level of adoption of technology	Access to farmers association Access to RITA system

	• Development of propagation, plant regeneration and transformation of naranjilla • Cryopreservation of cassava and tree tomato • *In vitro* propagation of soursop improved		
Activity 2.3 Identification of points of genetic intervention and mechanism of plant stress	• Genomics tools used to understand and exploit diversity for cassava starch and post-harvest deterioration • Characterization of genetic diversity and key pathway genes for carotene and mineral contents in cassava		
OUTPUT 3. Collaboration enhanced	•		
Activity 3.1 Organization of Networks, Workshops, training courses in biotechnology	• The Cassava Biotechnology Network was re-established • Contribution to training courses • Organization of a legume genomics meeting between CG and US universities. • Organization of the CG planning workshop on biofortification. • At least 70 people received training • Participation of team members to international, regional conferences	Reports	Funding available
Activity 3.2 Data Base and Genetic Stocks	• Database for bean microsatellites established • New version of Flora Map distributed • Database for gene constructs, plasmid and vectors established	Number of register users, report, access to databases, publications	Continued core support
Activity 3.3 Project proposals and Publications	• Five new projects approved and 11 proposals submitted	Number of projects approved	Continued core support
Activity 3.4 Donors contributing	• Twenty five donors contributed	Number of current donors	Continued core support
Activity 3.5 Project SB-2 staff	• 8.9 Senior Staff person/time • 41 Support Staff • 3 Adminjstrative Support Staff • 18 Graduate Students • 14 Undergraduate Students	Total number of staff	Continued core support

OUTPUT 1. Genomes and wild and cultivated species of mandated and non mandated crops, and associated organisms characterized

Activity 1.1 Characterization of genetic diversity

Main Achievements

- The fingerprinting of common bean genotypes with microsatellite was carried to determine the origin of the patented Enola variety. The similarities obtained are consistent with the hypothesis that Enola is a selection from one of the pure-line commercial cultivars of the Mayocoba market class, grown in Mexico for export to the USA.
- Diversity in the CIAT collection of tepary beans (*Phaseolus acutifolius*) analyzed with two combinations of AFLP primer pairs to distinguish taxonomic relationships with *P. parvifolius* as well as within the species between *P. a.* var. *acutifolius* and *P. a.* var. *tenuifolius*.
- Genetic diversity of microsatellite alleles determined for a third common bean parental survey that provide the basis for mapping and genetic tagging experiments for drought and abiotic stress tolerance. This information was incorporated into a new molecular genetics database constructed for microsatellite parental surveys and the Beangenes database.
- Phaseolin characterization of Caribbean common bean germplasm showing hybrid Mesoamerican -Andean origin to red and pink mottled "Andean" landraces.
- Interspecific progeny presented significant introgression of drought tolerance from P. *acutifolius* to common bean. These represent another potential source of tolerance genes to improve common bean, and may also be a source of heat tolerance.
- The identification of a Symbiotic Bacteria from Native Colombian Entomophagous Nematodes was obtained using sequence analyses of PCR amplified 16 S rDAn regions. The data showed that the 16s sequence from the nematode symbiotic bacteria *Bacillus* sp. is closely related to *B. cereus, B. thuringiensis* and *B. anthracis*.
- Molecular characterization was carried out on Fleminga, Cratylia, as part of a collboration with the forage project and on soursop, lulo, plantain, and avocado as part of the collaboration with Corpoica.
- Red rice accessions collected from Tolima and Huila, Colombia, were fully characterized using morphological, phenological, and microsatellite markers. The characterization allowed distinguishing red rice biotypes alike to cultivated varieties or the wild species *O. rufipogon*, or in between.
- Crop/wild/weedy specific microsatellite to be used for tracing gene flow and introgression were identified. Current methodology sensitivity allowed detecting 2% introgression in bulked DNA samples of different genotypes.
- Potential red rice accessions candidates to conduct gene flow/introgression analysis were selected based on susceptibility to RHBV, overlapping flowering with crop, presence of red/brown color stem, leaves, grain awn and husk.

1.1.1 Fingerprinting of common bean genotypes with microsatellite markers: The Enola variety

C. Quintero[1], O. Toro[2], E.Gaitán; D. Debouck[2] and J. Tohme[1]
[1]SB-2 Project; [2] SB-1 Project

Introduction

In December 2000 CIAT filed a formal request for reexamination of US patent no. 5,894,079, also known as the yellow or "Enola" bean patent with the US Patent & Trademark Office in Washington, DC (Debouck, personal communication, 2002). Cluster analyses of seed proteins and isozyme data on a group of 22 bean genotypes located the Enola variety in the largest group (CIAT, 2001). These genotypes were then analyzed with microsatellite markers.

Materials and Methods

Twenty bean genotypes from Mexico and Peru and the patented variety Enola were analyzed. One microsatellite marker per linkage group was chosen on the basis of a discriminatory power ranging between 0.74 and 0.94 (Gaitán, personal communication).

Five seeds of each genotype selected for this study were grown in the greenhouse. Leaf tissue was then collected in liquid nitrogen, ground and 5 g used for genomic DNA extraction, employing a CTAB-chloroform protocol according to the modifications made by Afanador et al. (1993).

PCR reactions were carried out in a final volume of 20 µl as follows: 20 ng of genomic DNA were added to a mix containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5-2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.1 µM of each forward and reverse primer, and one unit of *Taq* polymerase. The PCR profile included an initial denaturation step at 94°C for 3 min followed by 35 cycles of 15-sec steps at 94, 50-55 and 72°C, with a final extension step at 72°C for 5 min. Amplified products (3.5 µl) were resolved on 6% denaturing polyacrylamide gels with 5 M urea and 0.5X TBE, using silver staining protocol according to the manufacturer's guide (Promega Inc., USA)

In total 52 polymorphic alleles from the 21 genotypes evaluated in this study were scored for their presence or absence; and a cluster analysis was performed with the NTSYS-pc vers. 2.02 software package (Rohlf, 1994), using the UPGMA method, based on the Dice similarity coefficient.

Results and Discussion

Figure 1 shows the results of the scoring of the presence (1) or absence (0) of the 52 polymorphic alleles.

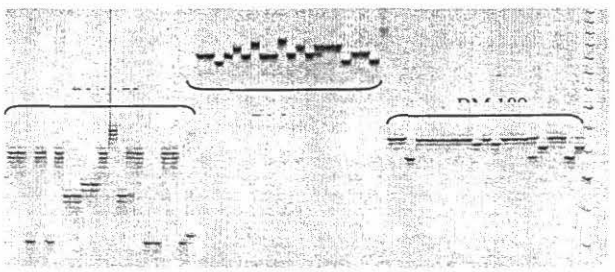

Figure 1. Microsatellite amplification of bean genotypes including the patented variety Enola.

Microsatellite markers were suitable for distinguishing genetic diversity among the bean genotypes selected for this study.

At a 0.38 similarity level, four groups were identified (Figure 2). Some similarity with the results obtained from the isozyme analysis was observed, but this time the microsatellites discriminated within the largest group, where the Enola variety was found. The group contained Canario-type Peruvian germplasm such as G5707, G5703 and G14024. The germplasm accessions, G13094 (Mayocoba) and G 11891 (Culiacan-11-57R-M-37-M-M) from Mexico were nearly identical for the loci analyzed in this study, and a high degree of similarity between them and the Enola variety was also observed (0.91 similarity level), which may indicates that the Enola variety is not unique.

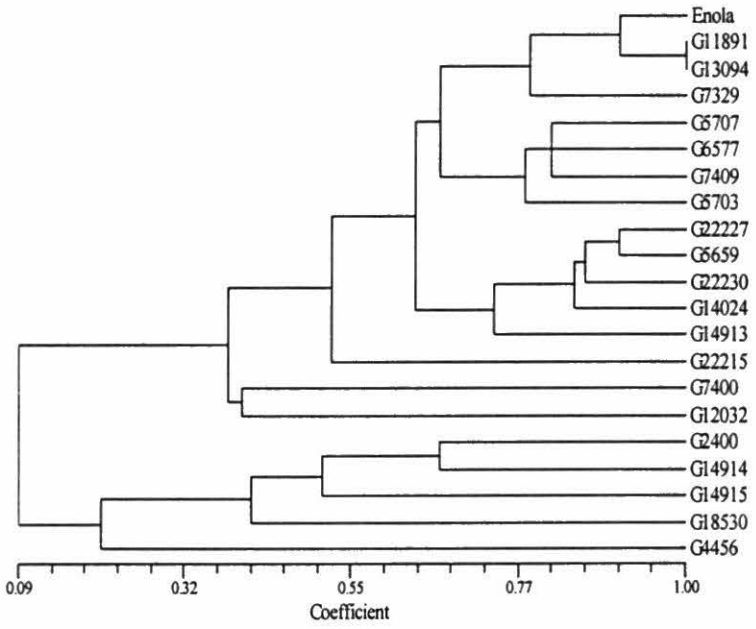

Figure 2. Dendrogram of similarities among some common bean genotypes, using UPGMA and Dice coefficient.

These similarities are consistent with the hypothesis that Enola is a selection from one of the pure-line commercial cultivars of the Mayocoba market class, grown in Mexico for export to the USA (Kelly, 2000).

The exclusive property claim to all bean cultivars with the Enola seed-coat color, based on the "invention" of that seed-coat color is therefore being contested on the basis of the argument that the program of several successive cycles of self-pollination and selection from yellow bean materials purchased in Mexico did not create or invent the seed-coat color, which has existed in Peru since ancient times (Debouck and Voysest, personal communication).

References

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Acknowledgments

We acknowledge the invaluable contribution of Fabio Pedraza (Bean Germplasm Characterization Laboratory) for providing the location of the microsatellite markers in the linkage map.

1.1.2 Genetic diversity of tepary beans uncovered with multiple AFLP combinations

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SB-2 Project

Introduction

The genetic diversity within tepary bean (*Phaseolus acutifolius* A.Gray) and related species (*P. parvifolius)* has been studied using isozyme markers and RFLPs (Scinkel and Gepts, 1988, 1989; Garvin and Weeden, 1994). The objective of this research was to use amplified fragment length polymorphism (AFLP) markers to study the patterns of diversity within the species and its placement relative to other *Phaseolus* species that were used as an outgroup. AFLPs tend to be evolutionarily conserved markers and serve to reference different species relative to each other, however to be accurate more than one combination of AFLP primers should generally be used. Last year we reported on the results of one AFLP combination and this year we compare a second AFLP primer combination to obtain more conclusive results. Additional objectives of the study were to determine if *P. acutifolius* and *P. parvifolius* merit being separate species and if molecular markers can distinguish between the botanical varieties var. *acutifolius* and var. *tenuifolius* within the species *P. acutifolius*. Another purpose of this research was to lay the groundwork for genetic improvement of tepary beans given that presently there are no improved varieties of tepary beans and a diversity assessment would be important for deciding on which crosses to make. Improved varieties of tepary beans would be a very useful and interesting crop especially for dryland agricultural systems because tepary beans are the most drought-adapted species of the genus. They are also known to have high heat and salinity tolerance and good nutritional quality. Reestablishment of tepary bean production would build on a tradition of cultivation in Mexico,

South western United States and Central America, that goes back 5000 years. New varieties of tepary beans could also be important for other desert regions where pulse production is needed.

Methodology

Genotypes and DNA extraction: A total of 108 genotypes from the Genetic Resources Unit of CIAT were analyzed in the experiments as described in last year's annual report. These included an outgroup of 9 genotypes from the *Phaseolus* genus including 4 *P. vulgaris* (common bean); 4 *P. lunatus* (lima bean); and 1 *P. glabellus* genotype.

AFLP analysis: Amplicon-template preparation, pre-amplification, and selective amplification were described in last year's annual report. To determine which were the best primer combinations based on the *Eco*RI (E) -*Mse*I (M) adapters and primers with 3 selective nucleotides each we conducted a survey with one common and one tepary bean accession. Based on this we decided to add the combination E-ACC/M-CTA to the combination E-AAG /M-CTT that we analyzed last year. PCR products were run on 4% silver-stained polyacrylamide gels for 1, 1.5 and 2 hours as described in last years report.

Data analysis: Genetic similarities between genotypes were determined with the Dice coefficient using NTSYS 2.02 (Rohlf, 1993). The similarity matrices were used to construct dendrograms with the same program. Principal component analysis was done with the software package SAS (SAS Institute, 1989). Once the groups were determine, mean similarity indices were estimated between the following groups: *P. acutifolius* cultivated, *P. acutifolius* var *acutifolius* , *P. parvifolius*, *P. vulgaris*, *P. lunatus*. Primer combinations were compared with Mantel "Z" statistic for the correlation of genetic distance matrices.

Results and Discussion

Both AFLP combinations used in this study had a good polymorphism rate, clear amplification profile and well-distributed range in PCR product sizes. The AFLP combinations produced a total of 262 bands (167 for E-AAG/M-CTT; and 95 for E-ACC/M-CTA 95 bands. In terms of numbers of polymorphic bands, the primer combination E-AAG/M-CTT was a lot less efficient that the primer combinations E-ACC/M-CTA. (Table 1). Considering all the bands produced in the two primer combinations, 99.2% of the bands were polymorphic across all species and 74.8% were polymorphic across the outgroup. However, polymorphism within *P. parvifolius* (16.8%) was low, within cultivated *P. acutifolius* (31.7%) was low to intermediate and within wild *P. acutifolius* (42.7%) was intermediate (Table 2).

Both monomorphic and polymorphic bands were used to determine the genetic similarity between genotypes. For the combined analysis of two AFLP primer combination only 99 tepary beans and their close relatives were analyzed, consisting in *46* cultivated *P. acutifolius* var. *acutifolius*; 32 wild *P. acutifolius* var. *acutifolius*; 11 *P. acutifolius* var. *tenuifolius;* and 10 *P. parvifolius* accessions. Figure 1 shows the principal component analysis derived from either AFLP combination separately or for the total number of bands. The combination E-AAG/M-CTT was efficient for grouping the genotypes by species while the combination E-ACC/M-CTA in addition to separating the species allowed a better distinction between groups within species. This was evidenced in the separation of four groups: cultivated *P. acutifolius*, wild *P. acutifolius* var. *acutifolius*, wild *P. acutifolius* var. *tenuifolius* and *P. parvifolius* with this second AFLP combination. The analysis of the combined dataset also coincided with the established taxonomic relationships for the group of species analyzed, with *P. glabellus* as the most distant from *P. acutifolius* followed by *P. lunatus* and *P. vulgaris*. The correlation between the two genetic matrices

for each AFLP combination was 0.83. The use of two AFLP primer combinations seems to have sampled different parts of the bean genome and gave us a more accurate picture of the relationships within and between species.

The structure of the dendrogram created for the full dataset of AFLP bands as shown in Figure 2, also agrees with known taxonomic relationships for the six species represented in the study. *P. glabellus* was the most distant group, followed by *P. lunatus*. *P. vulgaris* was the closest to the *P. acutifolius* - *parvifolius* clade. The level of similarity was around 35% between the five groups. Within both *P. vulgaris* and *P. lunatus* the distinction between Andean and Mesomerican genepools was clear. The level of similarity between genepools was lower in *P. vulgaris* (65%) than in *P. lunatus* (75%). Within the *P. acutifolius* - *parvifolius* spectrum, all the accessions shared up to 50% similarity. In the dendogram, a total of five groups could be distinguished within the *P. acutifolius* - *parvifolius* spectrum: 1) cultivated *P. acutifolius* from Central and North America 2) cultivated *P. acutifolius* from North America (mainly Sonora and Sinaloa), 3) wild *P. acutifolius* var. *acutifolius* 4) wild *P. acutifolius* var *acutifolius* and *tenuifolius*; and 45) *P. parvifolius*. These five groups could be organized hierarchically into a larger clade, consisting of groups 1 and 2 with the remaining as separate groups. The first clade contained all the cultivated *P. acutifolius*, while the remainding groups contained all the *P. acutifolius* var. *tenuifolius* and *P. parvifolius* accessions. The wild *P. acutifolius* accessions were distributed among the two clades, with some more allied to the cultivated accessions of the same species and others allied to the *P. parvifolius* group. Within the first clade, the two cultivated groups (1 and 2) were related at 85% similarity and these were related to the wild accessions (group 3) at 75% similarity. The *P. parvifolius* and *P. acutifolius* (both var. *acutifolius* and *tenuifolius*) were related at 64% similarity.

As mentioned above, the AFLP combinations accurately displayed the genetic structure of the *Phaseolus* genus, where tepary beans are in the tertiary gene pool of common (*P. vulgaris* L.) and scarlet runner (*P. coccineus)* beans but are fairly distant from other *Phaseolus* species such as lima beans (*P. lunatus, P. glabellus*). The analysis also showed that tepary beans were less diverse than common bean or lima beans. This is probably because tepary beans most likely have had a single center of origin from where they were distributed across the current range of the crop, while common and lima beans were domesticated in two centers of origin. The relationships within the *P. acutifolius* - parvifolius clade has been controversial. The AFLP data presented here suggest that the *P. acutifolius* and *P. parvifolius* probably do not deserve to be different species, but could qualify as possible subspecies or varieties within the species. The high amounts of diversity found in the wild *P. acutifolius* and *P. parvifolius* accessions are an interesting resource for breeding tepary bean cultivars. The high similarity among all the cultivated tepary beans, seems to indicate that the crop may have arisen from a single domestication event and have suffered a genetic bottleneck which limits diversity within the cultivars. From this study, there is very little evidence for introgression from wild relatives into the cultivated genepool after the initial domestication event. Tepary beans are known to have a very low crossing rate that limits the creation of new diversity within the crop. The lack of diversity within the cultivated tepary bean is a serious limitation for improvement of the crop. The lack of diversity within the cultivated tepary bean belies some of the variability found for disease and insect resistance within the species. However, that lack of diversity in other characteristics such as plant morphology, adaptation range has serious implications for improving the species agronomically and using the species in inter-specific hybridization.

Future plans

- Additional wild tepary bean genotypes will be analyzed, including representative of *Phaseolus parvifolius* and *P. acutifolius* var. *acutifolius*, *tenuifolius* and *latifolius*.
- Genetic studies will be conducted with crosses between individuals from the different groups identified in this study (see following section).

References

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Table 1. Number of monomorphic and polymorphic band obtained within each genotype group for two combinations of AFLPs.

	E-AAG/M-CTT			E-ACC/M-CTA		
	P. acutifolius Cult.	*P. acutifolius* Wild	*P. parvifolius*	*P. acutifolius* Cult.	*P. acutifolius* Wild	*P. parvifolius*
No. genotypes	50	55	9	49	48	10
monomorphic	139 (83.2)	116 (69.5)	159 (92.2)	40 (42.1)	34 (35.8)	59 (62.1)
polymorphic	28 (16.8)	51 (30.5)	18 (7.8)	55 (57.9)	61 (64.8)	36 (37.9)
Total	167			95		

Table 2. Number of monomorphic and polymorphic band obtained within each genotype group for the full set of AFLPs.

	P. acutifolius Cult	*P. acutifolius* Wild	*P. parvifolius*	*P. vulgaris*	*P. lunatus*	outgroup	total
No. genotypes	46	43	10	4	4	9	108
monomorphic	179 (68.3)	150 (57.3)	218 (83.2)	203 (77.5)	210 (80.2)	66 (25.2)	2 (0.8)
polymorphic	83 (31.7)	112 (42.7)	54 (16.8)	59 (22.5)	52 (19.8)	196 (74.8)	260 (99.2)
Total					-		262

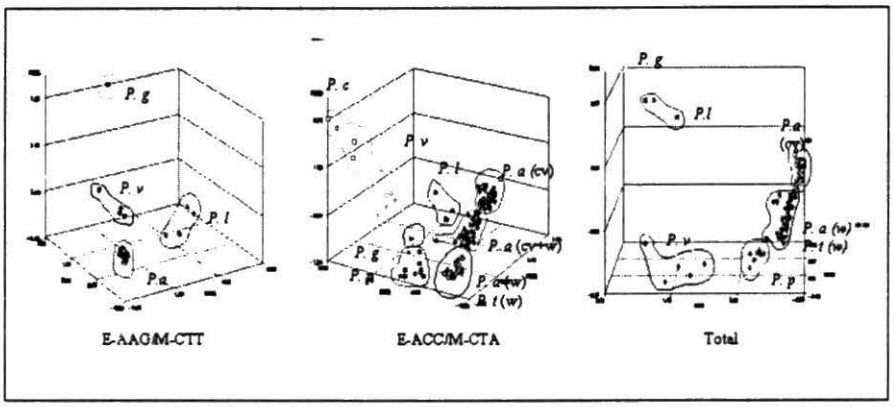

Figure 1. Principal component analysis for the AFLP primer combinations (E-AAG/M-CTT and E-ACC/M-CTA) separately and combined showing the relationship between 4 *Phaseolus vulgaris* (*P.v*); 4 *P. lunatus* (*P.l.*) 1 *P. glabellus* (*P.g.*); 46 cultivated *P. acutifolius* var. *acutifolius* (*P. a* (cv)), 32 wild *P. a.* var. *acutifolius* (*P. a* (w)) ; 11 *P. acutifolius* var. *tenuifolius* (*P. t* (w)); and 10 *P. parvifolius* (*P. p* (w)) accessions.

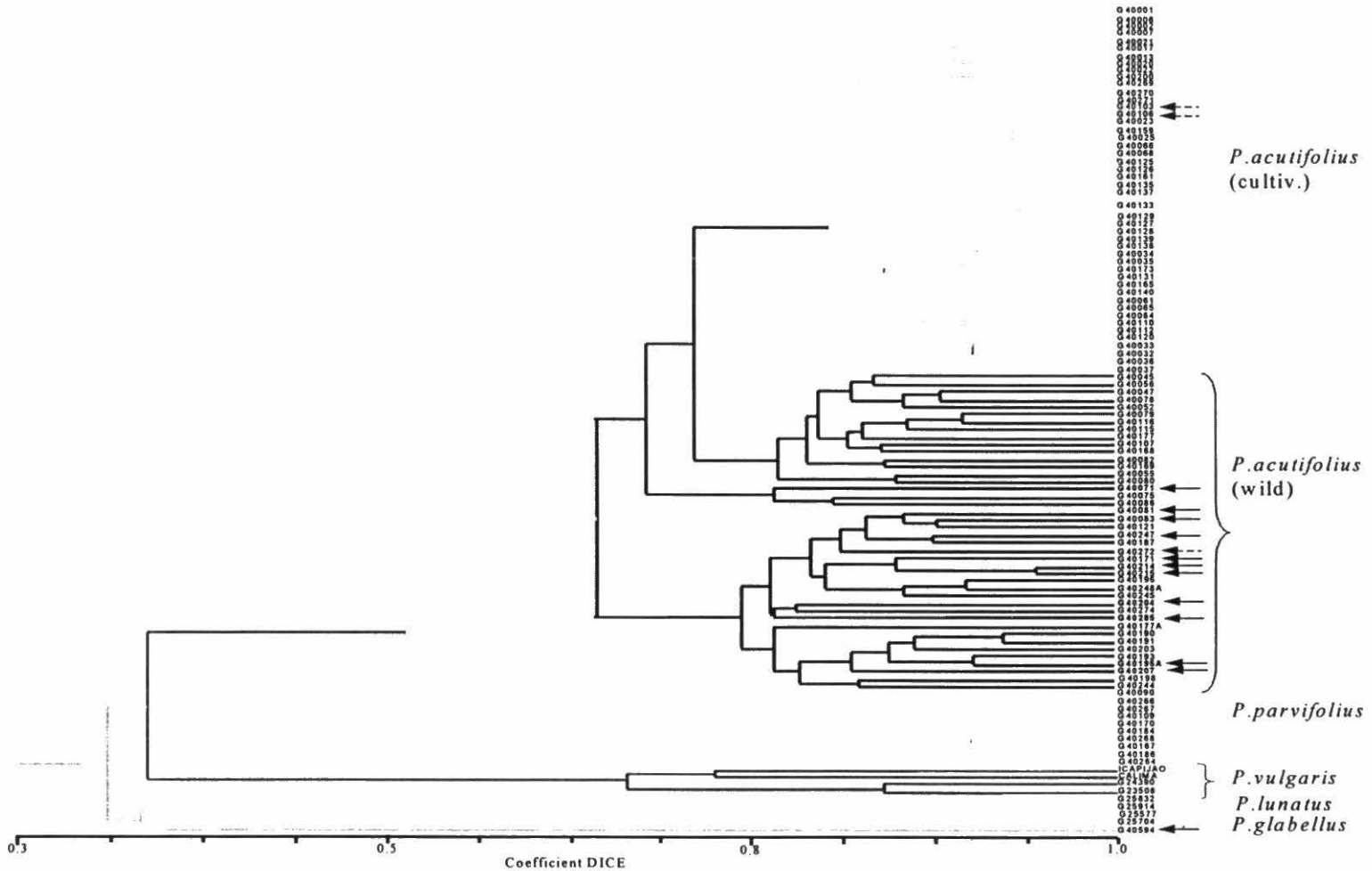

Figure 2. Dendrogram showing the associations among 108 accessions of cultivated and wild tepary beans (*Phaseolus acutifolius* and *P. parvifolius*) and three others bean species (*P. vulgaris, P. lunatus* and *P. glabellus*) using Dice genetic similarity coefficient for two AFLP primer combinations: E-AAG/M-CTT and E-ACC/M-CTA

1.1.3 Genetic analysis of crosses between cultivated tepary bean and wild *Phaseolus acutifolius* and *P. parvifolius*

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SB-2 Project

Introduction

Cultivated tepary bean (*Phaseolus acutifolius*) has several wild relatives. First are the wild accessions within the species itself, these include two variants (var. *acutifolius* and var. *tenuifolius*) and second there are the wild accessions belonging to the closely related species, *P. parvifolius*. The genetic diversity within cultivated tepary beans is small (see previous section), therefore the objective of this research was to study the variability generated by crossing cultivated tepary beans by several of their wild relatives. The crosses can be expected to incorporate added genetic diversity into the cultivated tepary beans which may be useful for breeding this neglected crop. These crosses are also being analyzed for polymorphism and segregation in the F2 generation using common bean microsatellites. The crosses will also be used to generate recombinant inbred lines that can be analyzed for biotic and abiotic stress tolerance genes segregating in the populations derived from these crosses. Finally, we also hope to identify any incompatibility factors in these intra- and inter-specific crosses and map them to help determine which species and variety designations are merited among the tepary beans.

Methodology

We used a set of six contrasting tepary parents (Table 1) to develop a total of seven reciprocal and non-reciprocal F2 populations (Table 2). Crosses were made with hand emasculation and both the F1 and F2 plants were grown in 9-inch pots in the greenhouse and single harvested. The F3 families from each of 120 F2 plants harvested from the greenhouse for the AP-1, AP-2, AT-1 and AT-2 populations were field-planted at CIAT headquarters in semester 2002 A (for the F3 of the AP populations) and semester 2002B (for the F3 of the AT populations). Seed scarification was used to increase the germination rate on both these crosses. The AP populations were advanced by single seed descent to the F4 generation in Semester 2002B. In each generation, data was collected on a series of phenotypic characteristics (Table 3) some of which are descriptors for the species (IPGRI, 1985). In the field, yield and yield component (number of pods per plant, number of seed per pod, etc) were evaluated for the F3 family and for the inidividual plant that was advanced to the F4 generation. In addition, for the AT population, rust and powdery mildew resistance were evaluated on a 1 to 9 scale (CIAT ref.). Leaf tissue was collected for each individual F2 plant grown in the greenhouse and DNA was extracted by the method of Afanador et al. (1993). Ninety-four plants were analyzed for each of the AP populations and fourty-six plants were analyzed for the AT populations. A total of 68 common bean microsatellite markers (BM, BMc, BMd, BMy and Clone series) were tested for polymorphism on the parents of each population. Polymorphic microsatellites were run on all individuals of the population along with the parents. Conditions for amplification and analysis of microsatellites are given in other parts of this annual report.

Results and Discussion

Phenotypic analysis showed that both the AP and AT populations were segregating for all the traits listed in Table 3. Several of the traits appear to be simply inherited, notably stem color, flower color (as evaluated in both the greenhouse for F2 plants and in the field for F3 families from all populations). Rust resistance (as evaluated in the field during a natural epidemic that occurred for the AT population in Semester 2002B) also appeared to be simply inherited. Meanwhile, growth habit, plant height, flowering date, maturation date, leaf size, leaf color, pod size, yield and yield components were more quantitative traits in all the populations. Leaf shape was probably an oligogenically inherited trait because there were gradations between the narrow leaf of the wild tepary bean and the wider leaf of the cultivated tepary bean. For the more narrow crosses, the AA population segregated for plant height, stem color, flower color and leaf shape in the greenhouse, while the AC populations showed very little segregation except in plant height in the greenhouse. Neither of these populations has been planted in the field yet.

For the molecular survey, the rate of parental polymorphism was roughly equivalent for both the AP (28 microsatellites or 40.6%) and AT (29 microsatellites or 43.3%) populations (Table 4). Of these a total of 25 and 9 microsatellites were selected to run on the AP and AT populations, respectively. Significant segregation distortion was observed for 48% (12 microsatellites), 76% (19), 11% (1) and 22% (2) of these markers in the populations AP-1, AP-2, AT-1 and AT-2, respectively. The average segregation distortion was much higher for the cross between *P. acutifolius* and *P. parvifolius* (62.0%) than between *P. a.* var. *acutifolius* and var. *tenuifolius* (16.5%).

The segregation distortion results suggest that there is a greater distance between the parents of the AP population than the AT population. Supporting this hypothesis was the observation of genetic incompatibilities and hybrid lethals and dwarfs in the cross between *P. acutifolius* and *P. parvifolius* but none between *P. acutifolius* var. *acutifolius* and var. *tenuifolius*. In each pair of reciprocal crosses the use of the cultivated *P. acutifolius* as the female parent reduced the amount of segregation distortion, while the use of the wild parent, either P. parvifolius or P. acutifolius var. tenuifolius increased the amount of segregation distortion (Table 4). This may reflect the possibility that a cytoplasmic factor for incompatibility is more significant when the wild tepary beans are used as females than when the cultivated tepary bean is used as the female parent.

Garvin and Weeden (1994) made a series of similar inter-varietal crosses between *P. a.* var. *acutifolius* and var. *tenuifolius* and studied the resulting populations with isozymes and RFLPs, finding a similar low level of 10% segregation distortion in this type of population. Our analysis is the first that we know of to reveal the high levels of segregation distortion in the inter-specific crosses between *P. acutifolius* and *P. parvifolius*. Segregation distortion and hybrid lethal incompatibilities are common among both intra-specific (eg. Andean x Mesoamerican *P. vulgaris*) and inter-specific crosses (*P. vulgaris* x *P. acutifolius*) within the genus *Phaseolus*.

Future plans

- Construct a full genetic map for the inter-specific and inter-varietal crosses using additional molecular (microsatellite and AFLP) or morphological markers.
- Tag traits of interest, such as rust and powdery mildew resistance, in the F2 populations.
- Single seed descent of F2 populations until the F7 generation to develop recombinant inbred line (RIL) populations.
- Conduct QTL studies in the RIL populations for drought and abiotic stress tolerance.

- Compare segregation distortion in the inter-specific and inter-varietal crosses and in F2 and F7 generations.
- Develop additional populations from potential tepary bean parents listed in Table 1.
- Leaf color variability among the segregating populations will be analyzed with aerial digital photography and color quantification as part of a separate project in collaboration with the Geographic Information System team.

Table 1. Parents used in crosses for genetic analysis of tepary bean.

Genotype	Species		Origin
G40006	**P. acutifolius**	Cultivated	Chiapas, Mexico
G40022	*P. acutifolius*	Cultivated	Arizona, USA
G40068	*P. acutifolius*	Cultivated	Arizona, USA
G40084	*P. acutifolius*	Cultivated	Durango, Mexico
G40106	*P. acutifolius* var. *acutifolius*	Wild	Jalisco, Mexico
G40110	**P. acutifolius**	Cultivated	Campeche, Mexico
G40113	*P. acutifolius* var. *tenuifolius*	Wild	Arizona, USA
G40185	*P. parvifolius*	Wild	Jalapa, Mexico
G40186	*P. parvifolius*	Wild	Jalapa, Mexico
G40240	*P. acutifolius* var. *tenuifolius*	Wild	Durango, Mexico

Table 2. F2 populations developed for genetic analysis of tepary bean.

Code	Type of Cross	Female	Male	No. Indiv.	No. Anal.
AP-1	Inter-specific (cultivated x wild)	G40022	G40186	120	100
AP-2	Inter-specific (cultivated x wild)	G40186	G40022	120	100
AT-1	Inter-varietal (cultivated x wild)	G40022	G40240	120	46
AT-2	Inter-varietal (cultivated x wild)	G40240	G40022	120	46
AA	Intra-specific (cultivated x wild)	G40022	G40106	120	--
AC-1	Intra-specific (cultivated x cultivated)	G40084	G40110	47	--
AC-2	Intra-specific (cultivated x cultivated)	G40110	G40084	120	--

Table 3. Characteristics evaluated on tepary bean populations.

Characteristics	Greenhouse	Field
Generation	F2[1]	F3[2]
Height	X	X
Growth Habit *	X	X
Leaf Shape *	X	X
Stem Color *	X	X
Flower Color *	X	X
Maturation Date	X	X
Dehiscence *		X
Flowering Date		X
Leaf Color *		X
Leaf Size (L x W) *		X
Pod Size (L x W) *		X
Powdery Mildew (1-9)		X
Rust (1-9)		X
Yield		X
Yield Components		X

* descriptors (IPGRI, 1985)

1/ Evaluated for both *P. acutifolius* x *P. parvifolius* and *P. acutifolius* x *P.a.. tenuifolius* populations

2/ Population of *P. acutifolius* x *P. parvifolius* was advanced to the F4 generation in the field

Table 4. Microsatellite segregation in four populations of tepary beans.

Population	AP-1 (P. acutifolius x P. parvifolius)				AP-2 (P. parvifolius x P. acutifolius)			
Allele	Cult %	Wild %	Het %	Chi-square	Cult %	Wild%	Het %	Chi-square
BM142	31.7	20.7	47.6	0.338	32.9	18.8	48.2	0.074
BM151	28.9	20.5	50.6	0.551	17.4	50.0	32.6	0.000
BM154	8.2	83.7	8.2	0.000	21.5	44.6	33.8	0.001
BM159	32.1	12.3	55.6	0.026	33.7	12.2	54.1	0.009
BM160	16.5	27.1	56.5	0.189	20.2	34.3	45.5	0.093
BM172	19.0	28.6	52.4	0.424	24.0	25.0	51.0	0.970
BM181	34.5	11.9	53.6	0.011	33.7	10.2	56.1	0.002
BM183	17.9	31.0	51.2	0.231	22.9	33.3	43.8	0.165
BM189	30.6	16.5	52.9	0.159	26.0	14.0	60.0	0.032
BM197	34.1	11.8	54.1	0.011	32.0	12.0	56.0	0.009
BM201	28.4	11.1	60.5	0.015	24.1	27.6	48.3	0.375
BMc5	18.5	21.5	60.0	0.256	26.0	28.6	45.5	0.053
BMd1	36.1	12.0	51.8	0.008	33.0	12.4	54.6	0.012
BMd11	21.4	27.4	51.2	0.725	18.2	27.3	54.5	0.295
BMd12	21.4	20.2	58.3	0.308	20.6	19.6	59.8	0.157
BMd17	35.7	23.8	40.5	0.066	22.4	28.6	49.0	0.670
BMd20	15.6	41.6	42.9	0.003	12.7	39.2	48.1	0.001
BMd36	21.2	24.7	54.1	0.674	22.2	16.2	61.6	0.048
BMd41	8.4	80.7	10.8	0.000	4.5	84.3	11.2	0.000
BMy2	36.8	26.5	36.8	0.045	31.7	31.7	36.6	0.018
BMy4	24.1	36.1	39.8	0.053	25.6	33.7	40.7	0.079
BMy6	23.5	21.2	55.3	0.592	19.2	24.2	56.6	0.333
Clon 7	41.9	41.9	16.1	0.001	54.0	31.7	14.3	0.000
Clon 410	25.0	36.3	38.8	0.048	15.5	60.7	23.8	0.000
Clon 454	25.0	28.6	46.4	0.725	25.3	16.5	58.2	0.114
Population	AT-1 (P. acutifolius x P. parvifolius)				AT-2 (P. parvifolius x P. acutifolius)			
D	Cult %	Wild %	Het %	Chi-square	Cult %	Wild%	Het %	Chi-square
BM172	26.1	28.3	45.7	0.822	13.3	28.9	57.8	0.195
BM183	14.6	34.1	51.2	0.207	31.0	23.8	45.2	0.667
BMc5	34.1	15.9	50.0	0.234	23.9	28.3	47.8	0.878
BMd11	34.9	9.3	55.8	0.045	23.9	26.1	50.0	0.978
BMy1	19.6	28.3	52.2	0.676	19.6	30.4	50.0	0.581
BMy2	22.2	22.2	55.6	0.757	26.1	39.1	34.8	0.054
BMy4	0.0	26.7	73.3	0.000	0.0	21.7	78.3	0.000
Clon 20	24.4	31.1	44.4	0.620	17.4	43.5	39.1	0.015
Clon 454	22.7	20.5	56.8	0.649	28.9	37.8	33.3	0.058

References

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1.1.4 Genetic diversity of microsatellites in common bean III

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[1]SB-2 Project; [1]IP-1 Project

Introduction

Microsatellites markers are based on short segments of DNA in which a specific simple sequence motif of 1-6 bases is repeated in tandem, multiple times. Due to the innate variability at microsatellite loci, these markers have been ideal for characterizing genetic diversity in crop species at the inter-specific, inter-subspecific, inter-varietal and even intra-varietal levels. Microsatellites have been found to vary in the polymorphism they detect depending on the length and sequence of the repeat motif they contain and their location along the chromosomes, specifically whether they reside in gene-coding or non-coding segments of the genome.

The objective of this study was to complement two previous parental surveys by evaluating all new *Phaseolus* microsatellite markers developed at CIAT and elsewhere for their allelic variability on a third panel of 20 common bean genotypes. This new panel of parents represents diverse cultivated germplasm of common beans, both Mesoamerican and Andean, which have been used as parents in the bean breeding program. Several accessions from Durango (type III climbing habit) and Guatemala (type IV climbing habit) races (groupings within the Mesoamerican genepool), are represented in a microsatellite survey for the first time.

Methodology

The genotypes consisted in 20 common bean genotypes including 4 Andeans and 16 mesoamericans (Table 1) which are the parents of 8 mapping populations being studied at CIAT for nutrient deficiency, aluminum and drought stress tolerance. Among the Andean accessions were representatives of the Nueva Granada race only, while among the Mesoamerican populations there were representatives of the Mesoamerica, Durango and Guatemala races. Several of the breeding lines from the SEA series are the result of pedigrees which combine parents from the Durango and Mesoamerica races. Among the genotypes used here were a mixture of type I (determinate bush), II (indeterminate bush), IIIa (prostrate bush) and IVa (climber) growth habits. The populations included 5 intra-genepool Mesoamerican x Mesoamerican crosses and 7 inter-genepool Mesoamerican x Andean populations (Table 2). The intra-genepool crosses included some that were between representatives of the same race or of different races (notably Mesoamerica x Durango-Mesoamerica hybrids). The genotypes were evaluated with a total of 166 microsatellite markers (of which 98 were derived from genomic libraries and 68 were derived from cDNA or gene sequences). The markers were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The amplification conditions are given in other parts of this annual report. The PCR products were resolved by electrophoresis for approximately one hour at 130 constant volts on silver-stained 4% polyacrylamide gels. Microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

Results and Discussion

The average rate of polymorphism was higher in the seven inter-genepool (Andean x Mesoamerican) crosses (55.1 %) than in the five intra-genepool (Mesoamerican x Mesoamerican) crosses (22.6 %) (Table 2a). Among the Mesoamerican x Mesoamerican crosses, the rate of polymorphism was higher in the inter-racial cross SEA5 x MD23-24 (25.9%) and in the intra-racial

cross BAT881 x G21212 (26.5%) than in the other crosses which were within a single race and between more closely related genotypes such as DOR364 x BAT477 (both Mesoamerican CIAT breeding lines). Among the inter-genepool crosses all were relatively similar in the level of polymorphism between the parents (from 55 to 59% on average), with the possible exception of BRBR191 x MAM38 (51.2%) and BRB191 x MAM49 (53%). Both of these trends for polymorphism rates in intra and inter-genepool crosse were equally evident when using genomic and cDNA derived microsatellites.

Genomic microsatellites detected more polymorphism (43.9%) than cDNA microsatellites (38.4%) overall. The difference was more noticeable in the intra-genepool crosses than in the inter-genepool crosses, where both types of microsatellites were about equally effective in uncovering polymorphism. Among the classes of markers, the BM, BMy and BMd microsatellites were the most polymorphic, while the Pv microsatellites were the least polymorphic (Table 2b). New microsatellites (clones) were intermediate in polymorphism, reflecting the fact that they are from a mix of genomic and cDNA clones.

Significantly fewer average alleles per locus were found for microsatellites from genes (2.9) than for microsatellites from non-coding sequences (3.7). Although the highest number of alleles was 12 for gene based microsatellites and 14 for genomic microsatellites, the gene-derived microsatellites frequently were bi- or tri-allelic and mostly distinguished the difference between Andean and Mesoamerican genepools. Meanwhile the genomic microsatellites detected more alleles and were thus able to resolve some within-genepool variation. The discriminating power (D) of the gene-derived microsatellites (0.478 among polymorphic, 0.349 among all markers, including monomorphic) was lower than for the genomic microsatellites (0.548 and 0.411, respectively). The discrimination power was positively correlated with the number of alleles produced at the locus. Null alleles were uncommon in both microsatellite classes. This study confirms that the more polymorphic genomic microsatellites may well become the mainstay of mapping studies since they will be more useful than the cDNA derived microsatellites in narrow intra-genepool crosses. Meanwhile the more conserved and stable cDNA-derived microsatellites may find their greatest utility in mapping in wide inter-genepool or inter-specific crosses.

Future plans

- Construction of another panel of common bean parents to survey for polymorphism in populations developed for disease resistance studies and marker assisted selection.
- Genotyping of many of the common parents and genetic sources used at CIAT, to allow us to implement whole-genome marker assisted selection that is specific to the genetic crosses made in our bean breeding program.

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

Table 1. Mapping parent genotypes used for assessment of genetic diversity of common bean microsatellites.

	Variety	Genepool	Race	Purpose	Growth Habit	Origin
1	BAT 881	Mesoamerican	Mesoamerica	Low fertility sensitive	II	CIAT line
2	G 21212	Mesoamerican	Mesoamerica	Low fertility tolerance	II	CIAT accession
3	BAT 477	Mesoamerican	Mesoamerica	Drought tolerance	II	CIAT line
4	DOR 364	Mesoamerican	Mesoamerica	Cultivar	II	CIAT line
5	G 3513	Mesoamerican	Mesoamerica	Low fertility tolerance	II	CIAT accession
6	G 19833	Andean	Nueva Granada	Low fertility tolerance	IIIa	CIAT accession
7	G 855	Mesoamerican	Guatemala	Climbing bean	IVa	CIAT accession
8	BRB 191	Andean	Nueva Granada	BCMV resistance	II	CIAT line
9	MAM 49	Mesoamerican	Durango	Cultivar, Aluminum tol.	IIIa	CIAT line
10	G 5273	Andean	Nueva Granada	Cultivar, Aluminum tol.	II	CIAT accession
11	MAM 38	Mesoamerican	Durango	Cultivar, Aluminum tol.	IIIa	CIAT line
12	SEQ 1027	Andean	Nueva Granada	Drought, Aluminum tol.	III	CIAT line
13	G 4090	Mesoamerican	Mesoamerica	Aluminum tolerance	III	CIAT accession
14	Tio Canela	Mesoamerican	Mesoamerica	Cultivar	II	Honduran
15	DOR 714	Mesoamerican	Mesoamerica	Aluminum tolerance	II	CIAT line
16	SEA 5	Mesoamerican	Durango-Meso	Drought tolerance	II	CIAT line
17	MD 23-24	Mesoamerican	Mesoamerica	Advanced line	II	CIAT line
18	SEA 15	Mesoamerican	Durango-Meso	Drought tolerance	II	CIAT line
19	G 685	Mesoamerican	Guatemala	Climbing bean	IVa	CIAT accession
20	SEA 21	Mesoamerican	Mesoamerica	Drought tolerance	II	CIAT line

Table 2. Polymorphism rate among 12 parent combinations for 166 microsatellite loci (68 cDNA and 98 genomic).

a) by marker class

Cross			cDNA		Genomic		Total	
			No.	%	No.	%	No.	%
BAT 881	x	G 21212	16	23.5	28	28.6	44	26.5
DOR 364	x	BAT 477	17	25.0	22	22.4	39	23.5
DOR 364	x	G 3513	10	14.7	23	23.5	33	19.9
DOR 364	x	G 19833	41	60.3	57	58.2	98	59.0
BRB 191	x	G 855	34	50.0	57	58.2	91	54.8
BRB 191	x	MAM 38	32	47.1	53	54.1	85	51.2
G 5273	x	MAM 38	37	54.4	57	58.2	94	56.6
BRB 191	x	MAM 49	33	48.5	55	56.1	88	53.0
G 5273	x	MAM 49	38	55.9	55	56.1	93	56.0
G 4090	x	SEQ 1027	32	47.1	60	61.2	92	55.4
Tio Canela	x	DOR 714	9	13.2	20	20.4	29	17.5
SEA 5	x	MD 23-24	14	20.6	29	29.6	43	25.9
Total evaluated			68		98		166	

b) by marker name

Cross			BM		BMd		CLONES		BMy		PV	
			No.	%	No.	%	No.	%	No.	%	No.	%
BAT 881	x	G 21212	22	37.3	9	17.6	6	18.2	6	60	1	7.7
DOR 364	x	BAT 477	16	27.1	8	15.7	6	18.2	6	60	3	23.1
DOR 364	x	G 3513	17	28.8	5	9.8	5	15.2	5	50	1	7.7
DOR 364	x	G 19833	38	64.4	29	56.9	14	42.4	10	100	7	53.8
BRB 191	x	G 855	39	66.1	28	54.9	12	36.4	8	80	4	30.8
BRB 191	x	MAM 38	36	61.0	26	51.0	12	36.4	7	70	4	30.8
G 5273	x	MAM 38	40	67.8	26	51.0	13	39.4	10	100	5	38.5
BRB 191	x	MAM 49	38	64.4	26	51.0	13	39.4	7	70	4	30.8
G 5273	x	MAM 49	39	66.1	26	51.0	13	39.4	10	100	5	38.5
G 4090	x	SEQ 1027	40	67.8	29	56.9	13	39.4	6	60	4	30.8
Tio Canela	x	DOR 714	14	23.7	7	13.7	4	12.1	4	40	0	0.0
SEA 5	x	MD 23-24	20	33.9	12	23.5	6	18.2	5	50	0	0.0
Total evaluated			59		51		33		10		13	

1.1.5 Phaseolin characterization of Caribbean common bean germplasm

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Introduction

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

The objective of this work was to characterize the phaseolin alleles found in the collected germplasm and thus help the University of Puerto Rico to determine the genepool to which the traditional varieties belong. A long term objective of this project is to conserve the genetic resources of this group of Caribbean varieties by using them in breeding programs.

Methodology

A total of 68 entries of common bean genotypes were genotyped for their phaseolin pattern. These included a total of 43 traditional varieties (or selections thereof) from the Caribbean (23 from Puerto Rico, 18 from Dominican Republic and 1 each from Haiti and Jamaica); bred lines from CIAT (6), University of Puerto Rico (15); as well as modern varieties from Colombia (ICA Palmar), Peru (Blanco Laran) and the United States (Montcalm, Redhawk). Total seed proteins were extracted from 0.10 g of peeled, finely-ground, oven-dried seed by a standard extraction technique used at the Genetic Resource Unit at CIAT. One microliter each of the protein extracts were separated with 6% separation / 12% stacking SDS-PAGE (polyacrylamide) mini-gels run for 50 minutes and stained with Coomasie Blue dye. The phaseolin pattern was compared to known standards provided by O. Toro of the Genetic Resource Unit of CIAT.

Results and Discussion

Only three phaseolin patterns were found among the Caribbean landraces: the most common being the "T" allele typical of many bush Andean beans. The "S" allele typical of Mesoamerican beans was the second most common allele while a third pattern, the "C" variant, was found for only a single traditional variety from Puerto Rico (Naranjito I). The "C" pattern is thought to be a hybrid

of "S" and "T" phaseolins. In both the Dominican Republic and Puerto Rico the "T" phaseolin was more common than the "S" phaseolin. It was notable that in Puerto Rico only one out of 23 varieties (4.3%) had "S" phaseolin, while in the Dominican Republic 5 out of 18 varieties (27.7%) had the "S" allele. Among the modern Andean varieties and breeding lines both "S" and "T" alleles were found. No additional diversity was observed at this locus for the germplasm studied and these results were confirmed with the use of phaseolin standards with known banding patterns.

Phaseolin allele has been associated with seed size in traditional varieties in Latin America (Castinieras et al., 1994) with "S" types generally being small seeded. The phaseolin locus has been associated in genetic studies with a QTL underlying seed size (Gepts et al., 1988). In this study, the "S" phaseolin pattern was found in many medium-seeded varieties although the largest seeded varieties did have the "T" phaseolin. In the modern varieties, the seed size was not correlated with phaseolin pattern.

It seems that the Andean type "T" phaseolin is more common in Puerto Rico than in the Dominican Republic due to its closer proximity to South America along the suspected route of introduction through the Leeward island chain into the central Caribbean. Conversely, the Mesoamerican type "S" phaseolin may be more common in the Dominican Republic than in Puerto Rico because of its proximity to Cuba, which may have acted as a bridge to Mexico and Central America, probable sources of Mesoamerican beans with "S" alleles. Indeed, two studies showed that Cuba had a mixture of "S", "Sb" and "T" phaseolins, with the Mesoamerican types predominating (Castinieras et al., 1994; Lioi et al, 1990). In that study phaseolin allele was correlated with seed size, while in this study, large seeded types had both phaseolin patterns, suggesting that hybridization and recombination between phaseolin type and seed size had occurred in some of these Caribbean "Andean" genotypes.

Therefore we may postulate a hybrid Mesoamerican-Andean origin for several Caribbean seed classes including the red mottled (Dominican Pompadour), pink striped (Jamaican Miss Kelly, Puerto Rican Colorado de Pais) and red speckled (Haitian Pompadour) types. In the Caribbean, seed size of landraces is often intermediate between Mesoamerican and Andean types, as is growth habit, leaf size and other phenotypic traits. This provides evidence of further mixing of the genepools in this region of the Americas. A similar area of genepool overlap and mixture is postulated to have occurred in Northern South America, especially in Colombia where many Andeans present Mesoamerican phaseolin alleles indicating possible past hybridization and introgression. These studies have been supported by other molecular marker assays and surveys. Meanwhile, breeding programs have encouraged the same sort of recombination between phaseolin types and seed size in their advanced lines. This was seen in this study where many of the CIAT and UPR large seeded "Andean" breeding lines were a mixture of either "S" or "T" phaseolin genotypes.

The advantages of hybridization between the genepools is evidenced in some Caribbean germplasm which although they have the medium to large seed size of the Andean types, have a greater adaptation to tropical lowland conditions to which the Mesoamerican types are better suited. Hybrid progeny that fit local preferences and had these advantages were probably selected by the farmers in the region and as such, the germplasm of the Caribbean shows promise for breeding efforts that try to adapt Andean beans to warmer climates.

Table 1. Phaseolin characterization of Caribbean germplasm

Entry	Name	Seed source	Origin	Color	Phaseolin
Caribbean Germplasm					
PT-40	B. VISTA	X049-93-94	Dom Rep	6M,K	T
PT-53	CHIJAR 35	X043-36	Dom Rep	6,M	S
PT-51	DERRUMBA 13	X043-24	Dom Rep	6,M	T
PT-54	H VALLE 24	X043-52	Dom Rep	6,M	S
PT-41	JB-178	X049-155	Dom Rep	6,M	T
PT-42	JB-569	X049-157	Dom Rep	6,M	T
PT-43	J BETA	X049-159	Dom Rep	6,M	T
PT-47	LARGA C	X043-15	Dom Rep	6,M	T
PT-48	LA CARMITO	X043-16	Dom Rep	6,M	T
PT-49	LA CHULA	X043-14	Dom Rep	6,M	T
PT-39	MAGUANA	X049-91-92	Dom Rep	6,M	T
PT-44	PC 50	X049-161	Dom Rep	6,M	T
PT-45	POMOR 17	X043-19	Dom Rep	6,M	T
PT-46	POMOR 19	X043-20	Dom Rep	6,M	T
PT-52	PACASAS 21	X043-28	Dom Rep	6,M	T
PT-56	PACASAS 29	X043-65	Dom Rep	6,M	S
PT-50	VASON 4	X043-30	Dom Rep	6,M	S
PT-55	VASON 25	X043-111	Dom Rep	6,M	S
PT-38	SALAGNAC 90A	X049-89-90	Haiti	6,M	S
PT-68	IND.JAMAICA RED	X049-137	Jamaica	6RK	T
PT-1	COAMO #2	X028-1	Puerto Rico	2RK	T
PT-2	COAMO #13	X028-2	Puerto Rico	2R,M	T
PT-3	COAMO #14	X028-3	Puerto Rico	5R,M	T
PT-8	COLORADO DEL PAIS 1	UPR	Puerto Rico	5R,M	T
PT-23	COLORADO DEL PAIS 2	RINCON	Puerto Rico	5R,M	T
PT-9	GURABO-1	X028-9	Puerto Rico	3K,G	T
PT-10	GURABO-2	X028-10	Puerto Rico	5R,M	T
PT-11	GURABO-3	X028-11	Puerto Rico	5R,M	T
PT-12	GURABO-4	X028-12	Puerto Rico	7M,M	T
PT-13	GURABO-5	X028-13	Puerto Rico	5R,M	T
PT-14	GURABO-6	X028-14	Puerto Rico	5R,M	T
PT-15	GURABO-7	X028-15	Puerto Rico	5R,M	T
PT-16	NARANJITO-1	X028-16	Puerto Rico	6M,M	C
PT-17	NARANJITO-2	X028-17	Puerto Rico	7M,M	T
PT-18	NARANJITO-6	X028-21	Puerto Rico	1K,G	T
PT-19	NARANJITO-7	X028-22	Puerto Rico	5R,M	T
PT-4	OROCOVIS IA	X028-4	Puerto Rico	6M,M	S
PT-5	OROCOVIS IB	X028-5	Puerto Rico	6M,M	T
PT-6	OROCOVIS IC	X028-6	Puerto Rico	7M,M	T
PT-7	OROCOVIS 2	X028-7	Puerto Rico	7M,M	T
PT-20	OROCOVIS-3	X028-23	Puerto Rico	3K,G	T
PT-21	OROCOVIS-2A	X028-24	Puerto Rico	6M,M	T
PT-22	OROCOVIS-2B	X028-25	Puerto Rico	2R,M	T
Non-Caribbean germplasm					
PT-57	A36	X049-45	CIAT	6,M	T
PT-62	AFR 285	X049-109	CIAT	5R,K	S
PT-63	AFR 619	X044-130	CIAT	6M,M	T
PT-64	AFR 699	X044-131	CIAT	6MK,G	T
PT-66	CAL 96	X044-134	CIAT	6MK,G	T
PT-67	DRK 57	X044-135	CIAT	6K,G	T
PT-60	MONTCALM	X049-95	USA	6,K	S
PT-61	REDHAWK	X049-125	USA	6,K	S
PT-58	ICA PALMAR	X049-53	Colombia	6,M	T
PT-65	BLANCO LARAN	X044-133	Peru	1,G	S

Future Plans

- Compare the phaseolin results with the RAPD data and evaluate the same germplasm with additional marker types especially microsatellites.

- Evaluate a larger number of genotypes from the Caribbean region for both phaseolin pattern and molecular marker diversity and compare these to collections from countries of the region that are held in the collection of common bean at CIAT.

- Determine whether the large seeded Caribbean germplasm traces back to the Nueva Granada

race and whether several races of Mesoamerican beans contributed genes to the this germplasm.

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1.1.6 Interspecific progeny evaluated for drought tolerance

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[1]SB-2 Project; [2]IP-1 Project

Introduction

The center of diversity of the genus *Phaseolus* is found in Middle America, and largely in Mexico. Thus some species of *Phaseolus* evolved in dry, near-desert environments. These dry-adapted species include *P. acutifolius* (Pa) both domesticated and wild forms, and *P. parvifolius* (Pp) which is either closely related to *acutifolius* or is a morphotype of the same. These species are considered to pertain to the tertiary gene pool of the common bean and crosses with *P. vulgaris* (Pv) are possible through embryo rescue. Work at CIAT has advanced in obtaining intermediate types that are nearly fertile with both parental species, thus facilitating gene transfer across the species barrier. One important objective of these crosses is to transfer drought tolerance from *acutifolius* to common bean.

Materials and Methods

Interspecific crosses were generated in previous years through congruity backcrosses (Haghighi and Ascher 1988), to transfer resistance to common bacterial blight (Mejía Jiménez et al. 1994). Several hundred interspecific progeny were available combining accessions of Pa and Pp with common bean cultivar ICA Pijao. Subsequently, as work advanced in improving fertility and gene introgression of interspecific hybrids, more complex crosses (Mejía Jiménez et al., 2001) were made involving other accessions of common bean (ICA Pijao, MAM38, A775, A800 and Bayo Madero) and cultivated (G40001, G40020, G40065) and wild (NI576) tepary beans.

F3 seed was planted in the field under drought conditions in the June season, 2001. Promising families were harvested in bulk and in F4, individual selections were made followed by another cycle of mass selection. Thus, in 2002, F6 families were evaluated in a yield trial with RBC design and three replications under severe drought conditions, as described above.

Results and Discussion

Highly significant differences in yield were registered among families and checks (Table 1). The tolerant heck, SEA 5 yielded 715 kg ha^{-1}, and the sensitive check (ICA Pijao) which figured as the common bean parent in many of the lines produced 468 kg ha^{-1} (significantly less than SEA 5). The best line derived from ICA Pijao yielded 798 kg ha^{-1}, which was significantly different than Pijao, suggesting effective introgression from acutifolius for drought tolerance. The best families, however, were derived from the cross BKI 11 of *P. acutifolius* with the race Durango common bean variety Bayo Madero from Mexico. Race Durango typically presents some level of tolerance to drought, and the combination of Bayo with *acutifolius* produced lines that yielded over a ton, or about 50% over the tolerant check. This is comparable to the yield advantage obtained in the intraspecific crosses which represented far more investment of time and breeding than in these crosses with *acutifolius*, although the interspecific progeny are still far removed from commercial common bean type with the necessary agronomic traits and grain. The hope is that the mechanisms that operate in *acutifolius* might be complementary to those in *vulgaris*, and permit the pyrimiding of even greater tolerance. Heat tolerance is still another trait that is required in combination with drought tolerance, and these crosses may offer this trait as well.

Table 1. Yield (kg ha^{-1}) of interspecific hybrids between common bean and P. acutifolius.

Entry	Cross Code	Yield kg ha^{-1}	Maturity	Pedigree
27	BKI 11	1065	77	Bayo Madero X [CBC5(CBC3X CBC3)*]
28	BKI 11	1038	74	Bayo Madero X [CBC5(CBC3X CBC3)*]
6	4V3A1-002	798	69	(((((Pv x Pa) x Pv) x Pa) x Pv) x Pa) x Pv
54	MMNNI 14	751	73	{(CBC5X CBC5)X (CBC5X CBC3) } X{(G40065 X NI576)X [(CBC5X CBC5)X (CBC5X (CBC3X CBC3)}**
64	INB 39	737	72	(((((Pv x Pa) x Pv) x Pa) x Pv) x Pa) x Pv
30	BKI 11	731	72	Bayo Madero X [CBC5(CBC3X CBC3)*]
29	BKI 11	728	77	Bayo Madero X [CBC5(CBC3X CBC3)*]
60	SEA 5 (ch)	715	71	
	ICA Pijao (ch)	468	72	
	LSD (0.05)	184	2.3	

- Cross between congruity backcross hybrids involving the genotypes G40020 and G40001 of tepary bean, and ICA Pijao, A775, MAM38 and A800 of common bean (Mejía Jiménez et al. 1994).

** Double congruity backcross hybrids (Mejía Jiménez et al. 2001) involving the genotypes NI576, G40065, G40020 and G40001 of tepary bean, and ICA Pijao, A775, MAR1, MAM38 and A800 of common bean

Conclusions

Interspecific progeny presented significant introgression of drought tolerance from *acutifolius* to common bean. These represent another potential source of tolerance genes to improve common bean, and may also be a source of heat tolerance.

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1.1.7 Identification of symbiotic bacteria from native Colombian entomophagous nematodes

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Introduction

Whiteflies are considered a major problem in cassava production in the Neotropics and Africa. Most whitefly species cause crop losses through direct feeding; some are very efficient vectors of several economically important plant viruses.

Despite intensive crop improvement efforts, current whitefly and virus control practices still depend heavily on pesticides. Despite the increase in pesticide use, whiteflies are not being controlled effectively because of pesticide resistance. Thus the use of pesticides for whitefly control is neither environmentally sound nor sustainable.

Host plant resistance offers an environmentally sound solution; however crop plant resistance to whiteflies is scarce. In the future the use of insecticidal genes to whiteflies by genetic transformation should be a good pest-control alternative. Genetic transformation of cassava with Bt genes has been identified as desirable for protection against stem borers. Recently a promising new group of toxins that might eventually be used instead of Bt or in combination with it were isolated—symbiotic bacteria (Enterobacteriaceae) from entomophagous nematodes, belonging to the genera *Xenorhabdus* and *Photorhabdus*. These bacteria, which live in a mutualistic association with the nematodes, are released from the gut of the nematode when the insect hemocoel is invaded by the nematode. These bacteria-secreted compounds are active against a wide range of insects from different orders and are as patentable as the delta-endotoxins of Bt and therefore may provide useful alternatives to the deployment of Bt toxins in transgenic plants.

In 1992 it was found that native nematodes are associated naturally with *Cyrtomenus bergi* in three different locations of Colombia. These nematodes were originally identified as three strains of *Heterorhabditis bacteriophora,* but their identification has been revised by another taxonomist who concluded that they correspond to a new species of nematode, order Rhabditidae, genus *Rhabditis*.

Therefore, assuming that the symbiotic bacteria-nematode association is specific, the possibility of identifying new bacteria species from the aforementioned native Colombian nematodes is probable and the identification of a new insecticide protein from these bacteria would be possible. Biochemical and morphological tests showed that the isolated bacteria probably belong to the genus *Bacillus*.

The purpose of this study was to identify the bacteria isolated in association with *Rhabditis* sp. by using sequence analyses of PCR-amplified 16S rDNAs. This novel insecticide could be used against a range of pests in addition to whiteflies. These include mealybugs, hornworm and soil pests such as burrower bugs and white grubs.

Materials and Methods

Strains. The following were used: Strain IJ3SQC92 from the symbiotic bacteria nematodes, *Bacillus* sp. and the control strain *Bacillus thuringiensis* (Laverlam).

DNA isolation. Isolates were grown in Medium Φ (3 ml) and incubated at 28-30°C on a shaking rack overnight. DNA extraction was done as described by Lumini (1996) with some modifications.

PCR amplification of 16S rDNAs. PCR amplification was done as described by Babic et al. (2000) with some modifications. The following two eubacterial-specific oligonucleotide primers (QIAGEN) were used: 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' sense and 5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3' antisense (Weisburg et al., 1991).

Nucleotide sequencing of PCR-amplified 16S rDNAs. The PCR-amplified 16S rDNAs of the aforementioned strains were sequenced. Amplified 16s rDNAs were purified with the QIAquick PCR Purification Kit (250) (QIAGEN). Single-strand sequencing was performed using a Big Dye kit (Perkin Elmer).

Results and Discussion

DNA isolation. We obtained a pure and concentrated DNA of two strains, *Bacillus* sp. and *B. thuringiensis* (Figure 1).

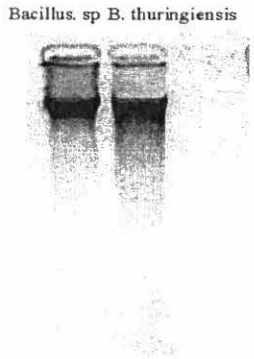

Figure 1. DNAs of *Bacillus* sp. and *B. thuringiensis* visualized in 1.5% agarose gel.

PCR amplication of 16S rDNAs. DNAs of two strains isolates were amplified with the two specific eubacterial primers used, two produced a single fragment. Two fragments were around 1600 bp, which was the size expected for 16S rDNAs (Figure 2).

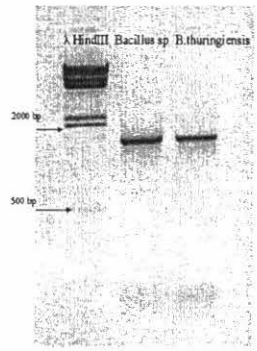

Figure 2. PCR amplifications of 16S rDNAs from *Bacillus* sp. and *B. thuringiensis*.

Nucleotide sequencing of PCR-amplified 16S rDNAs. The isolated sequences 16S rDNAs of *Bacillus* sp. and *B. thuringiensis* were compared to GenBank database sequences using the algorithm BLASTN to identify the higher scoring similarities. For *Bacillus* sp. there was a high percentage of homology (98%, data not shown) to different *Bacillus* spp., mainly to *B. cereus, B. thuringiensis, B. anthracis* and *B. mycoides*. The previous sequences along with other more distantly related species of *Bacillus* (*B. larvae, B. pasteurii, B. subtilis, B. pumilus, B. licheniformis, B. lentus, B. firmus, B. coagulans* and *B. badius*) were downloaded from the GenBank database to draw specific relationships between these species and the isolated sequence (*Bacillus* sp.).

These sequences were aligned using CLUSTAL-X. A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance-tree topologies. Graphic representation of the resulting trees was made using NJPLOT (Figure 3).

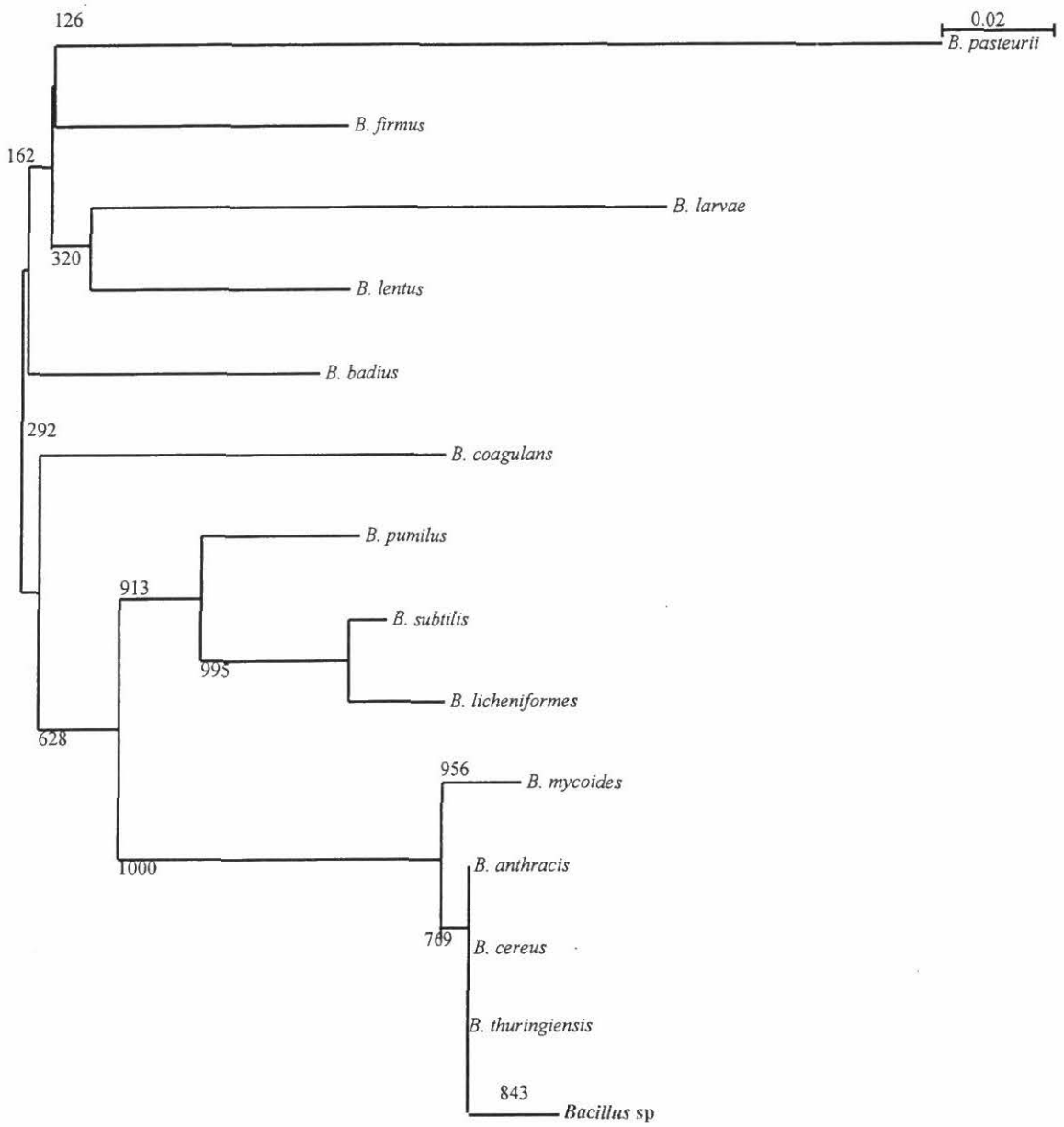

Figure 3. Neighbor-joining tree from *Bacillus* spp. partial 16S sequences based on bootstrap approach; to determine the reliability of the topology, numbers indicate bootstrap support for interior nodes; the bar indicates a distance of 0.002 substitutions per site.

The output tree showed that the 16S sequence of *Bacillus* sp. was grouped with *B. cereus, B. thuringiensis, B. anthracis* and *B. mycoides,* confirming the high percentage of homology presented by BLASTN. Groups formed in the tree were also consistent with the identification keys from *Bacillus* spp. according to the International Journal of Systematic Bacteriology. These keys are supported by traditional methods (morphological data, biochemistry tests) to classify the major species of *Bacillus* (Thiery & Frachon, 1997).

The data showed that the 16s sequence from the nematode symbiotic bacteria *Bacillus* sp. is closely related to *B. cereus, B. thuringiensis* and *B. anthracis.*

Future Plans

- Isolate more strains of the symbiont bacteria *Bacillus* sp. from nematodes
- Conjugate molecular, morphological and biochemical data to make a clear identification of the symbiont bacteria
- Perform DNA-DNA hybridization of different strains and *Bacillus*-related species to identify the problem species

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1.1.8 Analyzing the genetic diversity of the Colombian plantain (musacea) collection using microsatellites

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Summary

The Colombian collection of Musaceas (CCM), maintained on the farm "El Agrado" (Armenia, Quindio), has plantains adapted to high-altitude agro-ecosystems (elevation 1310 m). Of the 134 accessions, some of which were the first introductions to America, only a few have been characterized on the basis of agronomic and morphologic traits including growth habit, development and production (Belalcázar et al., 1991). Several ongoing studies on the morphological, biochemical and molecular characterization of the genus *Musa* will certainly contribute toward understanding the genetic diversity of the CCM. We propose to evaluate this collection further, using microsatellites to simplify the management of the collection by reducing, for example, *in vitro* and *ex situ* maintenance costs. DNA samples have already been collected. We will use 25 microsatellite primer pairs for analyzing the genetic diversity of the CCM.

1.1.9 Molecular and agromorphological genetic diversity characterization of soursop (*Annona muricata* Linn) and related species of the Anonaceae family

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Summary

We are characterizing, at the molecular and agromorphological level, the genetic variability of soursop and related species of the Anonaceae family, available in national germplasm banks as well as in other agricultural research centers. Of the 98 samples collected, the DNA extracted and analyzed with three AFLP primer combinations (E-ACT/M-CAA, E-AGC/M-CTC, E-AGC/M-CAA; AFLP's Análysis System I Kit - GIBCO-BRL). Samples were run on denaturing, polyacrylamide gels (4 or 6%, depending upon primer combination). AFLP patterns are being interpreted to understand genetic similarity relationships among samples.

1.1.10 Improving the breeding of lulo (*Solanum quitoense* Lam) through understanding the species' genetic diversity

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Summary

Lulo is a fruit crop in high demand in Colombia. To satisfy consumer requirements, the country has to import approximately 25% of the demand from Ecuador, which is equivalent to the production of 10,000 ha. Colombia has ecological niches suitable for growing the crop, genetic variability to develop new genotypes, germplasm collections of the species and related taxa, and breeding programs that have already released the first Colombian var. *Lulo La Selva.* To improve the breeding of lulo through an understanding of its genetic diversity requires the support of modern technologies such as molecular markers. We propose to study the genetic potential of lulo and related species available in the State Germplasm Bank System, coordinated by CORPOICA, in an attempt to establish and exploit the genetic diversity of the crop. For this purpose the collection is being morphologically characterized. Furthermore, DNA samples have been extracted from several individuals and will be analyzed using AFLP's Analysis System I (GIBCO-BRL).

1.1.11 AFLP characterization of *Capsicum* sp. germplasm from Guatemala and Cuba

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Summary

Home gardens are microenviroments containing high levels of species and genetic diversity within larger farming systems. These gardens are not only important sources of food, fodder, fuel, medicines, spices,construction materials and income in many countries around the world, but are also an important means for in situ conservation of a wide range of plant genetic resources. Home gardens are dynamic in their evolution, composition and uses. Their structure, function, and species and varietal diversity have been influenced by the changes in socioeconomic circumstances and cultural values of the users of these gardens. The understanding of these factors and the ways they change, according to the behavior and decision-making patterns of these users is crucial in shaping strategies for including home gardens as a viable option for in situ conservation of agrobiodiversity (IPGRI's Home Garden Projects). AFLP molecular characterization of the existing diversity in *Capsicum* sp. from home gardens in Guatemala and Cuba is the main purpose of this research. We collected 86 samples in Guatemala and 85 in Cuba. The DNA was extracted and samples analyzed with two primer combinations (E-AGC/M-CAA and E-AGG/M-CTT) with the AFLP's Analysis System I Kit (GIBCO-BRL). Then, the samples were run in 4% polyacrylamide gels. More than 80 polymorphic DNA bands were observed, which—after classification and statistical analyses—will provide information on the diversity maintained in home gardens.

1.1.12 Molecular analysis of the genetic stability of cassava clones (*Manihot esculenta* Crantz) cultured *in vitro* with silver nitrate to retard growth

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Introduction

Genetic stability of in vitro-propagated plants has been a concern for the conservation (in vitro or cryoconservation) of germplasm. The molecular criterion has been proposed as the method of choice to detect genetic changes. Thus our objective was to monitor the genetic stability of these plants at the molecular level, after they had been exposed to in vitro culture and growth retardants. We decided to use AFLPs (given their more ample coverage of the genome) to fingerprint *in vitro*-maintained plants of six cassava clones, which were subjected to treatments with two concentrations of silver nitrate to retard growth.

Materials and Methods

Plants were first micropropagated by meristem culture, grown in the greenhouse and planted in the field. Morphological, agronomic and isoenzymatic evaluations were run on all materials. The AFLP's Analysis System I kit (GIBCO-BRL) was used, with some modifications, to run the AFLPs. Two genomic DNA extractions from young leaves were performed for each clone to guarantee the reproducibility of the method (Table 1).

Table 1. Clones and silver nitrate treatments performed during the experiments.

ID for Molecular Analyses	Clone	Treatment
1	MARG 2	8S[1]
2	MARG 2	AG3[2]
3	MARG 2	AG4[3]
4	M BRA 337	8S
5	M BRA 337	AG3
6	M BRA 337	AG4
7	M COL 2056	8S
8	M COL 2056	AG3
9	M COL 2056	AG4
10	M NGA 16	8S
11	M NGA 16	AG3
12	M NGA 16	AG4
13	M VEN 329A	8S
14	M VEN 329A	AG3
15	M VEN 329A	AG4
16	CM 2177- 2	8S
17	CM 2177- 2	AG3
18	CM 2177- 2	AG4

[1] 8S: Controls, [2]AG3: 10 ppm silver nitrate, [3]AG4: 12 ppm silver nitrate.

Results and Discussion

Two primer combinations were selected for AFLPs. They gave the following banding patterns:

- E-AAC/M-CTA: generated 68 well-defined bands, monomorphic within each clone and polymorphic between clones
- E-AAG/M-CTG: generated 61 well-defined bands, monomorphic within each clone and polymorphic between clones

We have not detected band differences between controls and treatments in all six clones analyzed with these two primers. This may be an indication that treating plants with a growth retardant such as silver nitrate does not have a major effect on the genetic stability of the plants; at least not detectable with the methods reported here. More primer combinations, as well as other markers like microsatellites, may be used to expand the genome coverage throughout the cassava linkage groups so more data can be generated to corroborate our initial findings.

1.1.13 AFLP characterization of avocado (*Persea americana* Mill.) genetic diversity

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Summary

Avocado (*Persea americana* Mill.) is a subtropical fruit tree with 24 chromosomes and a haploid genome size of 8.83 x 10^8 bp. Genetic analysis and breeding of avocado is complex, mainly due to tree size, a prolonged juvenile phase and a lack of knowledge of its genetics. Efforts are underway to characterize the genetic diversity of germplasm collections available using molecular markers such as RFLPs, DNA fingerprints (DFP), microsatellites and RAPDs. Our objective is to use AFLPs to study the genetic diversity in a germplasm collection maintained ex situ (field plantation) by CORPOICA (Palmira, Valle), which contains 61 accessions from Colombia, Mexico, Guatemala and Trinidad and Tobago. DNA from all clones was extracted following a modified Dellaporta protocol. Currently we are testing primer combinations with the AFLP's Analysis System I Kit (GIBCO-BRL) to select those that give reproducible, polymorphic banding patterns.

1.1.14 Genetic diversity and core collection approaches in the multipurpose shrub legumes *Flemingia macrophylla* and *Cratylia argentea*

Genotypes of grasses and legumes with dry-season tolerance

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Introduction

CIAT's work on shrub legumes emphasizes the development of materials to be used as feed supplement during extended dry seasons. High-quality tropical shrub legumes are readily available for better soils, but germplasm with similar characteristics adapted to acid, infertile soils is scarce. *Flemingia macrophylla* and *Cratylia argentea* have shown promising results in such environments; hence work on these genera is part of the overall germplasm strategy of the CIAT Forages team. *C. argentea* is being increasingly adopted and utilized, particularly in the seasonally dry hillsides of Central America and, more recently, the Eastern Plains of Colombia. However, most research and development has been based on only a few accessions; hence activities to acquire and test novel *C. argentea* germplasm is of high priority. *F. macrophylla* is also a highly promising shrub legume with excellent adaptation to infertile soils. In contrast to *C. argentea*, whose adaptation is limited to an altitude below 1200 m, *F. macrophylla* can be grown successfully up to 2000-m altitudes. However, the potential utilization of *F. macrophylla* is limited by the poor quality and acceptability of the few evaluated accessions. The project aims to investigate the genetic diversity within collections of *F. macrophylla* and *C. argentea* with three main objectives:

- Identify new, superior forage genotypes based on conventional germplasm characterization/evaluation procedures: morphological and agronomic traits, forage quality parameters, including in vitro dry matter digestibility (IVDMD) and tannin contents
- Optimize the use, management and conservation of the collections. Test and compare different approaches to identifying core collections for each species based on (a) genetic diversity assessment by agronomic characterization/evaluation; (b) germplasm origin information; and (c) molecular markers (AFLPs).
- Create a basis for planning future germplasm collections with respect to methodology, geographic focus and genetic erosion hazards

Methods

Agronomic characterization and evaluation. Space-planted, single-row plots in RCB design with three replications were established in Quilichao in March 1999 (*C. argentea*, 39 accessions) and March 2000 (*F. macrophylla*, 73 accessions). Additionally two replications were sown for seed production and morphological observations. The following parameters were measured in the trials: vigor, height and diameter, regrowth, incidence of diseases, pests and mineral deficiencies, and dry matter (DM) yield during wet and dry seasons. Crude protein content and IVDMD of the entire collections were analyzed for their nutritive value. For the morphological evaluation, qualitative and quantitative parameters were measured such as days to first flower, days to first seed, flower color, flowers per inflorescence, flowering intensity, pod pubescence, seeds per pod, seed color, leaf area, peduncle length, etc.

For *F. macrophylla*, a more detailed analysis of nutritive value was conducted of a representative subset (25 accessions) that included high, intermediate and low nutritive value accessions, the selection based on crude protein content and IVDMD. The analysis comprised fiber (NDF, ADF, N-ADF), tannin purification, and condensed and hydrolyzable tannin contents. Additionally, another subset of 10 accessions [9 high-quality accessions (18437, 18438, 21083, 21090, 21092, 21241, 21580, 22082, 22327) and CIAT 17403] was sampled 2, 4, 6 and 8 wk after cutting to assess the effect of age on digestibility as well as on protein, fiber and tannin content.

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

Analysis of available origin information. Based on ecogeographical information of accession origins, a core collection will be created based on the hypothesis that geographic distances and environmental differences are related to genetic diversity. The analysis will be conducted with FloraMap™, a GIS (Geographic Information System) tool developed by CIAT, which allows the production of climate probability models using PCA and cluster analysis.

Genetic analysis by molecular markers (AFLPs). Genetic analysis will be conducted using the AFLP molecular marker technique. Based on the results a core collection will be created, using multivariate statistic tools (PCA and cluster analysis).

Data analysis and synthesis. Individual and combined data analyses of all generated information will be performed, including the use of GIS tools and multivariate statistics. In the analysis of each of the different approaches (agronomic characterization, origin information, molecular marker analysis), PCA and cluster analysis are used to create core collections. The eventual correlation among the different approaches and clusters is evaluated. The outcome is expected to help decide which of the three methods or combination thereof is most appropriate (i.e., timewise and cost

efficiency) for creating a core collection; e.g., if an agronomic evaluation of the entire collection is not feasible because of time constraints, a core collection may be created using origin information and/or molecular marker analysis.

Based on molecular marker similarities and the GIS analysis, suggestions will be made for focusing future collections on areas with particularly high diversity and for improving collection (= sampling) strategies (e.g., sampling frequency; roadside collections). Accession duplicates in the world collections will also be identified.

Results and Discussion

Agronomic characterization and evaluation. Three and two evaluations per season were carried out for *C. argentea* and *F. macrophylla*, respectively, indicating considerable phenotypic and agronomic variation in the collections. Data for *C. argentea* and *F. macrophylla* are presented in Tables 1 and 2, respectively.

For *C. argentea*, the IVDMD varied from 61-67% and crude protein content, from 19-23%. Mean DM production was 219 and 202 g/plant in the wet and dry season, respectively. The higher dry season yields indicate good adaptation of *C. argentea* to dry conditions and its sensitivity to excess moisture. According to these results, CIAT accessions 18674, 22406, 22408, 22409, 22375 and 18957 had the highest DM yields ranging from 247-319 g/plant. Productivity of these accessions was higher than yields of the material advanced for cultivar release in Costa Rica and Colombia (an accession mix of CIAT 18516/18668).

For *F. macrophylla,* IVDMD varied from 33-53% and crude protein content, from 15-24%. Mean DM production was 208 g/plant in the wet season and 118 g/plant in the dry season. In comparison to the results obtained for *C. argentea*, the lower yield and productivity in the dry season may indicate a lower drought tolerance of *F. macrophylla.* The most productive accessions were CPI 104890, CIAT 7184, 21090, 21241, 21248, 21249, 21519, 21529 and 21580 with a total DM production >300 g/plant. Among the materials with high digestibility (>45% on the average), CIAT accessions 18437, 20622, 20631, 20744, 21083, 21090, 21241 had DM yields higher than 200 g/plant.

Based on the forage quality results (IVDMD and CP content, Table 2), a representative subset (25 accessions: 10 erect, 11 semierect, 4 prostrate) was chosen for subsequent analysis of NDF, ADF, condensed and hydrolyzable tannin content (CIAT 17403, 17407, 18437, 18438, 19457, 20065, 20616, 20621, 20622, 20744, 20975, 20976, 21083, 21087, 21090, 21092, 21249, 21529, 21580, 21982, 21990, 21992, 22082, J001). Fiber analysis showed that the FND, FAD and N-FAD contents are higher in the dry season than in the wet season. The fiber content of high- and medium-quality accessions was nearly identical, while fiber content of the low-quality accessions was slightly higher (Table 3). A subset of 10 high-quality accessions was analyzed for wet season-forage quality (IVDMD, crude protein, fiber and tannin content) after 2, 4, 6 and 8 wk of regrowth. The IVDMD, crude protein and fiber content varied with time. IVDMD generally increased slightly and was highest after 6 wk. To verify these findings the study will be repeated with cuts in the dry season. Fiber content (ADF and NDF) seemed to be correlated with IVDMD and generally also had its highest values after 6 wk of regrowth. There was very little variation for crude protein content (Figure 1).

Table 1. Agronomic evaluation of a collection of *C. argentea* in Quilichao. Data for 6 evaluation cuts (3 in the dry season and 3 in the wet season). Grey underlaid: Accessions with DM yields higher than accession mix CIAT 18516/18668 (material advanced for cultivar release in Costa Rica and Colombia).

CIAT Treat-ment No.	Height (cm)	Diameter (cm)	Regrowth Points (No.)	Mean DM yields			IVDMD (%)	Crude Protein (%)
				Wet S. (g/pl)	Dry S.	Mean		
18516	118	103	20	247	238	243	63.35	22.16
18667	119	99	18	218	208	213	63.73	21.34
18668	108	107	18	203	217	210	63.25	21.90
18671	120	102	19	239	190	214	63.56	20.99
18672	102	85	14	185	150	167	60.50	21.10
18674	122	114	23	329	310	319	63.72	21.87
18675	117	95	16	226	208	217	62.68	20.55
18676	115	93	16	231	193	212	61.00	20.42
18957	118	102	18	251	244	247	62.49	21.06
22373	117	91	17	203	207	205	62.82	20.71
22374	124	104	18	247	234	241	64.89	20.96
22375	130	98	18	255	255	255	65.04	22.41
22376	105	78	12	155	146	150	62.51	19.78
22378	109	83	13	155	144	149	61.32	20.65
22379	118	92	16	231	205	218	63.92	20.64
22380	117	93	13	201	166	184	62.23	21.24
22381	111	88	13	178	149	163	63.04	20.59
22382	118	91	14	203	219	211	63.61	21.14
22383	109	95	14	197	163	180	62.00	20.47
22384	120	89	11	210	154	182	64.02	20.45
22386	121	86	13	195	177	186	65.28	20.03
22387	118	91	13	193	185	189	62.22	19.99
22390	104	94	14	174	158	166	64.30	20.07
22391	116	99	16	218	195	207	64.29	20.59
22392	121	88	15	176	150	163	63.96	22.37
22393	117	95	19	203	201	202	63.39	21.96
22394	117	88	15	181	165	173	64.36	22.35
22396	109	80	11	159	149	154	63.89	23.00
22399	109	89	14	160	147	153	66.19	20.93
22400	126	99	18	230	228	229	62.48	21.72
22404	120	97	15	212	226	219	65.68	21.97
22405	120	97	17	235	223	229	64.41	21.52
22406	119	112	22	297	271	284	63.25	21.07
22407	122	99	18	233	220	226	65.62	21.21
22408	129	105	18	278	277	278	67.25	21.54
22409	116	111	18	260	270	265	65.98	22.34
22410	125	96	18	237	214	225	63.07	20.75
22411	109	90	15	193	186	190	64.40	20.31
22412	121	93	13	234	231	233	63.58	19.06
Mean	116	95	16	219	202	211	62.9	21.1
Range	102-130	78-114	11-23	155-329	144-310	149-319	61-67	19-23

Table 2. Agronomic evaluation of a collection of *F.macrophylla* in Quilichao. Data from 4 evaluation cuts (2 in the dry season and 2 in the wet season). Growth habit: e = erect, s = semierect, p = prostrate. Grey underlaid: Accessions with digestibility >45% and DM yield >200 g/plant.

CIAT Treatment No.	Height (cm)	Diameter (cm)	Regrowth Points (No.)	Mean DM yields (g/pl)			IVDMD (%)	Crude Protein (%)
				Wet S.	Dry S.	Mean		
801 (e)	122	91	28	344	180	262	41.81	24.20
7184 (e)	116	98	34	357	266	312	40.01	22.72
17400 (s)	60	93	32	236	108	172	38.26	22.95
17403 (s)	68	98	34	262	153	208	40.29	22.05
17404 (s)	59	85	33	188	113	151	38.58	21.97
17405 (s)	59	79	30	188	131	160	40.63	21.50
17407 (s)	78	100	37	298	181	240	38.15	20.26
17409 (s)	57	104	36	295	157	226	38.22	20.85
17411 (s)	58	89	34	217	173	195	39.51	21.27
17412 (s)	76	98	41	267	167	217	40.37	20.84
17413 (s)	62	95	35	212	127	169	38.82	20.94
18048 (s)	32	48	23	55	26	41	45.05	20.42
18437 (s)	57	99	39	239	159	200	50.04	22.48
18438 (s)	66	79	40	204	84	144	53.41	21.65
18440 (s)	59	85	32	222	112	167	33.16	21.13
19453 (e)	95	71	18	162	93	128	42.44	21.60
19454 (e)	105	85	22	243	134	188	43.29	20.66
19457 (e)	112	86	25	215	175	195	39.10	21.56
19797 (s)	56	84	22	183	105	144	41.40	21.12
19798 (s)	61	93	27	228	153	190	43.27	21.39
19799 (s)	63	84	26	191	122	156	42.01	21.25
19800 (s)	70	92	35	208	150	179	37.21	21.33
19801 (s)	79	92	40	251	151	201	37.63	22.09
19824 (e)	64	96	36	237	164	200	42.10	22.21
20065 (p)	18	19	5	5	2	3	42.19	21.11
20616 (s)	70	104	37	292	149	221	38.79	21.94
20617 (s)	74	90	29	210	123	166	33.92	21.03
20618 (s)	72	91	31	209	136	172	35.62	21.74
20621 (e)	70	78	30	142	118	130	35.97	21.99
20622 (e)	142	90	29	323	223	273	45.36	22.35
20624 (s)	65	100	35	269	177	223	34.71	20.72
20625 (e)	122	87	25	305	185	245	44.28	23.30
20626 (e)	113	91	29	269	214	241	43.68	22.82
20631 (e)	110	89	24	321	204	262	45.22	21.84
20744 (e)	116	89	27	296	180	238	45.42	22.93
20972 (p)	26	54	32	62	36	49	41.45	21.14
20973 (p)	28	44	18	66	26	46	35.64	19.93
20975 (s)	48	77	41	134	71	103	46.32	20.09
20976 (s)	44	54	25	66	59	63	42.87	19.88
20977 (s)	30	32	9	18	12	15	43.21	19.13
20978 (s)	47	50	21	64	29	46	44.53	20.55
20979 (s)	48	77	41	142	78	110	39.89	20.60
20980 (s)	44	55	29	94	46	70	43.68	20.29
20982 (s)	50	62	28	89	57	73	43.33	19.64
21079 (e)	44	76	41	168	63	115	40.52	20.01
21080 (s)	38	58	13	94	32	63	39.49	17.20
21083 (e)	102	92	42	317	145	231	50.10	19.97

CIAT Treatment No.	Height (cm)	Diameter (cm)	Regrowth Points (No.)	Mean DM yields (g/pl)			IVDMD (%)	Crude Protein (%)
				Wet S.	Dry S.	Mean		
21086 (s)	26	44	9	43	30	36	37.13	15.11
21087 (s)	59	61	45	144	73	109	45.92	21.29
21090 (s)	93	112	59	520	201	361	49.81	19.73
21092 (s)	74	82	24	214	136	175	51.53	18.89
21241 (e)	131	95	26	362	195	278	45.30	22.20
21248 (e)	126	101	32	406	248	327	39.38	21.79
21249 (e)	127	106	34	508	257	382	41.42	21.71
21519 (e)	124	99	28	359	193	276	42.93	22.26
21529 (e)	128	98	31	420	200	310	43.31	22.24
21580 (e)	131	104	36	586	272	429	42.76	21.03
21982 (p)	21	58	35	98	30	64	41.74	21.52
21990 (p)	37	67	50	117	51	84	37.92	18.65
21991 (p)	31	53	25	64	29	46	39.35	21.00
21992 (p)	31	54	27	66	33	49	46.22	18.85
21993 (s)	40	71	40	117	59	88	43.17	20.02
21994 (p)	26	49	13	42	25	33	37.10	16.90
21995 (p)	32	56	32	77	27	52	38.12	19.80
21996 (p)	22	41	15	25	14	20	42.28	20.00
22058 (e)	81	59	12	135	86	111	38.15	18.69
22082 (s)	77	91	63	274	116	195	43.56	19.81
22087 (p)	24	46	16	44	14	29	42.33	18.12
22090 (s)	41	43	11	36	10	23	37.05	17.72
22285 (s)	46	73	46	144	62	103	38.20	19.72
22327 (s)	36	58	29	76	44	60	46.31	18.67
C10489 (e)	99	95	35	376	196	286	39.82	22.94
I15146 (e)	105	82	26	343	179	261	42.98	23.77
J001 (e)	120	88	31	315	175	245	41.64	23.52
Mean	69	78	30	208	118	163	41.06	20.91
Range	18-142	19-112	5-63	5-586	2-272	3-429	33-53	15-24

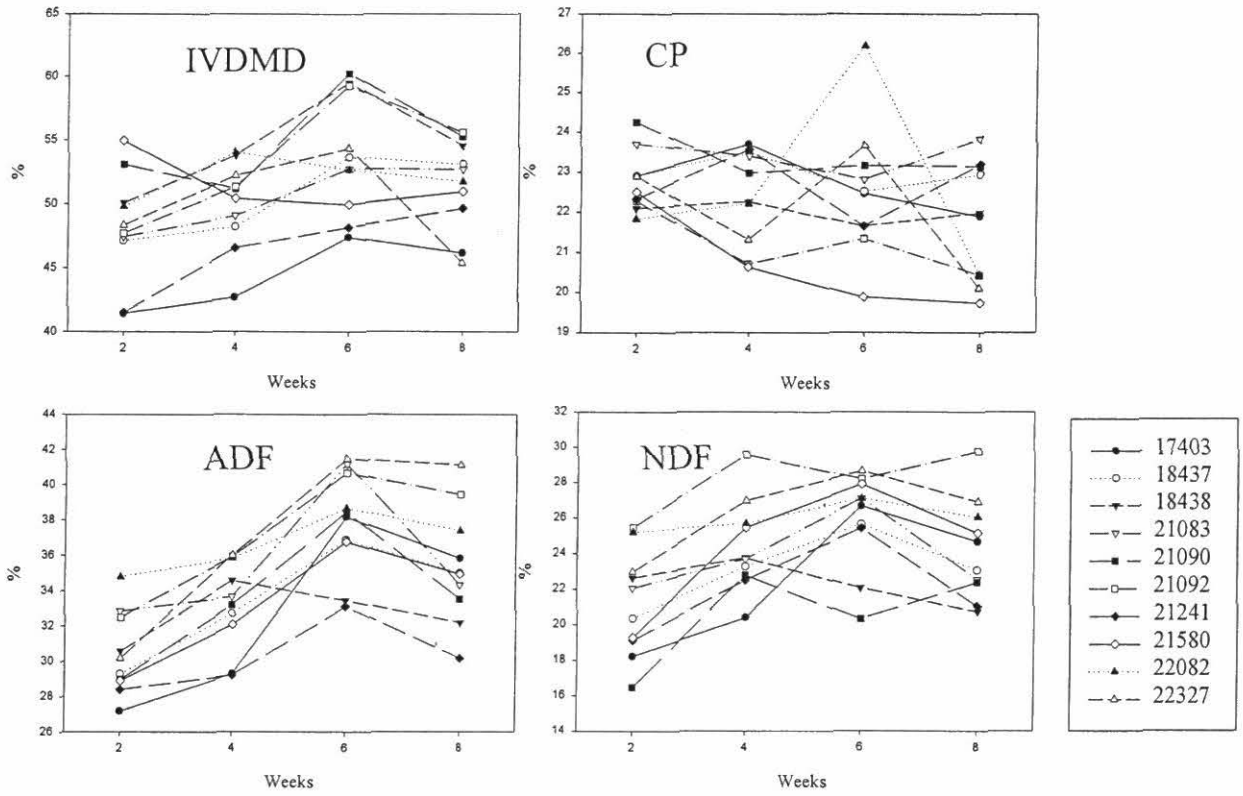

Figure 1. Variability in IVDMD, crude protein and fiber content of 10 high-quality accessions of *F. macrophylla* after 2, 4, 6 and 8 wk of regrowth. IVDMD = in vitro dry matter digestibility, CP = crude protein, ADF = acid detergent fiber, NDF = neutral detergent fiber.

Table 3. Fiber content of a representative subset (25 accessions) of *F. macrophylla*. Data from 3 evaluation cuts (2 in the wet season and 1 in the dry season). IVDMD = in vitro dry matter digestibility, NDF = neutral detergent fiber, ADF = acid detergent fiber, N-ADF = nitrogen bound to acid detergent fiber.

Forage Quality	IVDMD (%)		NDF (%)		ADF (%)		N-ADF (%)	
	Wet S.	Dry S.	Wet S.	Dry S.	Wet S.	Dry S.	Wet S.	Dry S.
High	48.1	44.9	37.6	40.3	28.9	28.3	1.5	1.5
Medium	46.3	39.8	37.5	41.2	28.2	30.1	1.5	1.6
Low	39.1	38.6	39.1	44.0	29.7	31.0	1.6	1.7
Mean	45.1	41.7	38.0	41.6	28.9	29.6	1.5	1.6

Morphological characterization and evaluation. The phenology study has not yet been completed, but available data allow some preliminary assessment using PCA. For *F. macrophylla*, the parameters included thus far are days to first flower, days to 50% flowering, length of inflorescence peduncle, flower color, length of bracts, seed color, inflorescence branching (terminal/axillar), flowering intensity, leaf(let) area, leaf peduncle length and pubescence, and data from a visual evaluation after 8 wk of regrowth (height, diameter, vigor, pests, diseases). PCA performed with the agronomic data of 71 *F. macrophylla* accessions revealed high correlations between seed color,

length of the inflorescence peduncle, inflorescence branching and height, as well as between days to first flower and days to 50% flowering (>70%). Cluster analysis (UPGMA) resulted in 8 groups. The first group contained 18 of the 19 erect-growing accessions. They were characterized by their height (160-290 cm), and by a terminal, pedunculate and branched, raceme-like inflorescence with light rose-coloured flowers (Photo 1) and black seeds. The average leaflet area was 40 cm^2 with an average peduncle length of 5 cm. The other erect-growing accession (No. 21249), with the same growth and inflorescence characteristics, was in a group of its own, distinguished by lower vigor and high disease attack. Group 3 (20 accessions) was composed nearly exclusively of semi-erect accessions with an average height of 134 cm and diameter of 205 cm. This group also included two prostrate accessions (19797, 20624) because of their extensive height and diameter. The inflorescences of these accessions were axillary and sessile, with dark pink flowers in dense, cylindrical racemes (Photo 2) with brown and mottled seeds. Accession No. 18437 with the same characteristics fell into a fourth group of its own because of higher disease attack, together with No. 21090, due to very late flowering (355 days to 50% flowering). The remaining 26 semierect and prostrate accessions, with the same inflorescence characteristics as mentioned before but with a lower average height (86 cm) and diameter (137 cm), fell into a large sixth group. Groups seven and eight differed from all other accessions with respect to the bracts and bracteoles. Generally in *F. macrophylla* the bracts and bracteoles are shorter than the inflorescence resp. flowers. In some accessions the bracts and bracteoles were longer than the inflorescence resp. flowers (Photo 3), falling into two distinct groups: Group seven with accessions 21080, 21994, 22058 and group eight with accession 21086. These accessions also had conspicuously larger leaves (avg leaflet area was 68 cm^2, while the avg of the whole collection was 34 cm^2) that resemble tobacco leaves. This growth type was therefore named "tobacco" in contrast to the three other aforementioned growth types: erect, semierect and prostrate.

Genetic analysis by molecular markers (AFLPs). Efforts made in genetic analysis showed as a preliminary result that the common manual DNA extraction methods do not work well with *F. macrophylla* and *C. argentea.* The modified protocol, which was used to extract DNA, showed promising results at the beginning, but frequent degradation, contamination and partial digestion occurred later due to secondary plant compounds, probably polyphenols. In preliminary trials with a commercial extraction kit, the DNA purity was higher and thus far problems have not occurred in either digestion or amplification.

Photo 1. Inflorescence of the erect growth type of *F. macrophylla.*

Photo 2. Inflorescence of the semierect and prostrate growth type of *F. macrophylla.*

Photo 3. Inflorescence of the "tobacco" growth type of *F. macrophylla.*

Progress Toward Achieving Milestones

- *Preliminary list of C. argentea accessions with superior performance relative to control (mixture CIAT 18516/18668) released in Costa Rica as cv. Veraniega.* Preliminary data indicate little variation in nutritive value among C. accessions. CIAT 18674, 22375, 22406, 22408 and 22409 had consistently higher DM yields than CIAT 18516/18668 over both drier and wetter seasons. Another set of *Cratylia* accessions was obtained from Costa Rica for comparison with the existing collection.

- *Preliminary list of F. macrophylla accessions with superior performance relative to CIAT 17403.* Preliminary data indicate several accessions with superior DM yield and/or better digestibility than CIAT 17403. Thus far the most interesting accessions are CIAT 18437, 18438, 21083, 21090 and 21092, followed by 19454, 20622, 20625, 20626, 20631, 20744, 20975, 21087, 21241, 21249, 21519, 21529, 21580, 22082 and I(LRI)15146 and J001. More detailed analysis of two subsets have shown that aceessions with higher feed quality in terms of

digestibility and DM production have lower fiber contents than low-quality accessions. A further set of accessions has been obtained from Viet Nam.

- *Four clearly distinguishable F. macrophylla growth types.* Morphogical studies clearly reveal four different growth types: erect, semierect, prostrate and "tobacco." Agronomically promising accessions are either of the erect or semierect type. Prostrate and tobacco-type plants generally have lower DM production and/or lower digestibility.

- *Preliminary analysis of origin information on F. macrophylla and C. argentea.* Passport data of the two collections were mapped with FloraMap and classified in clusters. The number of clusters utilized corresponds to the number of clusters employed in the agronomic study.

- *Core collection approaches in the multipurpose shrub legumes F. macrophylla and C. argentea assessed.* Preliminary analysis of origin and agronomic information did not identify correlations between the clusters obtained in the two approaches. Data will be further studied and compared to results of AFLP analysis.

1.1.15 Gene flow analysis from rice into wild/weedy relatives in the neo-tropics: Morphological and phenological characterization of red rice

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[1] SB2, [2] Fedearroz, [3] IP4. Funding from GTZ, Germany. Project No. 99.7860.2-001.00

Introduction

Hybridization between crops and their wild relatives sometimes brings genes into wild populations, occasionally resulting in the evolution of aggressive weeds and/ or endangerment of rare species. Transgenic crops may also result in similar outcomes. The likelihood of crop-to-wild hybridization depends on the out-cross rate, and on distance and direction between wild and crop populations. Cultivated rice, *O. sativa* L., is an autogamous plant, with a low out crossing rate of 0-1% (Roberts et al. 1961). Rice is an introduced species in the Neotropics from Africa and Asia, but with wild/weedy relatives including wild native species in Central and South America. Hybridization can be expected within the genomic group that includes *O. sativa*, viz., the AA group. The wild relatives of AA genome, which are found in Central and South America and may hybridize with the rice crop, include *O. rufipogon* and *O. glumaepatula* (Oka and Chang, 1961;Vaughan and Tomooka, 1999). Red rice (*Oryza sativa* f. *spontanea*) is weedy rice with a red pericarp and dark-colored grains. The seeds shatter readily and possess dormancy characteristics. The plants typically are tall, late maturing, and have pubescent leaves and hulls. In contrast to Asia where manual transplanting is still predominant, in tropical America direct seeding of red rice-contaminated seed source is common, making red rice the most serious weed problem. Genes from rice varieties may transfer quickly into red rice (1% to 52% hybridization rate)(Langevin et al. 1990). However, most of the hybridization rate estimates have been done under temperate conditions. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species.

Materials and Methods

Collection of Red Rice Samples in the Field. Various commercial rice field plots were selected at Tolima, the major rice-cropping area in Colombia. Plots with known cropping history were selected. Plots had been planted with the same variety for at least the last eight growing seasons (two years), and included one of the 4 more widely grown commercial varieties: Fedearroz 50 currently grown by 80% of farmers; Oryzica 1 the previous most popular variety cultivated before Fedearroz 50 was released; and Coprosem 1 or Cimarron. A total 158 red rice plants and their corresponding seeds were harvested from each plot. Samples were grouped in populations based on their origin according to the commercial variety grown in the field. The Fedearroz 50 population is comprised by samples from different locations. Plant and seed data was collected from field collections, and a sample was stored for herbarium record. Grain colors were coded using The Royal Horticultural Society (1966): brown (code 200), greyed orange (codes 164, 166 and 173), greyed purple (codes 183 and 185), greyed yellow (code 161), and yellow white (code 158). Seeds were increased for field experiments.

Morphological and Phenological Characterization. The morphological characterization was conducted using 16 qualitative and 9 quantitative descriptors. The phenology characterization included 6 descriptors. Plant and seed morphological evaluations were conducted using the samples collected from the field (F0 generation). The phenological characterization was performed in the greenhouse (F1 generation) and in the field (F2 generation) using progeny plants obtained through selfing of each F0 plant. The corresponding rice varieties grown in the field at the moment of the field sample collection, a transgenic Cica 8 rice variety, and the wild species *O. rufipogon*, *O. glaberrima*, and *O. barthii* were used as controls.

Data Analysis. Principal component and coordinate analysis were conducted using a SAS statistical package program.

Results and Discussion

Seed parameters allowed a better characterization of the red rice populations collected. The analysis of the 1650 seeds indicated broad husk color diversity, varying from greyed yellow color (alike the commercial varieties) to brown color (alike the wild rice species). The widest diversity was noted in the populations derived from the Oryzica 1 and Fedearroz 50 plots, where about 50% of the red rice biotypes showed grains with husk color alike the corresponding commercial variety, from 2% to 15% of biotypes had husk color alike the wild rice species, and the other biotypes with husk colors corresponding to either greywed orange stripes, or brown stripes. In contrast, about 40% of the red rice biotypes derived from the Coprosem 1 and Cimarron plots showed brown grains alike the wild species, 0% and 10% of the red rice with grains alike the varieties, and only one or two of the other husk color categories. Similar patterns were noted when the awn, the apiculus, and the pericarp were characterized. Some red accessions with colored grains, also showed greyed orange or greyed purple coloration in the leaves and tillers, which may facilitate the identification of hybrids since anthocyanin production in rice is encoded by dominant gene(s).

A correlation analysis between grain length and grain width also showed that the red rice populations derived from the Coprosem 1 and Cimarron field plots were the least diverse, and clearly distinct from the rice varieties. The red rice population derived from the Oryzica 1 plot was the most diverse. While some biotypes showed shorter and thicker grains alike the wild species *O. rufipogon and O. glaberrima*, others showed long and slender grains alike the varieties, and some biotypes were in between the wild species and the crop. Few of the samples from the Fedearroz 50 plot had long and slender grains. *Oryza barthii* had extra long and thick grains. Those red rice

biotypes undistinguishable from the variety by their grain traits still showed seed shattering characteristic of the weedy rice.

Principal component analysis using the 9 quantitative morphological descriptors indicated that 77% of the variability could be explained by 4 main components, where grain length, width and thickness, jointly with length between nodes, and width of the flag leaf and the previous leaf were the most important. The coordinate principal component analysis using the 16 qualitative morphological parameters allowed to group the 158 red rice biotypes in three major clusters mainly characterized by the presence or absence of grain awn, and brown husk color, respectively (Figure 1). Results indicated a significant similarity between some red rice biotypes and rice varieties. Likewise a tight association was noted between various red rice samples and the wild *Oryza* species, especially with *O. rufipogon* (Figure 1). Of the phenological traits evaluated days to flowering (DTF, 50% anthesis) is the most relevant when considering likelihood of gene flow. The Ryan-Einot-Gabriel-Welsch multiple range test ($p>0.005$) discriminated three groups: early flowering (*O.glaberrima,* mean DTF 85; and *O.barthii*, mean DTF 88), intermediate flowering (all red rice accessions, rice varieties and *O. rufipogon*, mean DTF from 91 to 111), except rice varieties Cica 8 and Fedearroz 50 which were classified as late flowering according to this test (mean DTF from 112 to 116). Most red rice biotypes collected from the Fedearroz 50 plots were earlier flowering respect to this variety, but flowering of various individual biotypes overlapped with both Fedearroz 50 and Cica 8. No clear influence on the flowering pattern was noted respect to the variety previously grown in the plot (Figure 2).

Conclusions

Seed traits significantly accounted for most of the variability found in the red rice populations collected. The presence/ absence of awn and husk color grouped the biotypes in three major clusters. Seed qualitative traits jointly with the relation grain length to width easily allows to identify the red rice varietal types and those similar to wild species from the regular weedy types. Large number of red rice biotypes overlap in flowering with cultivated varieties. No clear influence in flowering pattern was noted respect to the variety previously being grown in the field. Based on this morphological and phenological characterization, some red rice biotypes had been selected to conduct gene flow analysis and identify indicators for an easy trace of gene flow in the field over subsequent generations.

Future Plans

- Red rice biotypes representing the 3 major clusters with contrasting morphological characteristics easy to trace through hybridization, and overlapping in flowering respect to rice varieties were selected for evaluation to RHBV resistance.

- Of these biotypes, those susceptible to the virus and easily distinguishable by red rice specific microsatellite molecular markers (described in report 1.1.12 herein) will be potential candidates for the gene flow and introgression follow up analysis using either transgenic Cica 8 resistant to RHBV or other non-transgenic variety as pollen donor.

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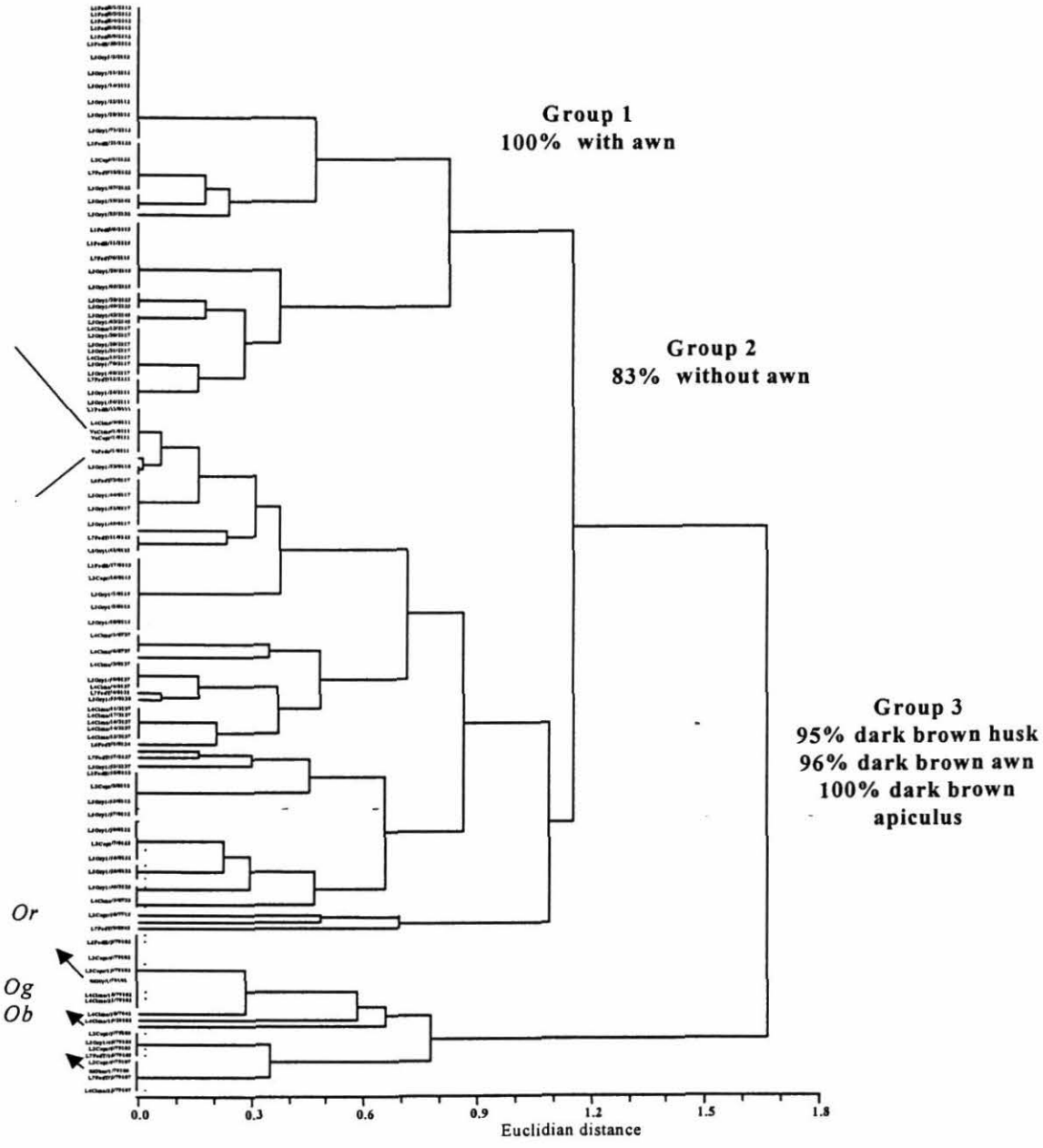

Figure 1. Coordinate principal component analysis using 16 qualitative morphological parameters.*Ob = O. barthii*; *Or = O. rufipogon*; *Og = O. glaberrima*

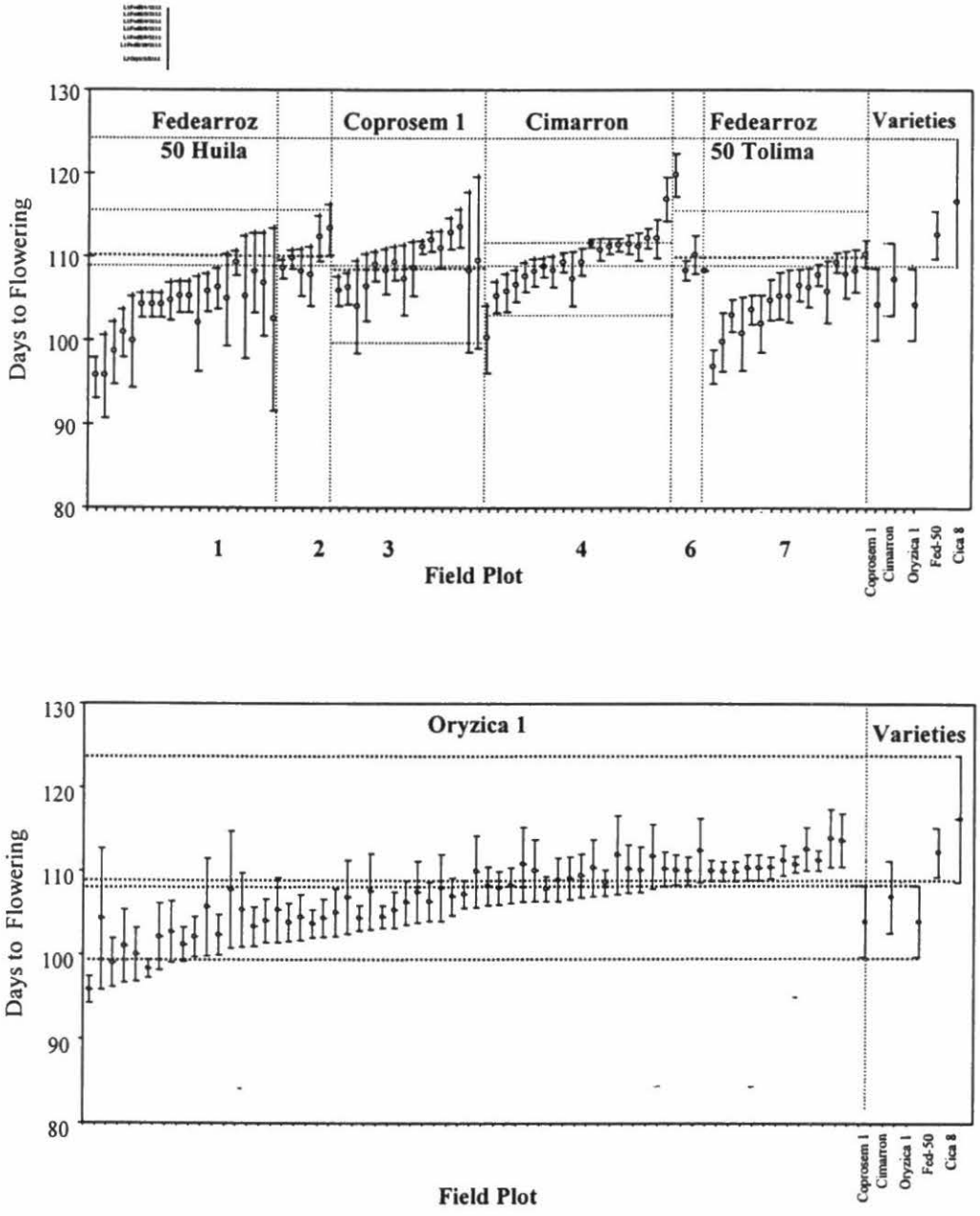

Figure 2. Days to flowering of each red rice biotype collected and corresponding variety being grown in the field at sampling time. Flowering of transgenic Cica 8 variety was included as a reference.

1.1.16 Molecular characterization of rice and wild/weedy relatives by microsatellites and their use to assess gene flow in the Neo-Tropics

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[1] SB2, [2] IP4 , [3] Fedearroz. Funding from GTZ, Germany. Project No. 99.7860.2-001.00

Introduction

A careful assessment of potential impacts of gene flow from transgenic plants on population genetics of natural crop plant biodiversity is needed in other to design strategies for the safe and durable use of these crops in the Neo-tropics. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives, and its effect(s) on the population genetic structure of the recipient species. Last year we reported a preliminary screening of 50 microsatellite markers to identify those polymorphic between a set of commercial rice varieties, wild *Oryza* species, and advanced transgenic Cica 8 RHBV resistant lines and hand-made crosses with selected varieties. The current report summarizes the progress on setting up the use of microsatellites markers to assess and trace gene flow from transgenic and non-transgenic rice into wild *Oryza* species and red rice under controlled confined field plots, and under local agricultural field conditions in Colombia. A genetic diversity analysis was first conducted in order to determine the genetic structure prior gene flow, and to select the best combinations of transgenic or non-transgenic rice, and wild/weedy populations to assess the gene flow.

Materials and Methods

Plant Material. The materials included: 9 rice commercial varieties (Cica 8, Cimarron, Fedearroz 50, Fedearroz 2000, Fedearroz Victoria 1, Iniap 12, Oryzica 1, Oryzica Llanos 5, and Palmar). Sixteen homozygous transgenic-Cica 8 rice lines, resistant to RHBV virus. Four handmade crosses each between one transgenic Cica 8 line and non-transgenic Cica 8, Iniap 12, Fedearroz 50 or Oryzica 1, respectively. One hand made cross each between non-transgenic Cica 8 and Iniap 12, Fedearroz 50 or Oryzica 1, respectively (controls). One hundred and fifty eight accessions of red rice collected from various commercial rice field plots in Tolima, the major rice-cropping area of Colombia, previously characterized morphologically and phenologically. One accession each of *O. barthii, O. glaberrima, O. glumaepatula,* and *O.rufipogon.* All these genotypes were included in order to select the microsatellites detecting the highest level of polymorphisms among genotypes, and the most polymorphic pairs from each class to conduct gene flow analysis.

Genetic Analysis using Microsatellite Markers. A set of 50 microsatellite markers (at least 4 per each chromosome) were screened. The microsatellite selection was based on their location in the chromosome (McCouch et al., 1997). At least two markers located distal from the centromere per each chromosome arm were chosen to increase the likelihood of finding recombination between the experimental genotypes. The markers 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). The genetic distance between samples was calculated using the Dice algorithm, and a dendogram was constructed using the Unweighted Pair-Group Mean Average (UPGMA). The genetic distances and dendogram were built using NTSYS-PC software version 2.02 (Rohlf, 1997).

Results and Discussion.

Genetic characterization of rice varieties, red rice, and wild Oryza species. The dendrogram obtained showed thirteen groups with a similarity of 0.43. O.glumaepatula was the most distant group, followed by O.glaberrima and O. barthii. O.rufipogon, was found within the red rice group (Figure 1). For the red rice population, in some cases it was possible to associate the genetic clusters with some phenological and morphological traits. The first group was mainly composed by red rice accessions with pale yellowish husk (91%) and early to intermediate flowering (64%). The second and third groups contained accessions with grains of pale yellowish husks (95%), but with intermediate to late flowering (93%). Most of the red rice-variety biotypes and commercial varieties are in this group. The groups 8, 9,10, and 11, were mainly composed by red accessions with grain awn (69%), and intermediate to late flowering (85%). Cluster 9 grouped the wild species O. rufipogon with some red rice biotypes with significant similarity on morphological and phenological traits. The fourteen most polymorphic microsatellites detected a high number of alleles (106). In general the size of the alleles ranged from 108 to 252 bp. This analysis allowed the identification of 46 specific alleles for red rice; 17 specific alleles for the wild species; and only two specific alleles for the rice varieties. O.glumaepatula showed larger number of specific alleles (8) compared to O.rufipogon, which shared all their alleles with red rice. A low number of heterocigotes was found in the red rice population as a whole, with a range of 0-19 heterocygotes per microsatellite. Total genetic diversity index of Nei (0.637) was intermediate to high. In contrast, the genetic diversity index between the red rice populations collected from the different field plots was 0.55, which is considered an intermediate value. The value obtained for Gst (0.136) reflects a lower genetic diversity inter-population (collected from different field plots) than intra-population (individuals collected from the same field plot analyzed as one population).

Tracing gene flow with microsatellite molecular markers. Clearly distinct combinations of crop, red rice, and wild species had been selected for gene flow and introgression follow up, based on the morphological, phenological, and molecular genetic characterization using microsatellite markers. Conditions had already been standardized for detection of 2% out-cross using genotype specific markers (Figure 2). Conditions are being optimized to detect 1% of out-crossing rate.

Conclusions

Specific microsatellite alleles were identified in different commercial varieties, red rice accessions and wild species, which can be used to trace gene flow and introgression. As expected, wild species *O. glaberrima* with *O. barthii* clustered together in a group, and in a separate cluster with *O. glumaepatula* of low similarity with the rest of the population. According to this cluster analysis, some red rice accessions showed high genetic similarity to the varieties and others to the wild species *O.* rufipogon. These genetic similarity coincide with the morphological and phenological characterization already conducted. The red rice-*O.rufipogon* biotypes will be subject of taxonomic classification to elucidate if they are introduced accessions of the wild species from Asia. The red rice-variety biotypes might be indicators of hybridization between red rice and the crop, and thus better candidates as receptors of gene flow. These materials could be ideal materials to trace transgene(s) flow. Clearly distinguishable red rice biotypes are recommended to trace gene flow from non-transgenic rice, in order to provide a broad understanding of the hybridization and introgression dynamic on this population over time.

Future Plans

- Best crop/ wild/ weedy candidates to conduct gene flow and introgression analyses will be selected based on the molecular characterization and the morphological/ phenological characterization presented in report 1.1.11 (herein).
- Crop/ wild/ weedy specific microsatellites will be used to trace hybridization and population genetic through generations in the field.
- The spatial distribution of alleles will be used to study local gene flow, including pollen dispersal distances.
- Results will be compared with those using transgenes as a tool for tracing gene flow.

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1.1.17 Use of chloroplast and mitochondria DNA polymorphisms for gene flow analisis in rice

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SB-2 Project

Introduction

Genetic information present in plant mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) has been of great used in phylogeny and in population genetics studies (maternal inheritance), because of its complementarities to nuclear Mendelian segregation. Organelle plant genomes are highly conserved in structure (Palmer and Stein, 1986). 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. This tool has been used in several interspecific taxonomical works to study the genetic organization in centers of diversity or to establish the relationships between the cultigens and their related wild taxa, in genera such as *Zea, Oryza, Musa, Solanum, Nicotiana, Lycopersicom* (Schmit et al., 1993). Sun et al. (2002) investigated the variation of nuclear, mitochondrial and chloroplast DNA among 75 cultivars and 118 strains of common wild rice from ten Asian countries using Southern Blot and PCR analysis. They were able to distinguish differences between the *Indica* and *Japonica* cultivars, where they detected variations in both the nuclear and the cytoplasmic genomes, confirming that the *Indica-Japonica* differentiation is of major importance in cultivated rice. Demuesure et al. (1995) designed a set of universal primers for amplification of polymorphic non-coding regions of mtDNA and cpDNA in plants. These primers could be used as a first approach to discern polymorphism among rice and wild/weedy relatives for use in gene flow and introgression analyses, thus facilitating the identification of the pollen donor source. The cpDNA and mtDNA polymorphisms analysis (maternal inheritance) complements the information generated with microsatellite markers (nuclear inheritance) giving a comprehensive understanding of the hybridization and introgression dynamic under field conditions.

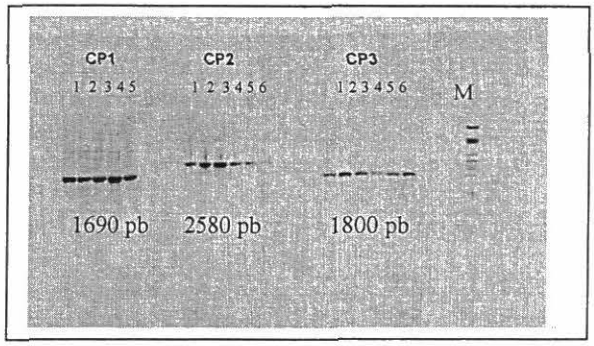
Material and methods

The experimental materials used in this preliminary study consisted of plants from *Oryza sativa* composed by one accession of red rice, the commercial rice varieties Cica 8 and Fedearroz 50, a hand-made cross between the transgenic Cica 8 RHBV resistant line A3-49-60-12-5 and Fedearroz 50, and two wild species *Oryza glumaepatula* and *Oryza rufipogon*. Total DNA was isolated from young leaves according to the method described by McCouch et al (1988). The PCR was carried out in 25 µL total volume containing the following components: 20 ng of genomic DNA, 0.4 µM of primers (Table 1), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 2.0 U Taq polimerase, 1X PCR buffer that consisted of Tris HCl 10 mM (pH 9.0), KCl 50 mM and Triton 0.1 %. The amplification was carried out using 1 cycle of 4 min at 94°C, 35 cycles of 45 s at 92°C, 45 s at 55°C, at 72°C for 4 min and one cycle of 10 min at 72°C. The PCR products were separated on agarose gel (1.0 %), stained with ethidium bromide and visualized by UV fluorescence. These amplification products were then digested by incubating 3µL of PCR product with four restriction enzymes at 37°C, for 4 h. The digested amplicons were then separated on 1.5% agarose gel.

Table 1. Pairs of primers sequences used to amplify plant cpDNA and mtDNA

Primer 1		*Primer 2*		Size pb. (Rice)
Choloroplast primer				
trnH [tRNA-His (GUG)] CP1	ACGGGAATTGAACCCGCGCA	trnK [tRNA-Lys (UUU) exon 1]	CCGACTAGTTCCGGGTTCGA	1690
trnK [tRNA-Lys (UUU) exon 1] CP2	GGGTTGCCCGGGACTCGAAC	trnK [tRNA-Lys (UUU) exon 2]	CAACGGTAGAGTACTCGGCTTTT A	2580
TrnC [RNA-Cys (GCA)] CP3	CCAGTTCAAATCTGGGTGTC	trnD [tRNA-Thr-(GGC)]	GGGATTGTAGTTCAATTGGT	1800
trnD [tRNA-Asp- (GUC)] CP4	ACCAATTGAACTACAATCCC	trnT [tRNA-Thr-(GGU)]	CTACCACTGAGTTAAAAGGG	NS
PsbC [pstI 44 kd protein] CP5	GGTCGTGACCAAGAAACCAC	trnS [tRNA-Ser-(UGA)]	GGTTCGAATCCCTCTCTCTC	1700
trnS [tRNA-Ser- (UGA)] CP6	GAGAGAGAGGGATTCCCGAAC C	trnfM [tRNA-fMet (CAU)]	CATAACCTTGAGGTCACGGG	1600
psa A [Psti p 700 apoprotein A1] CP7	ACTTCTGGTTCCGGCGAACGA A	trnS [tRNA-Ser-(UGU)]	AACCACTCGGCCATCTCTCCTA	NS
TrnS [tRNA-Ser- (GGA)] CP8	CGAGGGTTCGAATCCCTCTC	TrnT [tRNA-Thr (UGU)]	AGAGCATCGCATTTGTAATG	1100
TrnM tRNA-[tRNA-Met(CAU)] CP9	TGCTTTCATACGGCGGGAGT	rbcL[RuBisCO large subunit]	GCTTAGTCTCTGTTTGTGG	2800
Mitochondiral primers				
nad1 exon B	GCATTACGATCTGCAGCTCA	nad1 exon C	GGAGCTCGATTAGTTTCTGC	1700
nad4 exo1	CAGTGGGTTGGTCTGGTATG	nad4 exon 2	TCATATGGGCTACTGAGGAG	NS
nad4 exon 2	TGTTTCCCGAAGCGACACTT	nad1 exon 4	GGAACACTTTGGGGTGAACA	1500

NS= non standardized



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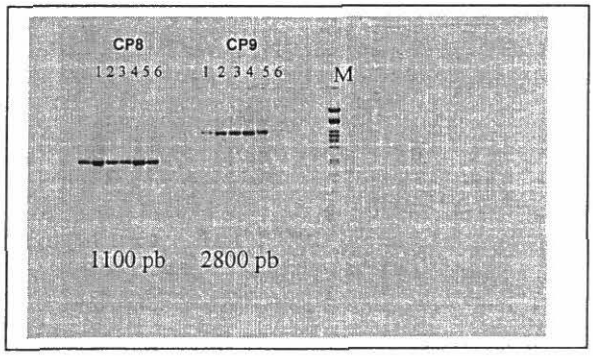

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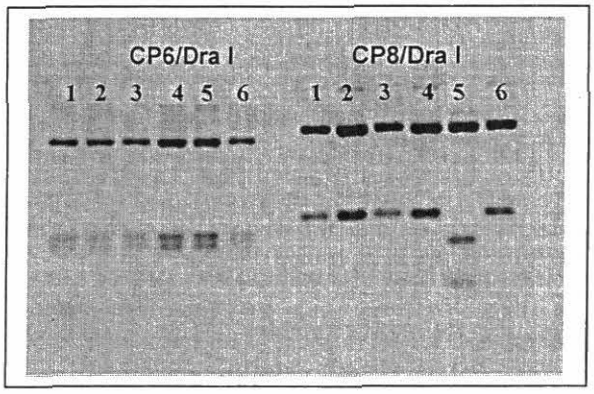

C

Figure 1. (A) and (B) Amplification of cpDNA. Lanes 1 = red rice; Lane 2 = Transgenic Cica 8 x Fedearroz 50; Lane 3 = Cica 8; Lane 4 = Fedearroz 50; Lane 5, *Oryza glumaepatula;* Lane 6 = *Oryza rufipogon.* (C) PCR products amplified with primers CP6 and CP8 and digested with *Dra I.*

Results and Discussion

The preliminary work included 12 pairs of primers, which amplify mostly non–coding sequences. Nine cpDNA primers amplified the corresponding regions in the six rice samples. These primes 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 (Figure 1). 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 aminoacid trnS [tRNA-Ser- (GGA)] and trnT [tRNA-Thr (UGU)]. A polymorphic fragment was observed for *O. glumaepatula* after the digestion with Dra I. This result needs to be confirmed using other DNA sample from another individual of *O. glumaepatula* (Figure 1).

Future Activities

- The evaluation of cpDNA and mtDNA amplified products using other additional restriction enzymes in order to identify specific polymorphic patterns between varieties, red rice and wild *Oryza* species.
- Determine if organelle polymorphism could be used as a tool to trace gene flow in rice.

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1.1.18 Molecular and Agro-morphological Characterization of the Genetic Variability of Soursop (*Annona muricata* L.) Accesions and related Annonaceus Species

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Introduction

The *Annonaceae* family (common name: anonas) presents 240 species of 30 genera in Colombia, and comprises several groups important as food products, commonly known as anones (*A. squamosa*), soursops (*A. muricata*), chirimoyas (*A. cherimola*), atemoyas (*A. squamosa* x *A. cherimola*) and "anones amozonicos" (*Rollinia mucosa*), among others (Murillo-A, 2001).

Soursop (*Annona muricata* L.), because of its exquisit taste, flavor and nutritive value is the most promisory anona species in Colombia (CIAT and C. BIOTEC, 2002). Like all the *Annonas*, soursop is native to the American tropics, possibly Colombia or Brazil (León, 1968). This is why Colombia may have the world's largest genetic diversity of soursop. However the extent of this diversity is not known , and has not been used in breeding programs to improve agronomic traits either. As a matter of fact, theres is only one germplasm bank in Colombia, C.I. Corpoica Palmira Germplasm Bank (43 anonas accesions), which has not been characterized at all.

This project is the first attempt to study the genetic diversity of annonaceus species from Colombia. The objective is to know the genetic variability of the germplasm bank by applying DNA molecular marker technology, like AFLP. We would like to know if this variability is representative of Colombian diversity. Corporación Biotec and CIAT (SB02 project) are in charge of the molecular characterization. Corpoica carries out the agro-morphological characterization and a support in population genetics. The results of this project will contribute to the development of more productive, genetically diversified planting material.

Methodology

For the genetic variability analysis, we evaluated 81 accesions from Corpoica germplasm bank and our own working collection. The 81 accessions were made of 45 entries from *A. muricata* and 36 of related *Annonaceae* species (Tables 1 and 2). We applied the method of Dellaporta et al. (1983) for DNA extraction, using PVP 1 g l^{-1} in the extraction buffer and a chloroform / isoamylalcohol (24/1) cleaning step before DNA precipitation. The AFLP protocol followed was that described by Vos et al. (1995), using AFLP Analysis System I Kit (Gibco BRL). In preliminary trials we evaluated 15 different combinations of selective nucleotides for AFLP amplification. Then, we selected three combinations that displayed the highest and "most readable" polymorphisms between soursop species and/or between Annonaceae species. They were E-ACT, M-CAA (combination F); E-AGC, M-CTC (combination M); E-AGC, M-CAA (combination N). Selective amplifications were size-fractionated on 6% or 4% polyacrylamide denaturing gels and visualized through silver staining. AFLP fingerprinting of each accesion were converted into a similarity matrix, based on Nei and Li index (1979). The similarity matrix was analyzed using NTSYS (Rohlf, 1994) computer program. Dendrograms were constructed by UPGMA method (Sneath and Sokal, 1973).

Results and Discussion

In the soursop germplasm, the 45 accesions showed 141 bands for combination F. The number of bands per individual ranged between 41 and 58, where only eight bands were monomorphic (Fig. 1), giving a 90 % polymorphism. Fourty-one accesions presented genetic similarities higher than 0.80 (Fig. 2). Four accesions, 1919, 1959, 1958 and 1920, which are currently identified as *A. muricata* species (C.I. Corpoica Germplasm Bank), showed specially different fingerprints. Genetic similarity between 1919, 1959 and the other 41 *muricata* accesions was 0.45. Similarly, genetic similarity between 1958, 1920 and the other 41 accesions was 0.30. As we can see in Fig. 3, 1959 looks more similar to *A. montana* than to *A. muricata*, while 1958 appears more similar to putative *A. glabra* accesions, and 1920 showed more similarity to putative *Rollinia mucosa* accesions (Table 2).

Table 1. Soursop and other anona-related species accessions of the C.I. Corpoica Palmira Germplasm Bank (August – 2002).

SOURSOP ACCESIONS				
	ACCESSION	ORIGIN	PROPAGATED BY	INTRODUCTION
1.	1994	El Bolo Valle	Seed	I – 89
2.	2045	Palmira Valle	Seed	X – 89
3.	H 2	Sonso Valle	Seed	IX – 89
4.	2512	Palmira Valle UNAL	Grafting	IX – 95
5.	2511	V. Gorgona Valle	Grafting	IV – 95
6.	H 1	Sonso Valle	Seed	IX – 89
7.	2020	Buga Valle	Seed	VII – 89
8.	2016	Buga Valle	Seed	IV – 89
9.	2017	Buga Valle	Seed	IV – 89
10.	1957	Caicedonia Valle	Seed	VIII – 87
11.	1995	V. Gorgona Valle	Grafting	I – 89
12.	1983	Palmira Valle	Seed	V – 87
13.	1985	Bajo Calima Valle	Seed	V – 88
14.	1946	Alcala Valle	Seed	VII – 87
15.	1943	Palmira Valle	Seed	VII – 87
16.	2014	Barinas Venezuela	Seed	I – 89
17.	2015	Fusagasuga Cundinamarca	Seed	II – 89
18.	2036	Sonso Valle	Grafting	IX – 89
19.	2042	Sonso Valle	Grafting	IX – 89
20.	1921	Ginebra Valle	Seed	V – 87
21.	1919	Samana Caldas	Seed	V – 87
22.	2040	Sonso Valle	Grafting	IX – 89
23.	2041	Sonso Valle	Grafting	IX – 89
24.	2039	Sonso Valle	Grafting	IX – 89
25.	6	Palmira Valle	Grafting	IX – 95
26.	1918	Manizales Caldas	Seed	IV – 87
27.	4	Palmira Valle	Grafting	IX – 95
28.	2037	Sonso Valle	Grafting	IX – 89
29.	2033	Sonso Valle	Grafting	IX – 89
30.	1	Palmira Valle	Grafting	IX – 95
31.	2513	C.I. Palmira Valle	Grafting	II – 86
32.	2514	Sonso Valle	Grafting	IX – 89
ANONAS SPECIES ACCESIONS				
33.	1959 Patrón	Sabaletas Chocó	Seed	VIII – 87
34.	1958 Patrón	Sabaletas Chocó	Seed	VIII – 87
35.	1920 Patrón	Victoria Caldas	Seed	V – 87
36.	Cabeza de Negro	Desconocido	Seed	VIII – 79
37.	Chirimoya	Palmira Valle	Seed	VII – 63
38.	Anona Colorada	Desconocido	Seed	X – 36
39.	Biriba	Brasil	Seed	V – 59
40.	Anona Blanca	Palmira Valle	Seed	V – 70
41.	Anona Glabra	Baudó Chocó	Seed	Pendiente
42.	Anona Montana	Baudó Choco	Seed	Pendiente

Note: Observations made on tree and fruit morphology (Dr Robert Tulio González M, U. del Pacifico, Valle), accessions 1959 and 1920 were moved from *A. muricata* to other anona species.

Tabla 2. Soursop and other related anonas species accesions from farms and national research centres, used in the molecular characterization and available at CIAT.

ACCESSION CODE	COMMON NAME	PLACE OF ORIGIN (place and department)	PROPAGATED BY	SPECIE
AMUR V1	Guanábano, Clon Elita	Vivero Profrutales, Candelaria (Valle)	Grafting	*A. muricata*
AMUR H2	Guanábano, Clon Rosa	Yaguará (Huila)	Grafting	*A. muricata*
AMUR H3	Guanábano, Clon Cristina	Yaguará (Huila)	Grafting	*A. muricata*
AMUR H4	Guanábana, Clon Francia	Yaguará (Huila)	Grafting	*A. muricata*
AMUR M5	Guanábano	Ciénaga (Magdalena)	Seed	*A. muricata*
AMUR A7	Guanábano	Turbo (Antioquia)	Seed	*A. muricata*
ASP V20	Anona Blanca Lisa	Finca la Esneda, Guacarí (Valle)	Seed	*A. squamosa?*
ASP GN31	Anón Amazónico	Margen Río Inírida (Guainía)	Seed	*Rollinia mucosa?*
ASP VA32	Anón Amazónico	(Vaupés)	Seed	*Rollinia mucosa?*
ASP GV34	Anón Amazónico	Finca la Primavera San José (Guaviare)	Seed	*Rollinia mucosa?*
ASP VA35	Anón Amazónico	(Mitú) Vaupés	Seed	*Rollinia mucosa?*
ASP V36	Anón Amazónico	Desconocido (Donado por Cartón Colombia)	Seed	*Rollinia mucosa?*
ASP Q38	Anón	La Tebaida (Quindío)	Seed	*?*
ASP V41	Atemoya	Finca la Esneda, Guacarí (Valle)	Seed	*A. cherimolia x A. squamosa*
ASP V42	Atemoya	Finca Venecia Caicedonia (Valle)	Seed	*Rollinia mucosa?*
ASP V43	Atemoya	Desconocido, Almacenes Exito Cali (Valle)	Seed	*A. cherimolia x A. squamosa*
AMON V51	Guanábana Cimarrona	Finca la Esneda, Guacarí (Valle)	Seed	*A. montana*
AMON Vi52	Guanábana Cimarrona	Cumaribo (Vichada)	Seed	*A. montana*
AMON H53	Guanábana Cimarrona	(Huila)	Seed	*A. montana*
AMON V54	Guanábana Cimarrona	Palmira (Valle)	Seed	*A. montana*
ASP V59	Anón	Almacenes Éxito, Cali (Valle)	Seed	*A. squamosa?*
ASP CV61		Costa Pacifica (Entre Valle y Chocó)	Seed	*A. glabra?*
ASP A62	Guanabanilla	Turbo (Antioquia)	Seed	*A. glabra?*
ASP CU63	Anón Liso	Mercado de Girardot (Cundinamarca)	Seed	*?*
ASP T64	Anón	Espinal (Tolima)	Seed	*A. squamosa?*
ASP BR60		Brasil	Seed	*?*
ASP VR65		Robles (Valle)	Seed	*?*
ASP VR66		Robles (Valle)	Seed	*?*
ASP V21	Chirimoya Imbanaco	Cali (Valle)	Seed	*A. cherimolia*
ASP Q22	Chirimoya	Armenia (Quindío)	Seed	*A. cherimolia*
ASP V45	Atemoya	Finca Varahonda, Pradera (Valle)	Seed	*A. cherimolia x A. squamosa*
ASP V46			Seed	*?*
ASP V47	Chirimoya	Bolo (Valle)	Seed	*?*
ASP V48		USA	Seed	*?*

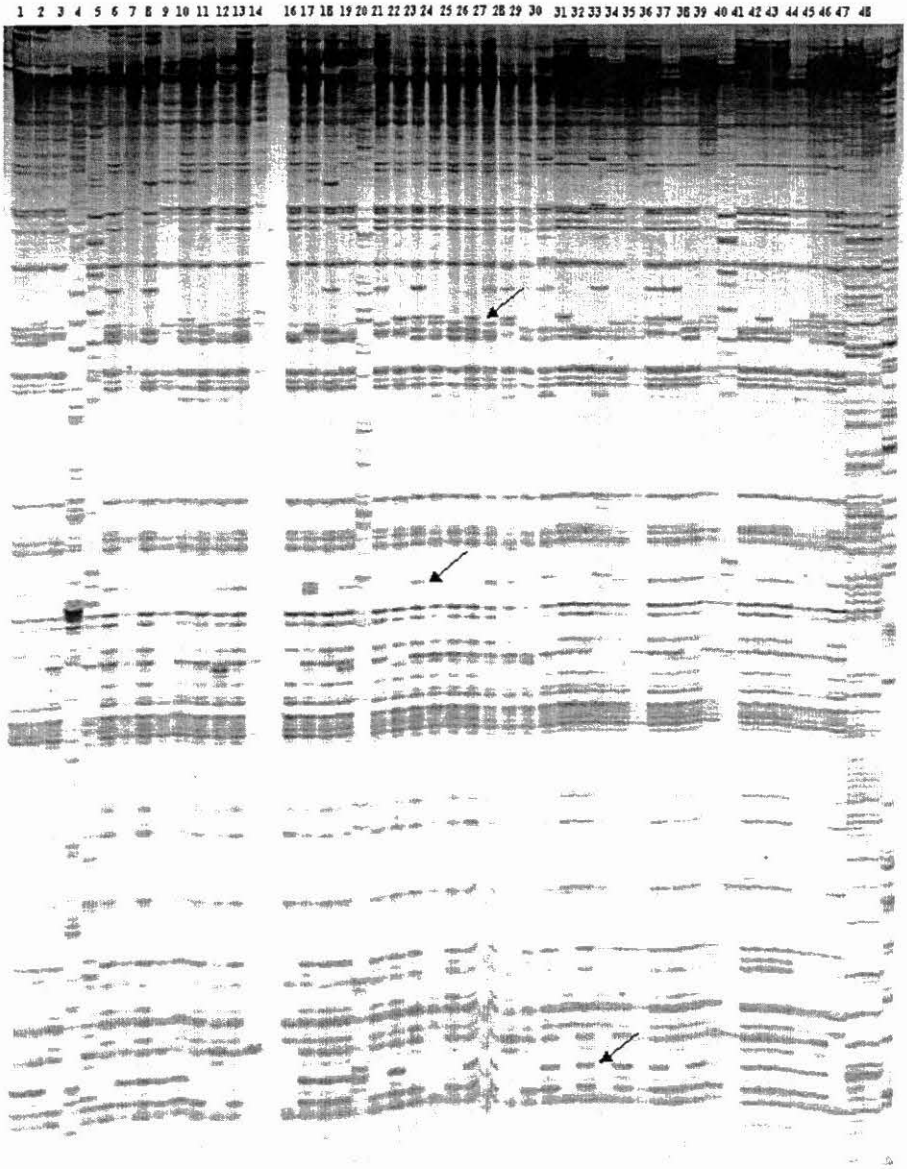

Fig. 1. Silver stained gel showing AFLP fingerprinting of *A. muricata* accesions with combination F: 1) 1985; 2) 2513; 3) 1957; 4) 1958; 5) 1959; 6) 1918; 7) C6; 8) 2014; 9) 2015; 10) 2016; 11) 2017; 12) 2040; 13) 2041; 16) AMUR V9; 17) AMUR A7; 18) AMUR M5; 19) 2033; 20) 1920; 21) 2020; 22) 2512; 23) 1994; 24) 2036; 25) 1946; 26) C1; 27) C4; 28) 2514; 29) 2037; 30) AMUR H3; 31) AMUR H21; 32) AMUR V1; 33) AMUR H35; 34) 1943; 35) 2039; 36) H2; 37) H1; 38) 1983; 39) 2045; 40) 1919; 41) AMUR V1; 42) AMUR H2; 43) AMUR H4; 44) 1921; 45) 1995; 46) AMUR H2; 48) ASP GV34. Arrows indicate polymorphic bands.

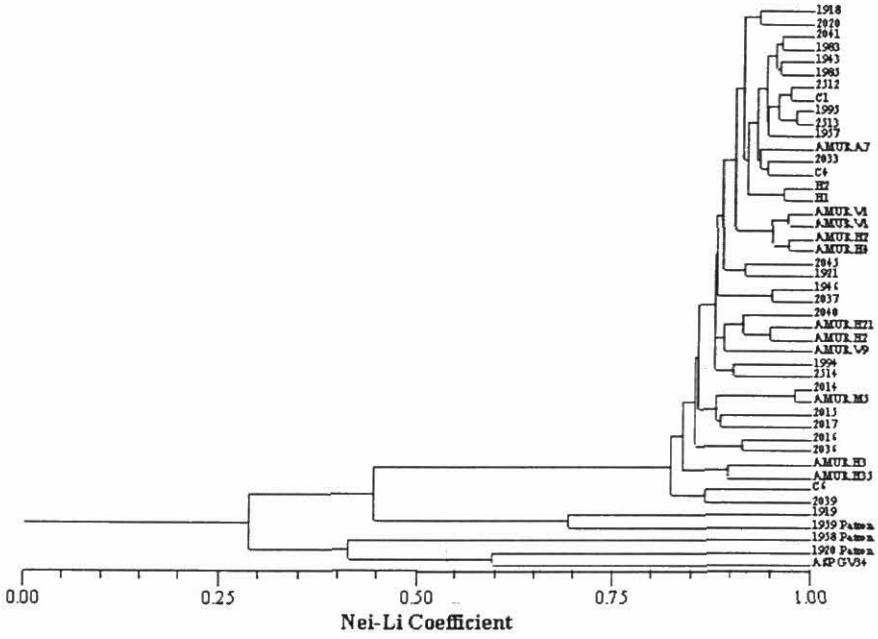

Fig. 2. Genetic similarity between *A. muricata* accesions, based on AFLP fingerprinting with combination F.

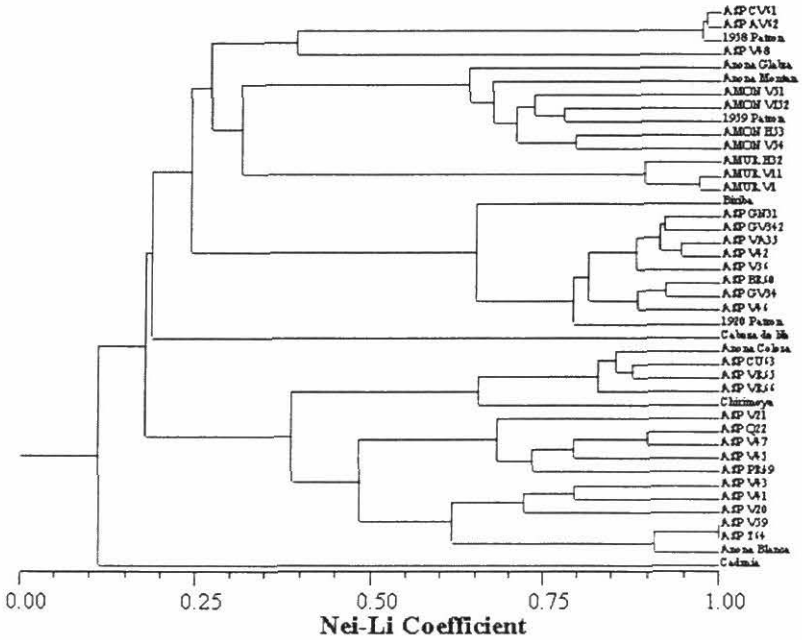

Fig. 3. Genetic similarity between anona accesions, based on AFLP fingerprinting with combination M.

Soursop accesions analysed with combination F presented low genetic variability, compared to the variability observed by *A. montana* and putative *R. mucosa* accesions (Fig. 2 and 3). The development of the crop in Colombia, and the exchange of germplasm by soursop growers, has made possible the trade of seeds adapted to different agroecological zones. This may be one reason why, in our analysis, 2014 and AMUR M5 from Barinas (Venezuela) and Ciénaga-Magdalena (Colombia), respectively, presented a similarity higher than 0.85 with 2015 and 2017, accesions collected from Fusagasugá-C/marca and Buga-Valle, respectively (Fig. 2).

We observed genetic variability between accessions that originated from seeds of the same tree (siblings) for instance there was variability between AMUR H2-1 and AMUR H2 as well as between AMUR H3 and AMUR H3-5 (Fig. 2). This observed variability could be attributed to natural out-crossing on account of the protogyny in the soursop flowers (Escobar and Sánchez, 1992). To arrive at a definitive confirmation of the status of variability between siblings in soursop however, it is suggested that an in-depth molecular marker analysis using AFLPs for instance, should be carried out.

In general, in the *Annonaceae* germplasm evaluated there was high genetic variability (Fig. 3). Four groups were related at *0.20-0.25 similarity: A. glabra*-like accessions; *A. montana* and *A. muricata* accessions; accessions similar to "anones amazónicos"; chirimoyas, atemoyas and anones-like accessions.

Parallel to molecular characterization analysis, we are evaluating *Annonaceae* species as potential rootstocks for *in vitro* propagation of selected soursop clones (see "Optimization of the *in vitro* propagation methodology of selected clones of soursop (*Annona muricata* L.) and evaluation of the compatibility between different combinations of scions and rootsocks micrografted *in vitro*", this Annual Report). Previous observations indicate that not all accessions are usable as rootstocks. For instance, soursop scions respond differently to different rootstocks of *Annona montana*. Therefore it might be convenient, due to the intrinsic genetic variability (Fig. 3), to select, based on fingerprints, those accessions best suited for rootstocks.

Finally, one accession of *Annona glabra* from C.I. Corpoica Germplasm Bank appeared more related to *A. montana* accessions (genetic similarity of 0.65) than to putative *A. glabra* (similarity around 0.30). It´s therefore convenient to review, based on fingerprints, the present classification of several accessions in the bank.

Conclusions

Each soursop accession showed a different AFLP fingerprinting with combination F. This molecular marker could be used for the identification of soursop clones and future varieties.
Soursop accessions conserved at C.I. Corpoica Palmira germplasm bank presented low genetic variability with combination F. It may be convenient to collect new germplasm.
It is reccomended to check the identification of at least four accessions: 1920, 1959, 1958 and *A. glabra*.

Future plans

- Complete the genetic variability analysis of anonas accessions with two different combinations of primers (combinations M and N).
- Evaluate the genetic variability of siblings by AFLP markers.

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1.1.19 Simple Sequence Repeat (SSR) Marker Diversity in Cassava (*Manihot esculenta* Crantz) Landraces from Nigeria

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Introduction

Nigeria is the world's largest producer of cassava with an annual production of 32 million tons a year (Nweke et al. 2001). This is a more than 200% increase from production twenty years ago. Reasons for the leap in production have been attributed to government policies that favor cassava, population increase and the adoption of improved cassava varieties (Nweke et al. 2001). Nigeria has the highest area in Africa planted to improved varieties, 60%, and cassava is more of an urban staple and cash crop than a food security crop. The impact of the increased commercialization of cassava in Nigeria is expected to lead to an even greater adoption of improved varieties and an erosion of land races and the inevitable loss of diversity. A high level of genetic diversity of cassava has been demonstrated in land races found in traditional Ameri-Indian and African farming communities (Doyle 1997; Fregene et al. 2002), principally a product of the allogamous nature of cassava, agricultural practices, and natural and farmer selection. This genetic diversity is an important resource and needs to be collected and preserved for future use. Besides a study of genetic diversity might reveal genetic differentiation amongst accession that might represent heterotic pools. A study to assess the genetic diversity of cassava land races in Nigeria was

initiated in July 2000, we describe here completion of the SSR characterization component and present insights gained from the study.

Objectives

To study the genetic diversity of cassava land races on a country-wide basis.
Assess genetic differentiation between the Nigerian and Latin American accessions, for example accessions from Guatemala.
Genetic crosses between Nigerian and Neo tropical land races that are highly differentiated to test for heterotic groups.

Methodology

The original study plan was to characterize by SSR markers the 148 accessions held at the cassava germplasm banks of the National Root Crop Research Institute (NRCRI) and the International Institute of Tropical Agriculture (IITA). The IITA collection was made during the collaborative study of cassava in Africa (COSCA) from 65 villages in the entire country. The lack of passport data for more than half of the NRCRI collection and the incomplete IITA COSCA collection lead to a decision to conduct a fresh collection in all 65 Nigerian villages surveyed by the COSCA study. The collection was carried out by 3 teams from IITA during the period of May through June 2001. All 65 COSCA villages were visited and an average of 4-5 of the most commonly grown varieties were collected. Farmers were also asked questions on where they got their varieties, disease and pest incidence and end uses. A total of 285 accessions were collected. The names and passport data of the land races collected can be seen at the following URL: **http://newwebciat/vuca/Molcas/index.htm**, under Nigeria country study. The collection was planted at IITA, Ibadan and will be maintained there.

DNA was isolated from young leaf tissue harvested from field plants according to a modified miniprep method of Dellaporta et al. (1983) at the biotechnology research unit, IITA, Ibadan. DNA was quantiated by flourimetry and shipped to CIAT for molecular analysis. A student from IITA participated in the molecular analysis as a means of transferring the technology to Nigeria. A total of 36 SSR markers, two from each of the 18 linkage groups of the cassava genome, have been defined based on their clear banding patterns and robustness across several SSR diversity studies, this set of markers were employed in characterizing the land races. PCR amplification, gel electrophoresis, and silver staining were as described earlier for these SSR markers (Fregene et al 2002). A previous study had shown high differentiation between African and Guatemalan land races, a set of 13 land races from Guatemala was therefore included to confirm the earlier observation. SSR allele data captured off the gels using the computer software "Quantity One" (Bio-Rad Inc.) Genetic distance, based upon the proportion of shared alleles (PSA), was obtained using the computer program "microsat" (Minch 1993). The distance matrix obtained was displayed graphically using a principal component analysis (PCA) using the compuater program JMP (SAS Institute 1997). Parameters of genetic diversity and differentiation were calculated from allele data using the computer packages GENSURVEY (Vekeman et al 1997) and FSTAT (Goudet 1990).

Results and Discussions

Data from a total of 31unlinked SSR loci was available for statistical analysis the other 5 markers had poor overall data quality requiring their elimination. Genetic diversity parameters, including

total heterozygosity (Ht) and genetic differentiation (Gst) ranged widely from locus to locus (Table1). The average number of alleles for each locus was roughly four and is similar to that found for a study of land races from Tanzania and 7 Neo-tropical countries (Table 2). The probability that 2 randomly selected alleles in a given accession are different, average gene diversity, was 0.5832±0.0482 and it is quite high as also found for the previous study. However, average gene diversity was higher for land races from Guatemala (0.62 - 0.650) compared to those from Nigeria 0.49 - 0.57). Cassava land races from the humid and sub humid regions of Nigeria had higher average gene diversity compared to those from the semi-arid region of the country. The results found here buttress earlier findings that agricultural practices of cassava farmers and the allogamous nature of cassava produces a large pool of volunteer seedlings that natural and human selection acts upon to maintain a high level of land race diversity of a clonally propagated crop (Doyle et al. 2001; Fregene et al. 2002).

Unique alleles were found in the Guatemalan accessions and pair-wise comparison of the genetic differentiation estimator F_{ST} revealed moderate to high genetic differentiation between the Nigerian and Guatemalan land races (Table3). Of particular interest are Guatemalan land races from the town El Progresso. The F_{ST} (theta) estimator) of genetic differentiation averaged over all loci average is low 0.82, but is comparable to that from the study of cassava varieties from Tanzania and 7 Neo tropical countries (0.091). The low level of genetic differentiation in cassava comes as a surprise given the "out of Brazil" hypothesis for cassava. Possible explanations would be a continuos exchange of germplasm and the active generation of genetic diversity by farmers.

Genetic distances between all pairs of individual accessions was calculated by the 1-proportion of shared alleles (1-PSA) and presented graphically by a principal coordinate analysis (PCA) (Fig1). The PC1 and PC2 accounted for 26% and 16% of the total variance respectively. The PCA clearly separates the accessions from Guatemala from those from Nigeria, but it also reveals a sub-structure in the accessions from the Semi-arid region of Nigeria. The presence of a defined sub-structure in the genetic relationship of cassava land races from Africa has been demonstrated before in Tanzania (Fregene et al. 2002). It is yet to be understood the underlying basis for the sub-structure. These results also agree with a previous AFLP marker study of 29 African and 11 Neo-tropics land races that placed African and Neo-tropics land races in two distinct cluster with a sub structure for the African accessions (Fregene *et al.* 2000).

The differentiation amongst land races from Guatemala and Nigeria observed in a previous study (Fregene et al. 2002) and confirmed here may well represent heterotic pools as have been found for maize (Shull *et al.* 1952). One of the principal reasons for this study was to assess genetic diversity in cassava land races as a first step to delineating heterotic pools for a more systematic improvement of combining ability via reccurent reciprocal selection. Activities ongoing include diallel crosses of representative land races from Nigeria and Guatemala.

Future perspectives

- Genotype a larger land race collection from Guatemala with the 36 SSR markers
- Analyze the SSR marker results
- Genetic crosses between Nigerian and Guatemalan land races

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Table1. Parameters of Genetic diversity, Ho, Hs, Ht, Dst, Gst and Gst' (correction for differences in sample size) by SSR locus

LocName	Ho	Hs	Ht	Dst	Dst'	Ht'	Gst	Gst'
SSRY4	0.874	0.729	0.751	0.022	0.027	0.756	0.029	0.036
SSRY12	0.751	0.678	0.691	0.014	0.017	0.695	0.02	0.024
SSRY19	0.538	0.606	0.708	0.102	0.127	0.734	0.144	0.174
SSRY20	0.878	0.812	0.821	0.009	0.011	0.823	0.01	0.013
SSRY21	0.692	0.534	0.555	0.021	0.026	0.56	0.038	0.047
SSRY34	0.328	0.412	0.45	0.039	0.049	0.46	0.086	0.106
SSRY38	0.125	0.152	0.171	0.019	0.024	0.176	0.112	0.136
SSRY51	0.34	0.652	0.655	0.002	0.003	0.655	0.004	0.005
SSRY52	0.61	0.612	0.653	0.041	0.051	0.663	0.062	0.077
SSRY59	0.526	0.646	0.716	0.07	0.088	0.733	0.098	0.119
SSRY61	0.482	0.527	0.548	0.021	0.026	0.553	0.038	0.048
SSRY63	0.264	0.523	0.503	-0.02	-0.025	0.498	-0.04	-0.05
SSRY64	0.621	0.643	0.663	0.02	0.025	0.668	0.03	0.037
SSRY69	0.707	0.645	0.68	0.034	0.043	0.688	0.051	0.062
SSRY82	0.775	0.725	0.779	0.055	0.068	0.793	0.07	0.086
SSRY10	0.823	0.703	0.752	0.049	0.061	0.765	0.065	0.08
SSRY10	0.507	0.472	0.501	0.029	0.036	0.508	0.057	0.071
SSRY10	0.303	0.36	0.373	0.013	0.016	0.376	0.034	0.043
SSRY11	0.284	0.314	0.315	0.001	0.001	0.315	0.003	0.004
SSRY13	0.798	0.585	0.634	0.05	0.062	0.646	0.078	0.096
SSRY14	0.626	0.571	0.634	0.063	0.079	0.65	0.099	0.121
SSRY15	0.837	0.744	0.789	0.045	0.056	0.8	0.057	0.07

SSRY15	0.605	0.551	0.624	0.073	0.091	0.642	0.117	0.142
SSRY16	0.873	0.589	0.711	0.122	0.152	0.741	0.172	0.206
SSRY16	0.5	0.655	0.718	0.063	0.078	0.734	0.087	0.107
SSRY16	0.621	0.496	0.502	0.006	0.008	0.504	0.012	0.015
SSRY17	0.12	0.251	0.325	0.074	0.093	0.343	0.228	0.269
SSRY17	0.793	0.637	0.702	0.065	0.081	0.718	0.092	0.113
SSRY17	0.817	0.767	0.814	0.047	0.059	0.826	0.058	0.072
SSRY18	0.726	0.678	0.75	0.072	0.09	0.768	0.096	0.117
SSRY18	0.807	0.725	0.79	0.065	0.082	0.807	0.083	0.101
Overall	0.598	0.58	0.622	0.041	0.052	0.632	0.067	0.082

Table 2. Intra-population and inter-population estimates of genetic diversity parameters of cassava land races from different agro-eclogies of Nigeria and Guatemala

Population	n	#loc.	#l oc_P	PLP	K	K_P	HO_p	HE_p	HEc_p
Nig-Humid	50	31	30	96.8	4.3	4.4	0.5823	0.5683	0.5742
Nig-Semi Arid	111	31	29	93.5	4.2	4.4	0.5517	0.4972	0.4995
Nig-Sub humid	81	31	30	96.8	4.5	4.5	0.576	0.5677	0.5713
GUA-pro	5	31	30	96.8	2.9	3.0	0.678	0.558	0.6212
GUA-otro	6	31	31	100.0	3.5	3.5	0.6044	0.5977	0.6501
Mean :				96.77	3.9	3.97	0.5985	0.5578	0.5832
std				2.28	0.64	0.66	0.0482	0.037	0.0573

n: number of genotypes per sample
#loc: number of SSR loci;
#loc_P: number of polymorphic loci
PLP: percentage pf polymorphic loci
K: average number of allele per locus
K_P: average number of allele per polymorphic loci
Ho: observed heterozygosity
He: Average gene diversity
Hec_p: Average gene heterozygosity corrected for small samples size

Table 3 Pair-wise estimates of genetic differentiation estimated by F_{ST} (theta) between cassava land races from the humid, sub humid and semi-arid regions of Nigeria and 2 regions of Guatemala

	Nig.Sub-humid	Nig. Arid	Semi Nig. Humid	Gua-pro	Gua-otro
Nigeria Sub humid	0	0.0715	0.0026	0.1287	0.0843
Nigeria Semi Arid	0.0715	0	0.0511	0.1741	0.1133
Nigeria Humid	0.0026	0.0511	0	0.1288	0.0844
Guatemala-pro	0.1287	0.1741	0.1288	0	0.0047
Guatemala-otro	0.0843	0.1133	0.0844	0.0047	0

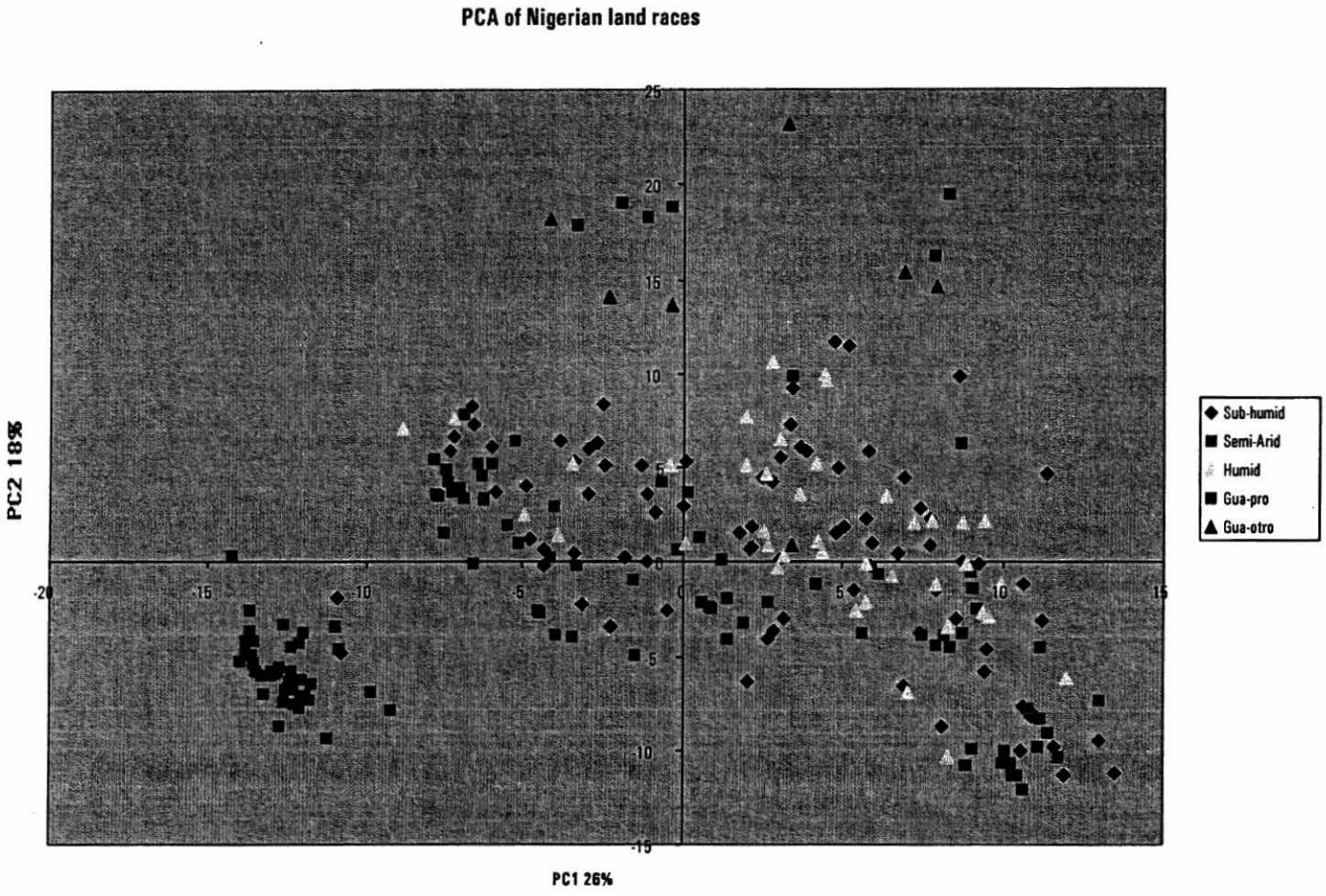

Fig. 1. PCA of cassava land races from Nigeria and Guatemala based on genetic distance (1-proportion of shared alleles) from 31 SSR markers

1.1.20 Simple Sequence Repeat (SSR) Marker Assessment of Genetic Diversity of Cassava Land Races from Guatemala

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Funding: IPICs, University of Uppsala, Sweden

Introduction

Two primary centers of diversity, one in South America and the other in Meso-America have been postulated for the genus *Manihot* (Roger and Appan 1973). Although several studies have demonstrated a likely South American origin for the cultivar sur (Allem, 1994; Fregene et.al 1994; Roa et al. 1997; Olsen and Schaal 1999), the diversity of cassava and its wild relatives in Memo- America is great enough to suggest a second center in Meso-America. Besides, the potential of Meso-American diversity in cassava improvement has not been properly assessed. Three recent studies of genetic diversity in land races from South America and Meso-America

(Chavariagga et. al. 1999; Fregene et. al. 2002; Raji et. al. unpublished data) have revealed unique alleles in land races from Guatemala at a frequency high enough to suggest a Meso American center of cassava diversity. The results of the three studies were based upon 6, 4, and 13 Guatemalan land races. The small sample size of the previous study could distort the allele frequencies and lead to wrong conclusions. A larger collection and SSR characterization of land races from Guatemala was therefore planned to confirm preliminary data of a Meso-American center of diversity and to secure the largely untapped diversity in Guatemala before it becomes extinct. In addition, a selection from the Guatemalan collection will be crossed to CIAT elite parents to evaluate the utility of the Meso-American diversity in cassava breeding.

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

Methodology

A collection of cassava land races was carried out all over Guatemala in May this year (Azurdia and Gomez 2002). A total of 128 accessions were collected in the departments of Baja Verapaz, Quiche, Huehuetenango, Alta Verapaz, San Marcos, Escuintla y Santa in Guatemala (Figura 1). For comparison with results of previous studies, DNA from 6, 11 and 12 cassava land races from Nigeria, Colombia y Brazil respectively were included. DNA from the Guatemalan accessions was isolated at the Facultad de Agronomia, Universidad de San Carlos de Guatemala using a micro-prep protocol of the Dellarporta (1983) methodology and transferred to CIAT. DNA from the other accessions was obtained from previous studies at CIAT.

Fig. 1. Collection sites of cassava germplasm in Guatemala May 2002.

The concentration and quality of DNA samples was accessed by flourimetry and agarose gel electrophoresis respectively. The DNA samples were diluted to10 ng/ml for subsequent PCR analysis. A set of 36 SSR markers, carefully chosen to represent a broad coverage of the cassava genome with moderate to high polymorphism information content (PIC) and robust amplification, were used in this study. SSR diversity studies PCR amplification, polyacrylamide gel

electrophoresis, and silver staining were as described earlier for these SSR markers (Fregene et al 2002).

Results

A total of 30 SSR markers have been analyzed to date in the Guatemalan germplasm. Results so far reveal a number of unique alleles in the Guatemalan accessions not found in those from other regions (Fig 1). The allele data was captured using the program "Quantity One" (Bio-Rad Inc) and entered directly into EXCEL (Microsoft Inc) in preparation for statistical analysis (Fig2). Statistical analysis to be carried out include: principal component analysis (PCA) of a distance matrix based upon 1-proportion of shared alleles, and parameters of genetic diversity and differentiation as described in Fregene et al (2002).

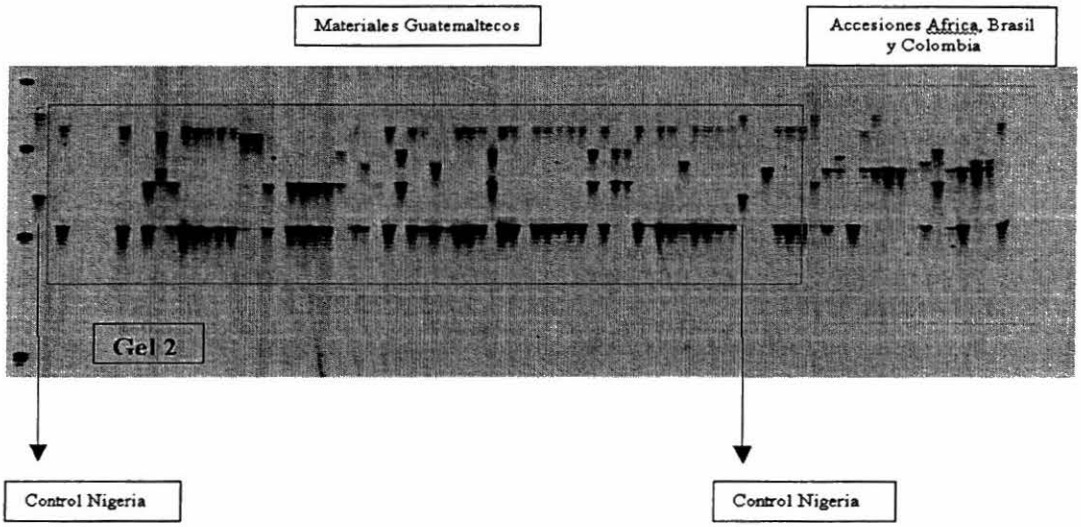

Fig 2. Silver-stained polyacrylamide gel of PCR amplification of cassava accessions from Guatemala, Colombia and Brazil with primers of SSR marker SSRY20. A unique allele can be observed in the Guatemalan accessions with a high frequency.

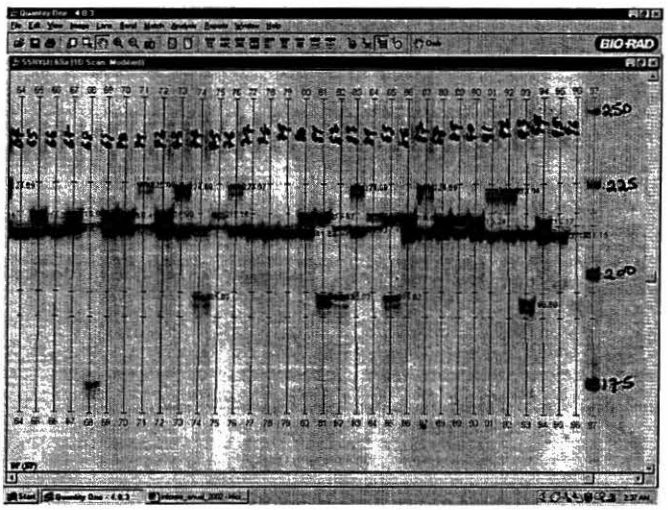

Fig2 . Determination of SSR allele sizes on silver-stained polyacrylamide gels using the software "Quantity One" (Biorad)

Future Perspectives

- Statistical analysis of the SSR data to estimate genetic diversity and differentiation
- Genetic crosses between representative accessions from Guatemala and elite cassava parents at CIAT

1.1.21 Report on the Molecular Characterization of Ghanaian cassava (*Manihot esculenta* Crantz) Land races and Predictability of Heterosis.

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Funding: IPICs, University of Uppsala, Sweden

Introduction

Cassava is an important food crop in developing countries where it is the fourth source of calories, after rice, sugarcane and maize, for more than 400 million people (El-Sharkawy, 1993). Africa is now the largest producer of cassava with a production of 90million metric tones in 1999(FAO 2000). It is cultivated mainly for its storage roots, which provide 390-400 calories/10g dry matter. The leaves when consumed as vegetable provide 7g protein per 100 g edible portion (IITA 1991). The collaborative study of cassava in Africa (COSCA) revealed that cassava serves as a family food staple, a famine reserve crop, and a cash crop.

Cassava was introduced from Brazil, its country of origin, to the tropical areas of Africa, the Far East and the Caribbean Islands by the Portuguese during the 16th and 17th centuries (Jones, 1959). In Ghana, the then Gold Coast, the Portuguese grew the crop around their trading ports, forts and castles. It was a principal food eaten by both the Portuguese and the slaves. By the second half of the 18th century, cassava had become the most widely grown and used crop of the people of the coastal plains (Adams 1957)

The spread of cassava from the coast into the hinterlands was very slow. It reached Ashanti region, Brong Ahafo and the northern Ghana, mainly around Tamale in the 1930.Until the early 1980s,the Akans of the forest belt preferred plantain and cocoyams and sorghum and millet in the north. Cassava became firmly established in most areas after the serious drought of 1982/83 when all other crops failed completely (Korang-Amoakoh,Cudjoe and Adams 1987).

Cassava ranks first in the area under cultivation and ultilization. Cassava contributes 22% of the agricultural gross domestic product AGDP compared to 5% for maize, 2% for rice, and 14% for cocoa (Al-Hassan, 1989;Dapaah, 1996). According to the Ghana Living Standards Survey (GLSS), for 1.73 million sampled households 83% were found engaged in cassava production. The spread of cassava into the upper west and upper east of Ghana is an indication of growing trend in cassava production through area expansion(MOA, 1990).

In the traditional bush-fallow system, some cassava plants are allowed to grow during the fallow period, which is long enough to allow cassava to flower and set seeds. The usual out crossing habit of cassava leads to the production of numerous heterozygous gene pools, which create phenotypic diversity. New hybrid combinations from self-sown seed from which farmers select

and propagate desirable types. This process creates pools of new land races, which are adapted to the different agro-ecological zones of Ghana. Coupled with this are the several names that farmers give to cassava as they distribute among themselves. Several land races have been found with the same name and morphological characteristics yet genetically different and the same land races could have different names in several places (Fregene et al., 2000). Doku (1969) recorded 30 such named local varieties in 1930 and by 1960 the number had increased to over 90. Selection for desirable traits has been done by farmers over 1000s of years. Hence the landraces possess higher frequencies of genes required for adaptation to biotic and abiotic stresses, food quality characteristics than unadapted materials. Vegetative propagation also leads to the accumulation of pest and diseases and good varieties susceptible to these biotic stresses disappear. These factors lead to a fairly high turnover of varieties and has implications for gene pool structure of cassava in any center of diversity. Selection is one of the principal factors at work in cassava's gene differentiation in Africa. Evidence for genetic drift has not been demonstrated given that cassava is vegetatively propagated crop, however the use of spontaneous sexual seeds by farmers has been documented (Mkumbira etal.2001). High heterosis for yield components, starch, and number of roots have been observed in cassava, and hence considered a promising method of genetic improvement (Easwari Amma and Sheela, 1996). Heterotis groups identified in maize in the early 20th century (Shull et al 1953) have been the basis of a very successful hybrid seed industry.

Objectives

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

Methodology

In January 2002 a collection of cassava land races from all the agro ecological zones in Ghana was done. A total of 45 villages visited during the collaborative study on cassava in Africa (COSCA) were visited. Another 28 villages, important for cassava production, were also visited. Farmers were assembled and asked to share information on cassava varieties grown by them, characteristics of their varieties, and reasons for keeping them. Farmers volunteered to give mature cassava stems, which were labeled.

Fresh young leaf samples of the accessions were collected on ice and used for DNA extraction. An amount of 0.1g of the fresh young leaf was ground in liquid nitrogen and the DNA extracted using the Qiagen kit. The extraction was carried out in IITA, Ibadan, Nigeria. The DNA was carried in absolute ethanol to CIAT. DNA quantification was done using the fluorometer. The DNAs were diluted to 10ng/ul and used for SSR reactions. A sub-set of 36 SSR markers, two from each of the 18 linkage groups of the cassava genome, was employed to obtain an estimate of genetic diversity and differentiation in the land races. PCR amplification, gel electrophoresis, and silver staining was as described earlier (Fregene et al 2002). An internal control of 10 genotypes was included to permit comparison between this study and other ones. The PAGE gels containing SSR data will be scanned and allele sizes determined using the computer software "Quantity One" (Bio-Rad Inc.) based upon an internal gel molecular marker size standard. Genetic distance, based upon the proportion of shared alleles (PSA), will be obtained from the raw allele size data using the computer program "microsat"of Eric Minch

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

Results

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

To date, seventeen out of a set of thirty-six primers used routinely for SSR characterization o cassava genetic diversity have been analyzed. The rest of the analysis is on going. Once the SSR marker analysis is completed, genetic distance and estimates of genetic diversity and differentiation will be calculated. A principal component analysis (PCA) will also be carried out to graphically display the genetic distance matrix. Based upon clusterings obtained above, genotyopes representative of the clusters will be selected as parents for a diallel experiment to search for heterotic patterns.

Future Perspectives

- Statistical Complete SSR marker analysis of the entire collection
- Obtain estimates of genetic diversity and differentiation from the SSR data
- Test heterotic patterns present within the collection or between the collection and others.

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Activity 1.2 Identification and mapping of useful genes and gene combinations

Main Achievements

- Over 150 new microsatellite markers have been developed from common bean small insert and cDNA libraries. These have been characterized for allelic diversity and some have been mapped on the core mapping population at CIAT (DOR364 x G19839) or on other populations (G2333 x G19839 and SEL1309 x DOR476). In addition, the microsatellites are being used to integrate genetic maps for QTL analysis of drought and abiotic stress tolerance in common bean RIL populations.

- Identification of quantitative trait loci and candidate genes for seed micronutrient content in two populations of common bean has shown the complex inheritance of iron and zinc accumulation. Further experiments are underway in collaboration with plant physiologists at USDA.

- Gene tagging has been successful for several insect and disease resistance genes in common bean. Bulk segregant analysis of thrips resistance has identified five candidate regions, while QTL analysis has identified two major genes for Apion resistance. Several microsatellites near the arcelin cluster are being tested for the selection of bruchid resistance. Resistance to bean golden yellow mosaic virus and common bacterial blight are being mapped in an additional population and will translate into new molecular markers for selection of these diseases.

- Marker assisted selection for the Cassava Mosaic disease (CMD) was conducted on a large number o f crosses made between CMD resistant progenies and elite parents and screened with the SSR marker NS158 that is tightly linked to CMD2 gene.

- Gene expression profiling of cassava responses to *Xanthomonas axonopodis* pv. *manihotis* infection was implemented using microarray. Six cDNA libraries were constructed from resistant and susceptible genotypes. Chip with 768 clones were constructed and hybridized and differential expression genes were detected.

- 312 DH lines derived from the Caiapo/*O.glaberrima* cross were phenotyped and screened with 100 SSRs. Seventy putative linkages were identified for yield and yield components.ALL DHs were homozygous either for Caiapo or *O.glaberrima* and positive transgressive segregation for most traits was detected. Significant associations were found between RM 127 on chromosome 4 and plant height, RM283 on chromosome 1 and panicle sterility, and RM292 on chromosome 1 and grain yield. Nearly 50% of the putative linkages(30) with a positive effect on agronomic traits was due to alleles derived from *O.glaberrima.*

- Twenty eight lines derived from the Bg90-2/*O.rufipogon* cross were planted in replicated yield trials in six locations in Colombia. Statistical analysis showed no significant difference in grain yield beteween BG90-2 and its progenies over locations. Although none of the progenies was excellent in all locations, a few lines performed better than Bg90-2 in each location. In summary, results obtained under greenhouse and farmer's fields confirm that *O.rufipogon* and *O.glaberrima* possess alleles with positive effects on yield, stress resistance(Rhizoctina solani, rice stripe necrosis virus), and grain quality. Molecular markers are being used to map QTLs associated with these traits and near isogenic lines are being developed for use in breeding programs.

- Male-sterile Nipponbare was developed through a backcross program as a pre-requisite to produce foundation seed of stable genetically-engineered Ds transposon lines.

1.2.1 QTL analysis of drought and abiotic stress tolerance in common bean RIL populations

MW Blair[1], MC Muñoz[1], S Beebe[2]
SB-2 Project; IP-1 Project

Introduction

Recombinant inbred line (RIL) populations are useful for genetic studies because they are made up of stable lines which can be grown over a wide range of environments in a consistent and statistically reliable fashion (large plots, many replicates). The objective of this research was to create an anchored, full-coverage genetic map for three populations of RILs developed to study abiotic stress tolerance in common bean. We were specifically interested in using the new microsatellite markers developed in our laboratory to anchor and fill in previously developed RAPD maps, both to compare genetic maps and to assure that all chromosomes are being assayed in the genetic study.
Our ultimate goal is to identify the regions of the genome which affect yield under phosphorous stress.

Methodology

Genotypes: Four RIL populations were developed from crosses between five abiotic stress tolerant susceptible and tolerant parents, all of which were bush bean types from the Mesoamerican gene-pool (Table 1). They were:

DOR364: an improved variety which is widely grown in Central America where it is known as 'Dorado', that is derived from the cross BAT 1215 x (RAB 166 x DOR 125). This genotype has type II growth habit, is inefficient for phosphorous and responds to fertilization with phosphorous.
BAT477: an advanced line from CIAT derived from the cross (G 3834 x G 4493) x (G 4792 x G 5694). This genotype has a type III growth habit and small cream colored seed. It has high nitrogen-fixation capacity and adaptation to drought conditions due possibly to a deep root system.
G3513: a landrace from Mexico (Comitán de Domínguez, Chiapas, 1635msnm) with type III growth habit and small black seed that yields well under low phosphorous conditions.
G21212: a landrace from Colombia (El Tambo, Nariño, 1360msnm) with small black seed and high efficiency under low phosphorous conditions.
BAT881: an advanced line from CIAT derived from the cross (G 3834 x G 2045) x (G 3627 x G 5481) which has low yield under phosphorous deficiency stress but high biomass production and good yielding ability under optimum conditions. It has very small brown colored seed with black hilum ring.

The seeds of all these genotypes are shown (Figure 1).

Table 1. Recombinant inbred line (RIL) populations derived from crosses between abiotic stress tolerant and susceptible Mesoamerican genotypes.

Tolerant Parent		Susceptible Parent	Population name	No. of RIL´s	Generation
BAT 477	x	DOR 364	BD	97	F5
G 3513	x	DOR 364	DG	95	F5
G 21212	x	BAT 881	BG	95	F5

DNA extraction: For the crosses DOR364 X BAT477 y BAT881 X G21212, eight seed per RIL line was germinated on humid paper in a dark growth chamber for five days until the first etiolated leaves could be harvested into 1.5mL eppendorf tubes. DNA extraction procedure was according to the protocol of Afanador et al. (1993). For the DOR364 X G3513, leaf tissue of two-week old seedlings was ground in liquid nitrogen and placed in 25mL centrifuge tubes for DNA extraction via a CIAT protocol (Gonzáles et al., 1995). DNA concentration was measured in a Hoefer "DyNA Quant 200" flourometer and diluted to a final concentration of 10 ng/µl, with a final volume of 500µl.

RAPD analysis: A total of 698 Operon primers were evaluated on DOR364 and BAT477; 603 on DOR364 and G3513; and 466 on BAT881 and G21212. RAPD reactions were carried out in 96-well plates on PTC-100 thermocylcers (MJ Research, Inc., Watertown, MA). The total reaction volume was in 25 µL. Annealing temperatures were 36 °C and extension was carried out at 72 °C. The PCR products were separated on 1.5% agarose gels that were stained with ethidium bromide and photographed with Polaroid film on a UV light box. The molecular weight standard consisted of *Pst*I digested phage DNA.

Microsatellite amplification: 50 ng of template DNA was used for PCR amplification in a 20 uL final reaction volume. PCR conditions are given elsewhere in the annual report. Amplification was carried out on PTC-100 or 200 thermocyclers (MJ Research). MgCl and annealing temperatures varied for different microsatellite markers. PCR products were run on denaturing 4% polyacrylamide gels run on SequiGen electrophoresis units (Biorad) and silver stained (Promega).

Phenotypic data: The RIL populations were planted in ten different environments (Table 2) and each RIL line was evaluated for yield, flowering and maturity date and 100 seed weight.

Linkage analysis and QTL detection: Genetic maps were constructed with MAPMAKER (v 3.0) for Windows. Markers were assigned with a minimum LOD of 3.0. QTLs were identified by a) single point analysis using simple linear regression which was conducted with the software Q-gene (Nelson, 1997) where the assumed model was for an *RI self* population; and b) composite interval mapping analysis which was conducted with the software QTL Cartographer v 1.5 or v1.21 (Basten *et al.*, 2001). Parameters for Model 6 analysis were a 10 cM window of analysis and 10 most significant markers used as control. Markers were detected by forward and backward multiple linear regression for each chromosomal position at 1 cM intervals with a global significance level of 5%. Both LR (likelihood ratio, - 2ln (L_1/L_0)) and LOD ($\log_{10}$ (L_1/L_0) were reported. QTL position corresponded to the point with maximum LOD within an interval. Determination coefficients (R^2 and TR^2) were used to determine the phenotypic variance explained by a single QTL (either alone or in conjunction with all other significant intervals). Additivity values were also estimated.

Table 2. Experimental locations in which three RIL populations were tested.

Name	Location	Year	Treatment[1]
APD7B	Darién (Valle del Cauca)	1997	High P
APD8A	Darién	1998	High P
APD8B	Darién	1998	High P
BPD8A	Darién	1998	Low P
BPD8B	Darién	1998	Low P
AFP9	Popayán (Cauca)	1999	High P
BFP9	Popayán	1999	Low P
S0A	Palmira (Valle del Cauca)	2000	Drought
S0B	Palmira	2000	Drought
RI0B	Palmira	2000	Irrigated

1/ Low or high phosphorous defined by fertilization level in kg/ha of super-phosphate.

Results and Discussion

The genetic maps for the three populations were enhanced by the inclusion of microsatellite markers, even though the rate of polymorphism was low for these crosses given that all the parents were Mesoamerican and therefore relatively similar. Polymorphism rate was highest in BAT881 X G21212 (36% of all microsatellites tested), then DOR364 X BAT477 (23%) and DOR364 X G3513 (23%). An advantage of the microsatellites was that the linkage groups made up of RAPD markers could be tied to chromosomes on the core map of common bean. For example, a total of 15 new microsatellites could be placed on the BAT881 x G21212 genetic map and this allowed the identification of eight chromosomes. In the DOR364 X BAT477 population seven linkage groups were assigned to chromosomes while in the DOR364 X G3513 only six linkage groups could be assigned.
Although fewer markers have been placed on the other two genetic maps, this work is continuing and will allow us to compare the positions of markers and QTLs from one population to the other. Therefore, for now we will present the results of phenotypic and QTL analysis of the BAT881 x G21212 population.

Phenotypic data collected for all three populations over the years 1997 to 2000 showed significant correlations between the same trait over locations and between some of the different traits within or across locations. Flowering data and maturity date were often correlated as might be expected. Yield was positively correlated with maturity date in some locations. It was surprising to see the correlation of yield data across different stress environments.
Quantitative trait loci (QTLs) were identified with single point analysis and the software Q-gene for all the characteristics measured. In single point analysis the level of global significance for QTLs was 1% ($p < 0.01$). However since there were 159 markers in the BAT881 X G 21212 map the per marker significance level was 6.28e-5 (0.01 / # markers), according to the Bonferroni correction factor (Lynch y Walsh, 1998). QTLs for yield in the different environments were found on chromosomes b03c, b05e, b06ga, b08fa y b08fb as described below:

Chr. b05e: a region defined by the markers AK1201 (RAPD) and BMd20 (microsatellite) was associated with yield under high and low P in Darién 1998a and explained more than 20% of phenotypic variance. The positive effect was from the BAT881 allele. In the same region, was a

QTL for maturity date under high P in Darién in 1997b and under drought in Palmira in 2000b. Although these QTLs only explained a little more than 10% of the variation, the association explains the correlation between yield and maturity date in those trials. The marker Y501identified another QTL for yield in high P in Darién 1998a.
Chr. b06ga: the marker L202 (RAPD) was associated with yield under low P in Darién 1998b and explained around 20% of variance. Another region with the markers BM137 y BM158 (both microsatellites) was associated with days to maturity in high and low P in Darién 1998a. For this QTL the longer maturity came from the G21212 allele.

Chr. b08fa and b08fb: QTLs were identified for the markers Clon638, T402, Q1701 and L201 for yield under high P in Darién 1998a and under low P in Darién in both semesters in 1998. Positive effects were associated with the G21212 allele. The consistency of this QTL leads us to believe that the region is associated with phosphorous use efficiency but affected by environmental variance. Several QTLs for maturity data under high P, low P and drought were found near Clon 007 and O1603 on this chromosome and most explained around 20% of variation. Other QTLs for yield under low P in Darién 1998a were found around the markers Q1801, Z1903, M1201 y Z701, while QTLs for maturity and flowering date under high P and drought were found near the microsatellite BMd25 and the RAPD´s V1602 y R2002.

Using composite interval mapping, we identified a total of 104 significant QTLs for 28 out of the 33 trait x location characteristics analyzed for the BAT881 X G21212 population. Minimum LOD for a significant QTL was set at the threshold 2.81 for any traits with a normal distribution. Traits with non-normal distribution were subjected to 1000 permutations with a significance level of 5% to find the empirical threshold for the experiment (Churchill y Doerge, 1994). Composite interval mapping had the advantage of identifying the location and effect of each QTL. A total of 33 QTLs were found for yield in the three locations under low P or drought stress conditions, which were the motivation for this study. Of these 19 were placed on linkage groups that could be assigned to chromosomes and 15 were on linkage groups that could not. The following is a description of the QTLs found per chromosome:

Chr. B05e: Seven QTLs, *r3, r5, r8, r9, r10, r18,* and *r32* were found on this chromosome. The first three were associated with yield under high P in Darién en 1997(b), and explained only 13 to 19% of phenotypic variation and were related to the BAT881 allele. The remaining QTLs were associated with yield under low P in Darién 1998(a), and were responsible for up to 37% of phenotypic variation related to the same parent. Another yield QTL was found near the marker BMd20, which was consistent across both high and low P in different seasons.
Chr. b06ga: Three QTL´s were found on this chromosome: including QTLs near R901 for yield under high P in Darién (1998a) and markers R101 and E104, at a distance of 34 cM, for yield under low P in Darién (1998a).

Chr. b08fa and b08fb: Eight QTL´s, *r6, r11, r13, r17, r22, r23, r29* y *r31* were identified on this chromosome. The QTLs *r13* (Clon007), *r23, r11* y *r6* (BM151) were significant for yield under irrigated conditions in Palmira (2000), explaining from 22 to 26% of the phenotypic variation and related to the G21212 allele. Two other intervals in the region, near C703 and BM151, also were associated with days to flowering under drought in Palmira (2000), explaining between 18 and 20% variation attributed to the G21212 allele. Another QTL (*dco5*), close to BM151, was associated with days to maturity under drought in Palmira (34%), as well as under high P in Darién 1997 (26%), low P in Darién 1998(b) (23%), with later maturity allele contributed by G21212. The markers W1603, L201, y U1001 (QTL´s *r31, r17* y *r29* respectively) were associated with yield under optimal conditions, including under high P in Darién (1998a). Finally, a QTL associated with G21212 near Q1701 explained 34% of the phenotypic variance for yield

under low P in Darien 1998. A final QTL, *r24*, was found on this chromosome segment for yield under drought stress in Palmira (2000b) (14%) related to the G21212 allele.

QTLs for other characteristics such as flowering or maturity date and 100 seed weight were found on chromosomes b03ca, b03cb, b05e, b08fa, b08fb, and linkage groups bg7, bg8, bg10 y bg15.

Future studies

Continue the integration of the three genetic maps with additional microsatellites.
Use the QTLs identified in this study as starting points for further genetic analysis via near isogenic line development.

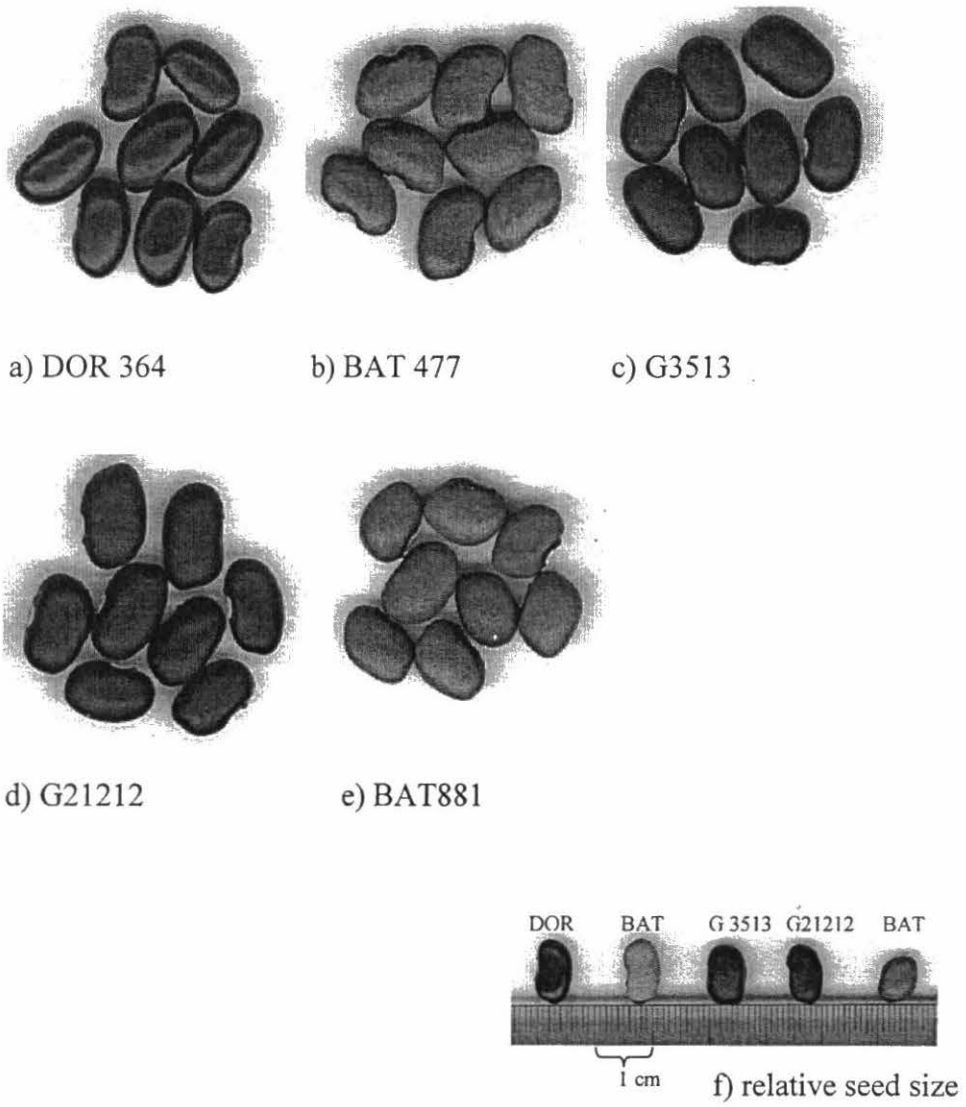

Figure 1. Seed types represented by the parents of RIL populations used in this study

1.2.2 Localization of candidate genes and QTLs for micronutrient content in two populations of common bean.

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Introduction

Legumes provide essential micronutrients that are found only in low amounts in the cereals or root crops. 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. The objective of our most recent studies has been to tag some of the quantitative trait loci (QTLs) controlling mineral content in beans and to characterize candidate genes for micronutrient accumulation in common bean.

Methodology

The two recombinant inbred line (RIL) populations, representing the Andean x Andean cross (G21242 x G21078); and a Mesoamerican x Mesoamerican cross (G14519 x G4825) that were used in this study are described in more detail in last year's annual report (CIAT, 2001). This year we added additional microsatellites to the genetic maps and expanded the QTL analysis to include both composite interval mapping (CIM) and single-point regression analysis (SPA). The mapping of microsatellites is also described in last year's report. Phaseolin analysis for the Andean population was conducted as described in a previous section of this report. Several SCAR primers were developed based on the sequence of common bean ferritin (Genbank entry X58274) and amplified with a range of PCR conditions. Segregating fragments were evaluated on the entire set of RILs. Genetic maps were constructed with Mapmaker and used for both single-point regression (SPA), simple interval mapping (IM) and composite interval mapping (CIM) QTL analysis. The interval mapping QTL analysis were conducted with the software QTL Cartographer v 1.5 (Basten *et al.*, 2001), using model 6 default parameters: 10 cM window of analysis with background of the 10 most significant markers, analyzing by forward and backward multiple linear regression for each chromosomal position at 1 cM intervals with a global significance level of 5%. QTL position corresponded to the point with maximum LOD within an interval. Determination coefficients (R^2 and TR^2) were used to determine the phenotypic variance explained by a single QTL (either alone or in conjunction with all other significant intervals). Additivity values were also estimated.

Results and Discussion

Of the 110 microsatellite markers tested on the parents of the populations, more were polymorphic and could be mapped in the Andean population (34) than in the Mesoamerican population (23). Although both crosses were with parents from a single genepool, the rate of polymorphism was higher in the Andean x Andean cross (40 %), than in the Mesoamerican x Mesoamerican cross (20.9 %). In addition a total of 81 RAPD bands were mapped in each of the populations. For the Mesoamerican population, 14 linkage groups could be detected, while the Andean population had a total of 12 linkage groups. As common bean has 11 homologous chromosomes, the present maps are likely to be representative, however some linkage groups remain sparse in coverage.
The total map length was 1210 cM for the Andean populations and 1235 cM for the Mesoamerican population. Across either population, up to six microsatellites were found per

linkage group. The single locus nature of microsatellite markers was useful for anchoring the RAPD markers to known chromosomes and for comparing between the two maps. The order of the microsatellites in each population agreed with that of the microsatellites in the core CIAT mapping population (DOR364 x G19833). Ten out of the eleven chromosomes of common bean could be identified in both the Andean and Mesoamerican populations based on the microsatellites or homologous RAPD bands that they contained. Three additional linkage groups were found for the Mesoamerican population that could not be tied to a chromosome because they lacked a microsatellite or a cross-referenced RAPD. The number of unlinked markers was 15 in the Andean population and 19 in the Mesoamerican population. Coverage was especially low for chromosomes b01, b03, b06 and b11 in the Andean populations and for chromosomes b01, b05, b09, b10 and b11 in the Mesoamerican population.

Segregation distortion occurred with 35% of the markers in the Andean population and was most notable on chromosomes b07 (where G21078 alleles predominated) and b10 (skewed towards G21242). In the Mesoamerican population, 22% of the markers showed distorted segregation and was most notable on chromosome b03 and with the unidentified linkage groups.

In both the populations both iron and zinc content in the RILs presented a continuos distribution, suggesting that mineral content behaved as a quantitative trait (Table 2). Iron content ranged from 33 to 98 ppm (average 57.9 ppm) in the Andean population RILs, and from 41 to 85 ppm (average 59.1 ppm) in the Mesoamerican population. Zinc content ranged from 25 to 49 ppm (average 34.5 ppm) in the Andean population and from 30 to 49 ppm (average 38.7 ppm) in the Mesoamerican population (Table 1). Highly significant correlations were observed among iron and zinc content in the Andean (r=0.63) and Mesoamerican (0.55) recombinant inbred line populations.

QTLs were found for iron and zinc content in both populations using SPA. However QTLs were only found in the Andean population using CIM analysis. The results of the SPA analysis were highlighted in last year's report so we will update the CIM results here and in Table 3: Significant QTLs (with a LOD > 2.5) were detected on chromosomes b01, b09 and b10 for iron and on chromosomes b01, b02, b08 and b10 for zinc. The positive markers varied in their level of significance and the proportion of variance in mineral content that they explained as indicated by the determination coefficient (R2). The most significant QTLs in composite interval analysis for iron and zinc, explained up to 32% and 38% of the variance, respectively. The majority of the positive QTLs were associated with alleles from the high mineral parent, G21242. However this was reversed for the case of one of the QTLs for zinc (Zn2 on chromosome b08) where higher zinc content was derived from the G21078 allele. Therefore, it appears that high mineral content parent provided most of the genes for high mineral content to their progeny, while the low mineral parent provide only one additional gene for mineral content. This may explain why the distribution of mineral content in the progeny was similar to the range between the parents and why transgressive segregation was minimal.

In some cases the QTLs for both minerals occurred jointly at the same marker and were consistent across both populations, therefore some of the QTLs for the accumulation of both minerals may be genetically linked or pleiotropic, controlling both traits at once. Joint analysis confirmed this by detecting QTLs for both minerals together on chromosomes b01, b02, b08 and b10. These results are promising for plant breeding of higher micronutrient content given that if the same QTLs contribute simultaneously to both iron and zinc content, it may be easy to select for these traits jointly.

We have also begun to study the common bean genes involved in getting iron and zinc from the root zone to the grain. Progress was made in mapping a single locus of the phytoferritin gene (probably a gene family) using a SCAR marker for the gene. The locus was identified on chromosome b07 near the phaseolin locus in the Andean population genetic map, which would be interesting given that both are seed proteins. Western blotting and analysis with ferritin antibodies showed similar protein molecular weights for the bean ferritins extracted from all the parents tested, indicating that it will not be possible to map the gene through an isoform approach. Phytoferritin is of interest because it is the major storage form of iron in all tissues including seeds, however other genes will also be analyzed in the upcoming year. Meanwhile, additional studies on the physiology of uptake are being conducted by collaborators at USDA-Houston to see if the mapping parents described above differ in their ability to adapt to iron deficiency. An assay for iron reductase activity in roots has shown that some varieties of common beans, as in most legumes tested, are more efficient than others in taking up iron.

The present work will hopefully permit us to focus on certain parts of the genome and certain physiological processes that influence higher mineral content in bean seeds. We plan to analyze additional populations and candidate genes in the upcoming year and integrate the information about the map locations of QTLs for micronutrients with those for other agronomic traits that we have been studying. The final objective is to be able to use marker-assisted selection to breed new varieties of common beans with commercial seed types and high micronutrient content.

Future Plans

- Expand the genetic mapping effort in the Mesoamerican population for which marker coverage is presently inadequate
- Compare maps generated for the two populations to discover all common QTLs.
- Fine map regions of the genome with desirable alleles for higher mineral content
- Detect QTLs for the amount of sulfur containing amino acids (SAA), as well as for the amount of the other minerals analyzed in the ICP study, which include Mn, Ca, Mg, K, P and S.

Table 1. Origin and characteristics of parents used to develop recombinant inbred line populations

Parent	Origin	Genepool	Seed Color	Growth habit	% protein	% phaseolin	Phaseolin pattern	Lectin pattern	Iron ppm	Zinc ppm
G21242	Colombia	Andean	Cream Mottled	IV	26.2	46.5	C	M	89.3	49
G21078	Argentina	Andean	Cream	IV	19.7	45	T	T	36.6	28.5
G14519	USA	Mesoamerican	Brown	IV	24.7	39.5	SB	V	83.4	38.7
G4825	Brazil	Mesoamerican	Carioca	II	22.4	30	B	M	35.2	33

Source: CIAT, Genetic Resource Unit database.

Table 2. Descriptive statistics for iron and zinc content in the Andean (G21242 x G2178) and Mesoamerican (G14519 x G4825) RIL populations.

Population	Trait	No. lines	Mean	Skeweness (K3)	Kurtosis (K4)	Signif.
G21242 X G21078	Iron	90	57.900	0.665	0.314	5.860**
	Zinc	90	34.522	0.421	0.108	2.702**
G14519 X G4825	Iron	110	59.131	0.446	0.033	3.663**
	Zinc	110	38.678	0.179	-0.301	1.006**

**s<9.21 corresponds to p<0.01.

Table 3. QTLs for iron and zinc content identified in the Andean populations G21242 x G2178 with both simple and composite interval mapping.

QTL	Chromosome	Marker	Position	Method	LOD	Source	Additivity	R2
Fe1	10	BM157	64.7	CIM	3.80	G21242	4.35	14.19
	10	W1201A	82.0	CIM	3.77	G21242	4.19	12.96
	10	H1801A	89.0	CIM	2.65	G21242	3.38	8.50
	10	AI1401A	64.7	IM	3.03	G21242	4.35	14.83
	10	L0204A	55.1	IM	3.01	G21242	4.26	14.21
Fe2	1	L0401A	46.1	CIM	2.62	G21242	3.26	7.98
	1	R0402A	92.6	IM	2.88	G21242	6.55	32.40
	1	P0901A	59.8	IM	2.79	G21242	4.83	17.09
Fe3	9	M1201B	56.4	CIM	2.49	G21242	3.73	9.29
Zn1	1	P0901A	59.8	IM	4.02	G21242	2.48	24.22
	1	R0402A	92.6	IM	3.86	G21242	3.15	37.70
	1	L0401A	46.1	IM	3.74	G21242	2.30	21.42
Zn2	8	I0601A	45.5	CIM	3.94	G21078	1.75	11.97
	8	CLON638	31.1	CIM	3.73	G21078	1.72	11.73
Zn3	A2b	BM156	44.6	IM	2.75	G21242	2.45	24.38
Zn4	10	W1201A	89.0	IM	2.49	G21242	1.82	13.21

Abbrv: IM (Simple Interval mapping), CIM (Composite interval mapping)

1.2.3 Genetic mapping of root traits and climbing ability in the cross G2333 x G19839.

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Introduction

Climbing beans are an important traditional component of agriculture in Central America and the Andean Region. Climbing beans have a higher yield potential than bush beans but this is often dependent on soil fertility levels and ability to recover nutrients from the soil. In this research we analyzed a population of recombinant inbred lines derived from the cross of G2333 x G19839, for yield, yield components, climbing ability and other morphological traits. G2333 is also a source of low phosphorus tolerance due to enhanced adventitious rooting capacity. Therefore the population was grown under high and low phosphorus treatments which were compared for root characteristics. Phosphorus deficiency is very important in Latin America and East Africa affecting 60 and 50% of soils in these regions, respectively. We will conduct a QTL analysis for these traits in the upcoming year.

Materials and Methods

Plant Material and Phenotyping: A total of 84 F5:8 recombinant inbred lines (RILs) from the cross G2333 x G19839 were grown under high and low phosphorus conditions in Darien (Valle) during the rainy season in semester 2002A. G2333 ('Colorado de Teopisca') is a climbing bean from Mexico that has a type IVa growth habit, while G19839 is a landrace from Peru with a type IIIa growth habit. The RILs were grown in two experiments, one at high phosphorus (fertilization of 300 kg/ha TSP (45 kg P2O5)) and one at low phosphorus (fertilization of 50 kg/ha TSP (7.5 kg P2O5)) levels. The experiment was a split-plot design, in a randomized complete block design with two repetitions under trellises and two replications without trellises. The non-trellised plots were planted at different times one week apart to allow for root sampling. .For the trellised plots, the following variables were evaluated for two plants each within a plot and averaged to produce plot values: plant height (PH), internode length (IL), number of vines per guide (NV), raceme length (RL), pod length (PL), number of pods per raceme (P/B). All these measurements were taken on the middle portion of the plant. Climbing ability (CA) was evaluated on a 1 to 9 scale (where 1=highly aggressive climber and 9 = no climbing ability). Climbing ability was measured both at flowering and again in pod filling stages. Data was also collected for yield per plant (Y/P), and the yield components: pods per plant (P/P), 100 seed weight (100s), days to flowering (DF), days to maturity (DM) and harvest index (HI) based on stem and pod weight. Data was analyzed with a combined ANOVA in which sources of variation were: environment (high or low P), genotype (RIL) and genotype x environment. Pearson's correlation coefficients were estimated for the combination of climbing ability with yield and all the yield components. For the root experiment, two plants were analyzed per plot for adventitious root number, basal root number, shoot dry weight, root dry weight. Derived variables were root-shoot ratio, specific root length. Root length was analyzed with the software program WinRhizo.

Marker analysis: DNA was extracted for the parents and the 84 RILs by the modified miniprep procedure used in the Germplasm Characterization Laboratory. A parental survey was conducted to identify SCARs, RAPDs and microsatellite markers that were polymorphic. The

SCARs evaluated so far include SCPO5, SAP6, ROC11, SK14, SI19, UR11GT2, SAS13, RBG303, SH18, H20, SBD5, SW19, SW13, SBB, SAB3, and BAC6. To reduce the risk of false positives all the SCARs were run on six random individuals form the populations in addition to the two parents. The RAPD primers used were ten of the most polymorphic used on other populations (AA19, AK06, H19, I-07, L04, 0-20, V10, W-06, X-11). A total of 110 microsatellites, including the BM, BMy and BMd series, were used in the mapping exercise. All the polymorphic markers were run on the entire population of RILs. Segregation was scored and the dataset was introduced into the program Mapmaker to construct linkage groups.

Results and Discussion

For the root traits, the G2333 x G19839 RIL population showed genotypic variation for all the variables. Analysis of variance is pending. Meanwhile, phenotypic results showed significant affect of genotype x environment for most of the traits measured on the aerial portion of the plant. For the analysis of climbing ability, there was significant GxE in the traits having to do with plant biomass: branch length, branch number, internode length, climbing ability at flowering, number of pods per plant and yield as indicated in the frequency histograms (Table 1; Figure 1). This demonstrates that phosphorus levels had a differential effect on the genotypes, whereby some genotypes responded better to high phosphorus while others were more severely affected by low phosphorus. This suggests that many of these traits are of low heritability and controlled by many quantitative genes. Meanwhile, the variables for pod length, pods per branch, height, climbing ability at pod fill, days to maturity and harvest index showed strong genotype and location effects but no significant interaction of genotype x environment. This suggested that these traits were less sensitive to phosphorus levels. Some of the same genotypes that performed well for these traits at high phosphorus were also good at low phosphorus levels. Therefore, the heritability of these traits is likely to be higher than for the ones mentioned above.

Yield and yield components, except for 100 seed weight and pod length, were highly correlated with climbing ability, (Table 2). These results help to confirm the high yield potential observed in climbing beans. Beans with good climbing ability also had a slight advantage in terms of pod length and seed weight but this was not highly correlated with climbing ability especially in low phosphorus. Climbing ability measured at two different stages were significantly correlated with each other and also with related traits, such as plant height and internode length (Table 3). This suggests that the visual scale created for measuring climbing ability is a good indicator of plant height and can be used as a substitute for time consuming physical measurements thus facilitating the evaluation of this variable. The importance of internode length in contributing to climbing ability is indicated by their high correlation. Meanwhile, the number of branches was less highly correlated with climbing ability and therefore was not as important a contributor to overall plant height. Note that because climbing ability was scored on a scale where 1 was the highest plant and 9 the lowest plant, climbing ability was negatively correlated with plant height.

It was interesting to find that correlations between climbing ability and its other component traits were higher under high phosphorus than under low phosphorus treatment. Meanwhile the correlations between climbing ability and yield components were similar in both environments, except for branch number which was correlated with several yield component traits under low phosphorus more than under high phosphorus. This suggests that branch number is important for reaching the yield potential of climbing beans under low fertility conditions because pod distribution becomes more important under this stress. Meanwhile under both high and low fertility levels, climbing ability affected pod length, seed weight, pods per plant and yield per plant in a similar manner.

Progress has been made on the genetic mapping. A total of 70 microsatellites was polymorphic for the two parents and were used to construct a framework map for the population. Of the full set of polymorphic markers, 66 could be placed into ten linkage groups. No microsatellites could be assigned to chromosome 10. The total map length was 1050 cM with an average interval between markers of 15.7 cM. Segregation distortion was observed for a small percentage of the markers. The 10 RAPD primers generated an addition 28 markers which are in the process of being integrated into this population. In addition, five out of the 16 SCARs were polymorphic and could be mapped to the predicted location for the SCAR (Table 4) based on previous studies. The preliminary QTL detection found three significant loci, on chromosomes b02 (for specific adventitious root length), b07 (for low P shoot dry weight) and b08 (for adventitious root number in high P).

Conclusions and Future Plans

We plan to saturate the genetic map described here with up to 150 single copy markers and a set of 10 additional RAPDs. Once the full genetic map is constructed we plan to conduct single point analysis and composite interval mapping to determine the genomic locations of quantitative trait loci controlling the characteristics described in this report. This is one of the first studies to investigate the inheritance of climbing ability in common bean in a recombinant inbred population. Apart from the determinacy and photoperiod response genes, no other loci which affect plant architecture in climbing beans, have been studied. This study will address this by providing the biological material to dissect the physiology and genetics of climbing ability. To understand better the role of genotype x environment interactions, we will evaluate the RIL population in several new environments (Palmira and Popayán). This way we hope to test the interaction of climbing ability with high or low temperatures found at these different altitudes. Once QTLs are identified we hope to have a better understanding of the inheritance of climbing ability and associated traits. The genetic map will also be useful for exploring the role of adventitious rooting for low phosphorus stress in common bean. The population is being phenotyped in hydroponic experiments in the greenhouse in Pennsylvania State University that we hope to describe in the upcoming year.

Table 1. Significance (a) (F statistic) for location, genotype and genotype x location effects for the trials grown in Darien, Colombia, in 2002 A.

SOURCE	DF	LR	LV	AVA	VR	ALT	NG	MDC
Rep (Loc)	2	7.89***	31.82 ***	2.11ns	0.05 ns	1.85ns	3.11*	0.79ns
Loc	1	12.86***	150.25***	0.95ns	68.33***	112.11***	42.71***	35.57***
Ril	84	4.51***	1.97***	4.65***	2.31***	3.33***	1.89**	7.95***
LocxRil	79	2.03***	0.95 ns	0.82ns	1.25ns	1.22ns	1.54*	1.01ns

SOURCE	DF	LE	CT	CT2	P100S	IC	NVP	PGP
Rep (Loc)	2	2.16ns	1.76ns	0.68ns	0.43ns	2.03ns	6.58**	10.05***
Loc	1	93.08***	73.6***	90.90***	2.49ns	7.34**	218.95***	323.98***
Ril	84	6.27***	4.16***	3.66***	2.22***	3.05***	2.94***	3.01***
LocxRil	79	1.45*	1.42*	1.35ns	0.11ns	1.14ns	1.52*	2.47***

(a) Significance at p = 0.001 (***),0.01(**),0.05(*),or not significant (ns)
(b) trait abbreviations given in text.

Table 2. Correlations between climbing ability and yield and yield components under high and low phosphorus treatment.

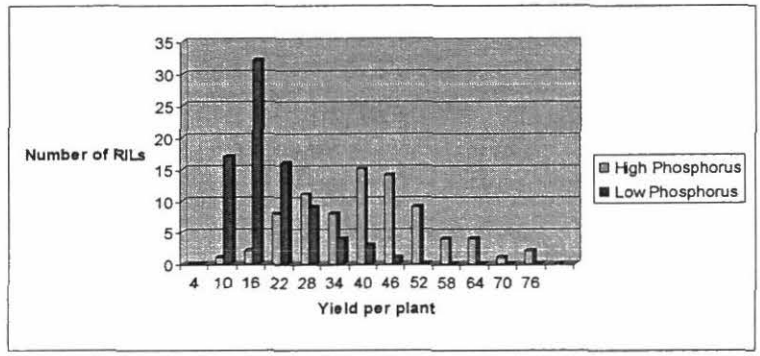
High phosphorus treatment					
	NV	p100s	PGP	LV	VRAC
CT1	-0.4484***	-0.2204 ***	-0.5878***	-0.3867***	-0.4327***
CT2	-0.4098***	-0.1990 *	-0.5425***	-0.3470***	-0.3782***
ALT	0.4959***	0.1239 NS	0.5415***	0.2498***	0.5204***
LE	0.4423***	0.2267 ***	0.5117***	0.2932***	0.5928***
NG	0.2762***	-0.0028 NS	0.2391***	0.0967 NS	0.1130NS
Low phosphorus treatment					
CT1	-0.5463***	-0.0406 NS	-0.5662***	-0.3744***	-0.4386***
CT2	-0.5303***	-0.0485 NS	-0.5536***	-0.3773***	-0.4577***
ALT	0.5544***	0.0835 NS	0.5263***	0.3815***	0.5085***
LE	0.4154***	0.0575 NS	0.3550***	0.3399***	0.5462***
NG	0.5802***	0.0989 NS	0.4949***	0.1781*	0.4159***

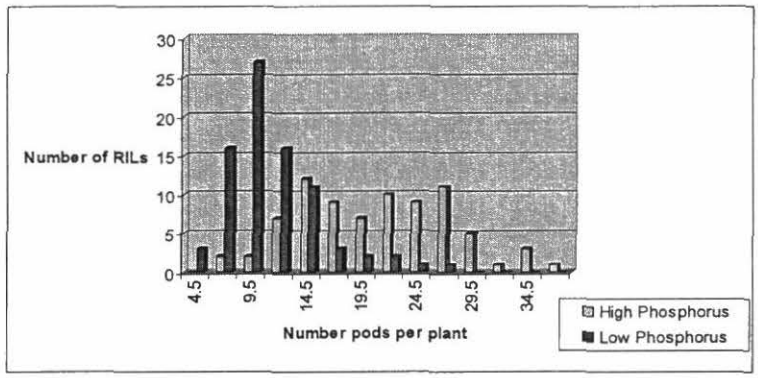
*** = p <0.001 ** = 0.01 * = 0.05 NS= not significant

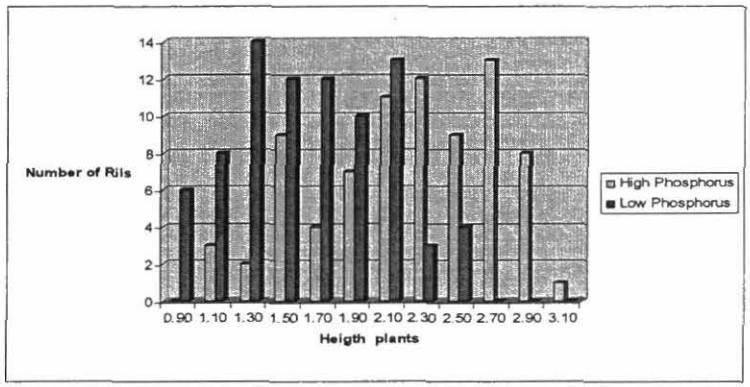
Table 3. Correlations between climbing ability and its component traits under high and low phosphorus treatment.

High phosphorus treatment					
	CT1	CT2	ALT	LE	NG
CT1	1.0000	0.9467***	-0.8386***	-0.7048***	-0.3382***
CT2		1.0000	-0.7807***	-0.6687***	-0.3659***
ALT			1.0000	0.7103***	0.4106***
LE				1.0000	0.2159***
NG					1.0000
Low phosphorus treatment					
CT1	1.0000	0.9390***	-0.8178***	-0.6604***	-0.3886***
CT2		1.0000	-0.8482***	-0.6447***	-0.4003***
ALT			1.0000	0.7101***	0.4301***
LE				1.0000	0.2552***
NGS					1.0000

*** = p <0.001 ** = 0.01 * = 0.05 NS= No Significativo







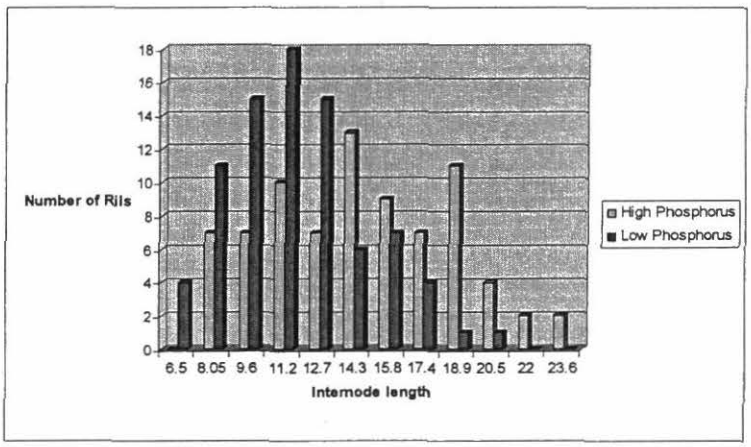


Figure 3. Frequency distribution of four traits in the G2333 x G19839 population under low and high phosphorus treatments.

Table 4. SCARs that segregate in the G2333 x G19839 population.

SCAR	Trait	Gene	Size	Chr.	AA	BB	AB	total
BAC6	CBB	Major QTL	Co-dominant 1250	B10	56	26	-	84
SAB3	Anthracnose	Co-5	Dominant 400	??	58	-	-	84
SAS13	Anthracnose	*Co-4*[2]	Co-dominant ???	??	52	31	1	84
SBB14	Anthracnose	*Co-4*[2]	Co-dominant 1150/1050	??	49	30	5	84
SBD5	BCMV	*bc-1*[2]	Dominant 1250	B03	-	37	-	84
SW13	BCMV	*I*	Dominant 690	B02	12	-	-	84

1.2.4 Bulk segregant analysis of thrips resistance

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Introduction

Thrips palmi is a damaging insect pest of common bean and other dicotyledenous crops that was introduced from Asia (Java, Indonesia) into the Americas during the last decade. Starting in the Caribbean, (Cuba, Dominican Republic, Haiti and Puerto Rico) the species spread rapidly into the United States and northern South America (Brazil, Colombia, Ecuador and Venezuela). The greatest damage inflicted to common bean production in Colombia is seen in climbing bean varieties that are grown for the fresh market (including snap beans and Cargamanto dry beans). Sequential plantings, common in the production of snap beans is very conducive to heavy infestations of thrips and whiteflies, which are synergistic in the damage that they inflict. Misuse of insecticides also can lead to resurgence in thrips populations.
In the 2000 Annual report we described a preliminary QTL study using RAPD analysis of a recombinant inbred line (RIL) population derived from the cross BAT881 x G21212. This year we used microsatellite markers to increase the precision of QTL identification and to associate these QTLs with specific chromosomes. The specific objectives of this research were to 1) construct bulks of lines differing in resistance and susceptibility based on the QTLs detected in that population; 2) conduct a bulk segregant analysis 3) screen the bulks with all existing microsatellite markers that were polymorphic for the two parents of this population; and 4) study the importance of each positive microsatellite with a single point regression QTL analysis.

Methodology

The BAT881 x G21212 population, consisting in 139 F7 generation RILs was evaluated over two seasons at a field site in Pradera, Valle, Colombia. In the first season (semester 1999A - April), the population was planted as an un-replicated trial; while in the second season (semester 1999B - July) it was planted in a randomized complete block design with three repetitions. The inoculation conditions were as described in Annual Report 2000. The lines were evaluated on a per row basis for both thrips damage and reproductive adaptation (RA) using a 1-9 scale

according to the CIAT standard evaluation (1=resistant, good pod set; 9=susceptible, poor pod set). In our previous work, quantitative trait loci (QTL) were identified through single-point regression analysis of the phenotypic data onto the one hundred and fifty one RAPD markers which had been run on the progeny. Based on the RAPD results, five DNA bulk pairs were created with five each of the most resistant and most susceptible progeny lines from the population that presented the correct allele at the QTL locus (Table 1). One of the bulks, 1R, only had four individuals due to few resistant genotypes with the appropriate allele at the QTL locus. These bulks were screened with 107 microsatellites, most of which had already been showed to be polymorphic for the parents. Any microsatellites showing a polymorphism in one of the bulks were run on a set of 94 out of the 139 RILs developed for this population. The microsatellite segregation data was compared to the phenotypic data (damage and RA in 99A and 99B) to establish whether there was an association with resistance based on a single-point regression QTL analysis done with the software program qGENE.

Results and Discussion

The bulk segregant analysis identified 20 positive microsatellites from the survey of 107 markers (18.7%) (Table 2). The bulks were effective at capturing a large number of potential microsatellites to screen and this number of lines per bulk resulted in relatively easy to read polymorphisms (Figure 1). Despite the fact that the bulks were determined based on separate RAPD linkage groups that showed QTL associations, several of the bulks included the same individual progeny lines. This may have led to some of the microsatellite assays detecting more than one positive bulk as was seen with the markers, BM143 and Clon037. Most of the microsatellites were tested against the bulks twice to confirm their repeatability and only a few showed differences in the bulk genotype. The bulks detected different numbers of polymorphic markers: 11 were associated with BG1, seven with BG7, ten with BG3, five with BG9 and four with BG6.

Resistance source were G21212 for the bulks BG1 (Damage), BG3 (Damage), BG9 (RA and Damage) and BG6 (RA); versus BAT 881 for BG7 (RA) eventhough among the parents, BAT 881 usually shows slightly better resistance than G21212. The QTL results may explain why many of the RIL's outperformed either parent evidence of transgressive segregation.
Segregation analysis of the positive microsatellites showed that segregation distortion was uncommon and most microsatellites (17 out of 20) had normal segregation. As expected for the F5:7 RILs, heterozygotes were relatively infrequent in the population (Figure 2).

The single point regression QTL analysis showed a total of nine significant marker x trait associations. For damage score, two microsatellites were significantly associated with the trait 1999A, while three were associated in 1999B. For reproductive adaptation, one marker was significant in 1999A and three were in 1999B. Two markers from chromosome b6, Clon037 and Clon410 were significant for both damage score and reproductive adaptation in 1999B. Clon037 was also significant for damage score in 1999A. The variation explained by any single marker ranged from a low R2 of 6.2% to a high R2 of 27.7%.
The consistency of QTL results across seasons and across traits and the high R2 value for the two markers on chromosome b6, indicate the importance of this QTL for durable thrips resistance. We will associate these results with the original RAPD linkage groups that were the basis for designing the bulks, when we map the microsatellites and RAPD markers together.

Future Plans

- Continue mapping with additional microsatellites to achieve complete map coverage in the BAT881 x G21212 population
- Composite interval QTL mapping with entire map dataset.
- Repeat the field analysis for a third season to measure thrips resistance

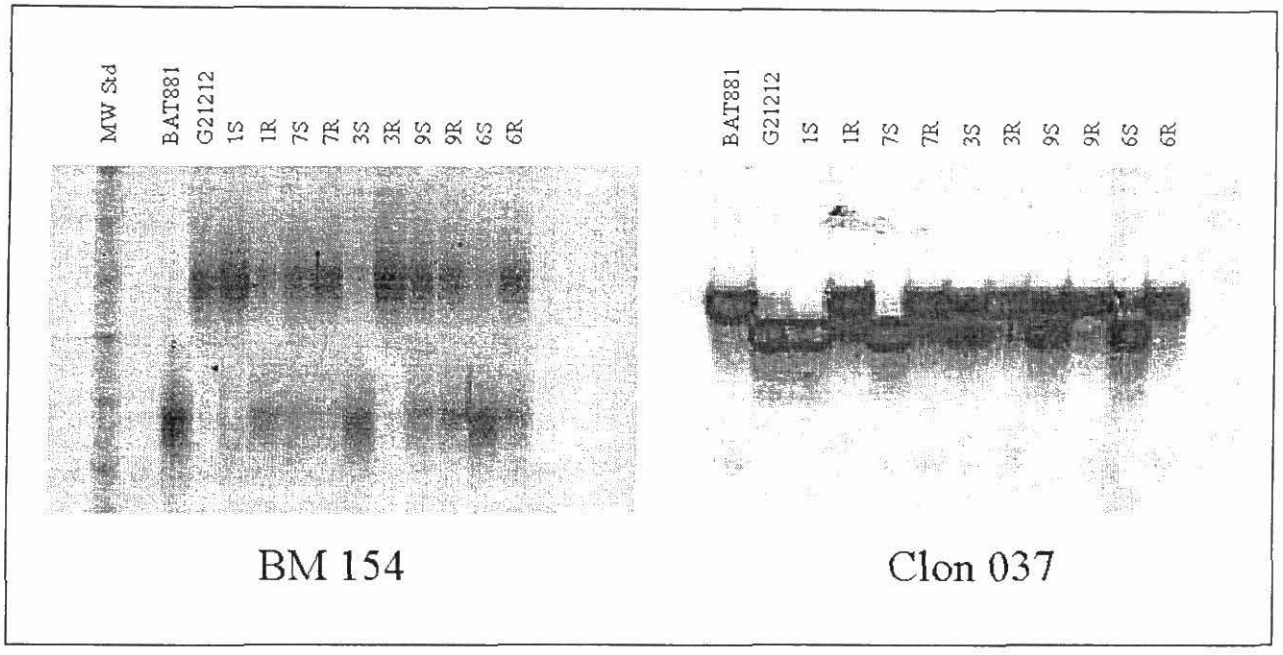

Figure 1. Bulked segregant analysis of thrips resistant and susceptible progeny of the population BAT881 x G21212 using two common bean microsatellites

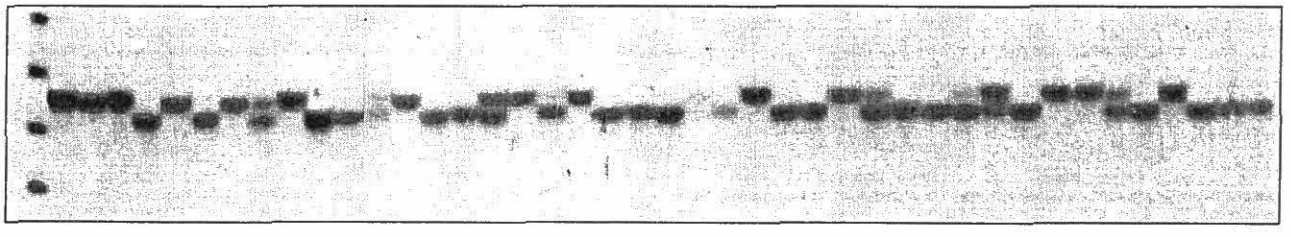

Figure 2. Segregation of Clon037 in the progeny of the population BAT881 x G21212.

Table 1. Individuals in each Bulk (Adaptive Reproduction (RA) in old scale)

Bulk 1 - BG1 susceptible (99B)						Bulk 1 - BG1 resistant (99B)					
No	Material	Dam 99A	RA 99A	Dam 99B	RA 99B	No	Material	Dam 99A	RA 99A	Dam 99B	RA 99B
55	BH 21134-74-1-1	8	9	8.3	8	35	BH 21134-46-1-1	7	5	6	6
72	BH 21134-110-1-1	7	8	8.3	8.7	45	BH 21134-60-1-1	5	6	4.3	5.3
74	BH 21134-113-1-1	5	5	8.7	8.3	81	BH 21134-128-1-1	4	4	6	5.7
77	BH 21134-124-1-1	6	5	8.7	8	88	BH 21134-144-1-1	4	5	5.3	6
89	BH 21134-147-1-1	5	6	8.7	8						
Bulk 2 - BG7 susceptible (99B)						Bulk 2 - BG7 resistant (99B)					
14	BH 21134-19-1-1	8	7	8.7	8	5	BH 21134-5-1-1	4	3	6.3	5.3
65	BH 21134-90-1-1	6	6	8.3	8	7	BH 21134-7-1-1	5	4	6.7	5.7
72	BH 21134-110-1-1	7	8	8.3	8.7	45	BH 21134-60-1-1	5	6	4.3	5.3
74	BH 21134-113-1-1	5	5	8.7	8.3	81	BH 21134-128-1-1	4	4	6	5.7
89	BH 21134-147-1-1	5	6	8.7	8	83	BH 21134-130-1-1	4	4	7	5.3
Bulk 3 - BG3 susceptible (99A)						Bulk 3 - BG3 resistant (99A)					
22	BH 21134-29-1-1	9	9	6.3	6.3	4	BH 21134-4-1-1	4	4	7.3	6.7
53	BH 21134-70-1-1	8	7	8.3	7	5	BH 21134-5-1-1	4	3	6.3	5.3
57	BH 21134-78-1-1	8	8	8	7.7	8	BH 21134-8-1-1	4	4	7.3	7.3
58	BH 21134-79-1-1	8	9	8	7.7	82	BH 21134-129-1-1	3	3	7	6
92	BH 21134-150-1-1	8	8	7.7	6.7	83	BH 21134-130-1-1	4	4	7	5.3
Bulk 4 - BG9 susceptible (99A)						Bulk 5 - BG9 resistant (99A)					
22	BH 21134-29-1-1	9	9	6.3	6.3	4	BH 21134-4-1-1	4	4	7.3	6.7
36	BH 21134-47-1-1	8	9	7.7	7	7	BH 21134-7-1-1	5	4	6.7	5.7
55	BH 21134-74-1-1	8	9	8.3	8	8	BH 21134-8-1-1	4	4	7.3	7.3
56	BH 21134-76-1-1	8	9	7.7	7	9	BH 21134-9-1-1	5	4	7.3	6.3
58	BH 21134-79-1-1	8	9	8	7.7	82	BH 21134-129-1-1	3	3	7	6
Bulk 5 - BG6 susceptible (99A)						Bulk 5 - BG6 resistant (99A)					
22	BH 21134-29-1-1	9	9	6.3	6.3	2	BH 21134-2-1-1	5	4	7.3	6.3
25	BH 21134-34-1-1	8	9	8	7.3	3	BH 21134-3-1-1	5	4	6.7	7
26	BH 21134-36-1-1	8	9	8	6.7	4	BH 21134-4-1-1	4	4	7.3	6.7
48	BH 21134-65-1-1	8	9	8	7.7	5	BH 21134-5-1-1	4	3	6.3	5.3
55	BH 21134-74-1-1	8	9	8.3	8	82	BH 21134-129-1-1	3	3	7	6

Table 1. Polymorphic markers selected from Bulked Segregant Analysis and evaluated on entire BAT881 x G21212 population showing QTL results for thrips damage and reproductive adaptation scores in semesters 1999A and 1999B in Pradera, Valle.

Marker	Chr.	Bulk					Allele MW		Segregation distortion				QTL results 99A Damage	QTL results 99A RA	QTL results 99B Damage	QTL results 99B RA
		1 (BG 1)[1]	2 (BG 7)[1]	3 (BG 3)[1]	4 (BG 9)[1]	5 (BG 6)[1]	B	G	BB	GG	BG	ChiSq	R2	R2	R2	R2
BM 053	1			X*			321	340	42	36	3	0.46	0.023	0.022	0.004	0.002
BM 137	6	X**		X-			117	177	30	61	1	10.56**	0.020	0.039	0.016	0
BM 143	2	X	X	X	X		132	145	46	46	2	0.0	0.035	0.035	0.015	0.075**
BM 151	8			X			146	151	38	51	5	1.89	0.029	0.033	0.011	0.014
BM 154	9			X			248	270	33	36	5	0.13	0.000	0.010	0.016	0.001
BM 156	2	X*				X-	218	225	40	49	5	0.91	0.001	0.002	0.002	0.004
BM 181	3	X*	X*				188, 182	182, 179	21	29	0	1.28	0.052	0.065	0.006	0.004
BM 184	9			X**	X-	X-	151	159	--	--	--	--	--	--	--	--
BM 200	1			X		X	300	335	45	36	5	1.0	0.031	0.032	0.005	0
BM 202	--	X*	X				151	153	39	45	7	0.42	0.063*	0.076*	0.001	0.001
Bmd 01	3	X					188, 172	181, 175	--	--	--	--	--	--	--	--
Bmd 16	4	X			X		105	n.a.	47	19	7	11.87***	0.045	0.013	0.026	0.027
Bmd 25	8	X*	X*				120	115	35	45	14	1.25	0.000	0.000	0.060*	0.018
Bmd 36	3			X*		X	175	180	45	44	5	0.01	0.013	0.001	0.007	0.001
Bmc 08	--	X-			X*		111	117	--	--	--	--	--	--	--	--
Bmy 01	4	X**			X-	X-	159	152	47	36	7	1.45	0.028	0.020	0.022	0
Bmy 11	4	X-		X*	X*	X*	240, 202	239, 208	30	52	12	5.9*	0.011	0.003	0.006	0.001
Clon 007	8		X	X			168	173	45	43	6	0.04	0.014	0.010	0.003	0.008
Clon 037	6	X	X		X	X	187	182	40	44	10	0.19	0.062*	0.034	0.277***	0.215***
Clon 410	6	X	X	X			101, 104	104, 106	43	49	0	0.39	0.040	0.013	0.196***	0.105**

* = confirmed twice; ** = confirmed three times; - = differences observed

[1] = initial linkage group designated Annual Report 2000

na = not amplified, RA = reproductive adaptation, 99A = semester 1999A, 99B = semester 1999B

1.2.5 Tagging Apion resistance in the population J117 x Jamapa

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Introduction

This year we continued a project begun last year to tag resistance to the bean pod weevil (*Apion goodmani* Wagner) which damages beans grown in Mexico and Central America. Resistance is controlled by two possible mechanisms – either antibiosis involving a hypersensitive response that encapsulates the oviposition site – or antixenosis that affects the preference of oviposition sites. Epistasis between two independent genes, *Agr* and *Agm*, has been suggested to control the hypersensitive response. The fact that a few genes control resistance may explain why it has been relatively easy to transfer resistance from Mexican landraces where it is found to new breeding lines with Central American grain types. The objectives of this research were to identify additional markers linked to the genes controlling resistance in the recombinant inbred line (RIL) population derived from Jamapa x J117 and to try to identify the chromosomal position of the resistance QTLs identified so far.

Methodology

An additional set of 54 F5 derived recombinant inbred lines was created for the Jamapa x J117 cross. These have been sent to a field site in Mexico for testing and have been used for DNA extraction. This brings to a total of 104 RIL lines the full set analyzed for this characteristic. Screening of susceptible and resistant bulks (of 4 lines each) has continued from last year with additional RAPD and microsatellite markers. A total of 131 RAPD primers and 200 (58 BM, 16 BMc, 51 BMd, 8 BMy, 53 Clon, 8 Pv and 6 VM) microsatellite markers have been evaluated so far. In addition the RAPD and SCARs that were polymorphic from last year's survey were run on the additional set of lines, however the microsatellite remain to be mapped on the full set of RILs for the upcoming year. In addition, the RAPD primers which were reported last year as polymorphic for the bulks were also mapped on two core mapping populations, DOR364 x G19833 and BAT93 x Jalo EEP558. A genetic map was constructed with the new dataset of 104 lines and all the polymorphic RAPD markers using the program Mapmaker.

Results and Discussion

In the bulked segregant analysis an additional three RAPDs and 27 microsatellites proved to be polymorphic for the parents and the bulks. All 28 RAPDs and the SCAR SK16 that were polymorphic for the bulks were run on the entire population of 104 individuals. The microsatellites will be run this upcoming year. As in last year's results, most of the markers were linked to each other in three tight linkage groups with four or more markers each. The genetic mapping was consistent between the first set of 50 individual recombinant inbred lines and this additional group of 54 individuals.

Among the RAPDs that were significant in the bulk segregant analysis, a total of nine were polymorphic in the DOR364 x G19833 population and one was polymorphic in the BAT93 x Jalo EEP558 population. The RAPD markers mapped to the first of these populations identified chromosomes b01, b02, b05, b07 and b08 as potentially important for Apion resistance, while the RAPD marker mapped in the second population identified chromosome b01 as important. This

agrees with the mapping results with single copy microsatellite markers that showed that three linkage groups namely chromosomes B1, B8 and B11 were important for the resistance.

Future Plans

- All positive microsatellites will be mapped on the full set of RILs
- QTL analysis will be carried out when phenotypic data is available for the new set of recombinant inbred lines.
- Two-way interaction tests will be done to confirm epistasis between the genes or QTLs identified above.
- Implement marker assisted selection for Apion resistance at CIAT, for which we will need to test the validity of using the microsatellite markers.
- Develop a SCAR marker from some of the positive RAPD markers.

1.2.6 Marker assisted selection of Arcelin-derived bruchid resistance.

MW Blair[1], S Prieto[1] ;C Cardona[2]
SB-2 Project; IP-1 Project

Introduction

The most common storage pests of common bean are the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman), and the bean weevil, *Acanthoscelides obtectus*. These cosmopolitan weevils belong to the Bruchid family and cause an estimated 13% loss to bean crops worldwide (Cardona and Kornegay, 1999). Zabrotes is especially important in warm tropical regions below 1000 m altitude, while Acanthoscelides is more common in cooler climates. While Zabrotes is only found in storage, Acanthoscelides also lays its eggs on the beans in the field (Schoonhoven et al. 1988). Researchers have found that a special seed protein named Arcelin, which was discovered in wild accessions of common bean from Mexico, provides high resistance to Zabrotes and slight resistance to Acanthoscelides (Osborn et al., 1988).

Arcelin and related proteins, including alpha amylase inhibitors and phytohemaglutinins (all members of the APA family of proteins) provide resistance to bruchids through antibiosis by reducing the adult emergence, female fertility and insect growth and lifecycle (Posso et al., 1989). These proteins are all synthesized only in the embryonic axis and cotyledons during seed formation. Arcelin is inherited as a monogenic trait and to date, there are 7 variants of arcelin (ARC 1-4 identified by Osborn et al (1986), ARC 5 by Lioi and Bollini (1989), ARC 6 by Santino et al (1991) and ARC 7 by Gallegos et al (1998)). These variants are all highly similar (Sparvoli and Bollini 1998) but provide different levels of resistance. Within the allelic series the level of resistance is progressively lower in the variants ARC5 > 4 >1 > 2 > 6 > 3 when in the background of the wild progenitor. However in the cultivated background the alleles that provide the most resistance are ARC1 > 2 > 5 > 3 > 4 (Cardona and Kornegay, 1999). Differences in resistance level are thought to be due to sequence variability or carbohydrate content (Harmsen et al. 1988). Arcelin is known to be a partially dominant gene, which provides its highest level of resistance to bruchids when in the homozygous form. Heterozygous Arc+/Arc- individual seeds are less resistant than Arc+/Arc+ seed. CIAT researchers have used the ARC 1 variant widely in their breeding programs to create resistant breeding lines such as the RAZ series through backcrossing and gene transfer (Cardona et al, 1990). However, no Arcelin-derived bruchid-resistant variety has ever been released.

The method of selecting for Arcelin based resistance has been to assay for the protein in the seed. A serological method has been implemented which detects arcelin in small quantities of ground seed tissue. The process requires arcelin-specific antibodies and protein electrophoresis equipment. Arcelin based selection of bruchid resistance has been very useful. For example in the breeding of the RAZ lines, two generations of backcrossing and selfing were generally sufficient to obtain resistant genotypes that were true to seed type when arcelin was evaluated during the backcrossing process. This represents one of the first true uses of marker-based selection in common bean although with a protein marker rather than a DNA based one. One limitation of protein based selection that it is time-consuming and is not compatible with other marker systems.

The objective of this research was to replace the protein based selection of arcelin with a genetic assay using closely linked microsatellite or SCAR markers for arcelin or related APA proteins. To do this, we are screening two DNA extraction techniques based on alkaline lysis and organic separation, to explore the potential of establishing a high-throughput DNA marker system to screen for arcelin based bruchid resistance. The long-term objective of this work is to increase the efficiency of breeding for multiple constraint resistance and facilitate the pyramiding of bruchid resistance with other biotic and abiotic stress resistances.

Methodology

A total of 63 genotypes were used in the experiments. These included the 7 wild accessions of common bean that were the sources of the seven variants of arcelin known to exist (Table1); 28 advanced breeding lines from the bruchid resistance program (either RAZ or GG designations) (Table 2); and 28 bruchid-susceptible parents used in crosses with Arc1 or Arc 5 containing lines (data not shown). Two DNA extraction techniques were used. One was a rapid, high-throughput "microprep" method based on alkaline lysis developed in the laboratory of N. Weeden. The other was the standard organic-solvent (phenol/chloroform) based "miniprep" used in our laboratory and based on the method of Afanador et al (1998). Tissue was harvested in the greenhouse as leaf disks cut with a hole-puncher for the microprep or newly emerging trifoliates for the miniprep. Another set of 791 F_4 and F_5 progeny derived from crosses between RAZ lines and the susceptible parents was grown in the field and DNA was extracted from leaf disk tissue by the alkaline extraction microprep technique. The DNA was used for microsatellite amplification which was conducted according to standard PCR protocols.

Results and Discussion

A total of seven microsatellites were selected based on either map location and proximity to the arcelin locus (BMd26, Clon41) or because they were part of the sequence of APA gene family members. This second group included BMd9 (derived from the D-Lec 2 gene Phytohemaglutinin-L; Genbank entry X06336), BMd15 (Erythroglutanating Phytohemaglutinin; K03288), BMd16 (Leucoaglutinating Phytohemaglutinin; K03299), BMy11 (Phytohemaglutinin pseudogene; X04649) and Pv-atct01 (Arcelin; M68913). It is interesting to note the large number of microsatellites in the phytohemaglutanin members of the APA family, but the lack of microsatellites in alpha amylase which have also been extensively sequenced.

In terms of the practical experiments, DNA quality was high for miniprep DNA but low for alkaline microprep DNA. Samples extracted with the second technique were easily degraded and could not be stored for more than a few days. This affected microsatellite amplification, with consistent results only obtained with the miniprep DNA. Five of the microsatellites have been amplified to date.

Microsatellite allele diversity varied with each of the markers. The marker detecting the most alleles (11) on the germplasm survey was Clon41, while the markers detecting the least were Bd15 and BMd26 (3 each). Within each of the subgroups of germplasm diversity observed with the different microsatellites also varied. The pattern of diversity for the alleles indicated that linkage disequilibrium exists between all the microsatellites tested and the Arcelin locus as discussed below:

Within the wild beans that were sources of resistance, BMd15 detected only one allele, BMd26 detected two alleles, Clon41 revealed three banding patterns, Pv-atct01 detected six alleles and BMy11 revealed seven band patterns (Table 1). Although BMd15 detected only one allele, this allele was different from any of the alleles found in the susceptible cultivated genotypes and therefore would make a good diagnostic marker for the presence of introgression from the wild accessions. Indeed this allele was found in all the Arc 1 containing RAZ lines (Table 2). Similarly, the two alleles for the microsatellite BMd26 that were found in the wild sources were unique to the wild accessions and the derived RAZ or GG lines and not present in any of the susceptible lines (data not shown). The alleles of the other markers also presented this pattern of association with arcelin introgression. However, the markers BMd26 and Clon41 which are not directly at the arcelin locus were associated with arcelin resistance in only 14 and 12 out of the 26 RAZ lines tested, respectively (Table 2). The two markers co-segregated in all the lines except for four indicating only moderate crossing over between the two markers during the development of the RAZ lines. The higher level of linkage disequilibrium between arcelin and BMd26 versus arcelin and Clon41 confirm the mapping results which indicate that BMd26 is more closely linked to arcelin than is Clon41. It is interesting to note that for BMy11 we found a different allele for each wild accession source of arcelin alleles and that the alleles associated with the Arc1 and Arc5 variants were perfectly diagnostic in the corresponding Arc1 and Arc5 containing RAZ and GG lines (Table 2). Therefore, this marker would be useful for diagnosing the arcelin variant present in a segregant.

Another interesting point to note is that the presence of multiple bands for some of the markers may indicate that these are duplicate loci. This is to be expected for some of the hemaglutinin-based markers (BMd9, BMd15, BMd16 and BMy11) because this protein is known to be encoded by a gene family.

Table 1. Microsatellite allele polymorphism in seven sources of Arcelin resistance identified in wild accessions of common bean

Source	Arcelin allele	Bruchid resistance	Marker and Molecular Weight				
			BMd 26	Pv atct 001	BMd 15	Clon 41	BMy 11
G 12882	ARC1	AR	139	187	164	171, 178	184, 195, 207
G 12866	ARC2	I	137	196	164	170, 176	207 (*)
G 12922	ARC3	S	137	189, 192	164	-	203, 207, 242 (*)
G 12952	ARC4	AR	144	-	164	169, 173	195, 203, 207, 242 (*)
G 02771	ARC5	AR	144	191	164	169, 173	184, 203, 207
G 11051	ARC6	S	-	-	164	169, 173	184, 195 (*), 203, 207
G 24584	ARC7	AR	137	200	164	171, 178	207, 242, 245

*faint signal

Table 2. Bruchid resistance and microsatellite alleles of RAZ lines and other Arcelin 1 containing breeding lines

Genotype	Genepool	Bruchid res.	Allele	Marker (Molecular Weight)				
				Clon41	BMd15	BMd26	BMy11	Pv-atct01
G 12882 (ARC 1) - check		AR	1	171, 178	164	139	184, 195, 207	187
RAZ 1	PVA1025 / WI-85-5	AR	1	167, 171	164	143	184, 195, 207	187
RAZ 12-1	859446-67 / G76	AR	1	171, 175	164	143	184, 195, 207	187
RAZ 24-6	859446-67 // G76	AR	1	-	164	140	184, 195, 207	187
RAZ 44	EX-RICO 23 /// G12882	AR	1	166, 170	164	143	184, 195, 207	187
RAZ 75	RAZ12-4 / XAN252	AR	1	166, 170	164	143	184, 195, 207	187
RAZ 82	RAZ12-4 / XAN252	AR	1	170, 175	164	139	184, 195, 207	187
RAZ 86	RAZ12-4 / XAN252	AR	1	170, 175	164	-	184, 195, 207	187
RAZ 91	RAZ12-4 / XAN252	AR	1	170, 175	164	139	184, 195, 207	187
RAZ 90	RAZ12-4 / XAN252	AR	1	170, 175	164	139	184, 195, 207	187
RAZ 109	RAZ 1 / CAP3	AR	1	170, 175	164	137	184, 195, 207	187
RAZ 138	RAZ / AND885	AR	1	168, 172	164	143	184, 195, 207	187
RAZ 111	RAZ / AND885	AR	1	168, 172	164	143	184, 195, 207	187
RAZ 106	RAZ 24-5 / AND885	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 4-3	859446-67 / G 76	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 15	EMP175 /// 859446-67 // XAN105	AR	1	-	164	143	184, 195, 207	187
RAZ 38	EX-RICO 23 /// G12882	AR	1	168, 174	164	139, 143	184, 195, 207	187
RAZ 73	RAZ12-4 / XAN252	AR	1	-	164	-	184, 195, 207	187
RAZ 80	RAZ12-4 / XAN252	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 85	RAZ12-4 / XAN252	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 87	RAZ12-4 / XAN252	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 89	RAZ12-4 / XAN252	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 101	RAZ12-4 / XAN252	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 136	RAZ1 / AND885	AR	1	168, 172	164	143	184, 195, 207	187
RAZ 163	PIJAO /// G12882	AR	1	171, 178	164	139	184, 195, 207	187
RAZ 105	RAZ24-5 / AND885	R	1	171, 178	164	139	184, 195, 207	187
RAZ 24	859446-67 // G76	AR	1	171, 178	164	139	184, 195, 207	187

Table 3. Bruchid resistance and microsatellite alleles of Arcelin 5 containing breeding lines derived from Talamanca as recurrent parent.

Genotype	Genepool	Bruchid resistance	Allele	Marker y Molecular Weight				
				Clon41	BMd15	BMd26	BMy 11	Pv-atct01
G 02771 (ARC 5) - check		AR	5	169, 173	164	144	184, 203, 207	191
GG 564-2(3)	TALAMANCA/// G02771	R	5	-	166, 200	-	207, 242	191
GG 564-2(7)	TALAMANCA/// G02771	R	5	-	166, 200	-	184, 203, 207	191
GG 564-3	TALAMANCA/// G02771	R	1	170, 175	166	143	184, 203, 207	187
GG 564-4	TALAMANCA/// G02771	R	5	170, 175	166, 200	143	184, 203, 207	191
GG 564-6	TALAMANCA/// G02771	R	1	170, 175	166	-	184, 203, 207	187

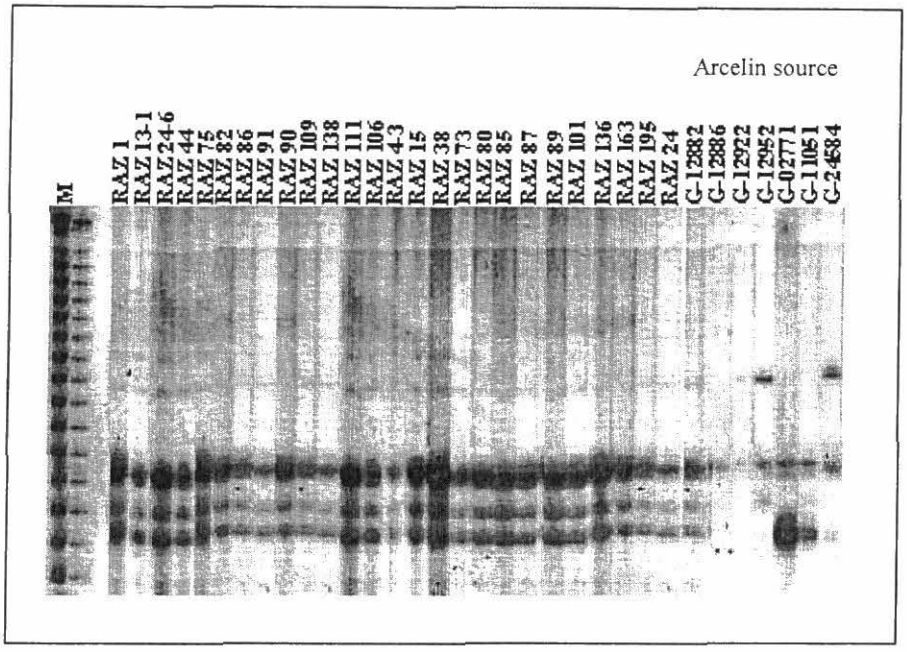

Figure 1. Microsatellite alleles at the BMy11 locus for seven sources of arcelin resistance and derived bruchid resistant RAZ lines.

Future work

Test the remaining two microsatellite markers in the region of the arcelin locus and improve the DNA extraction technique.

Determine the level of linkage disequilibrium between the markers and the arcelin locus in breeding populations.

Use the markers to select for greater recombination around the arcelin locus and break the linkage drag associated with this locus which has a negative affect on plant vigor of arcelin-derived lines.

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1.2.7 Tagging BGMV and CBB resistance in the population SEL1309 x DOR476

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Introduction

Common bacterial blight (CBB) is an important foliar and seed-borne disease of Andean beans grown in tropical lowland and mid-elevation areas of Africa, Central America and the Caribbean. The disease is also important in the subtropic and temperate regions of the Americas and Africa during hot, humid summer weather. The disease is caused by the pathogen *Xanthomonas campestris* pv. *Phaseoli* (Xcp), which is widespread and part of a complex of Xanthomonad bacterial pathogens attacking many broadleaf and vegetable crops. Bean golden yellow mosaic virus (BGYMV) is the most serious viral disease of beans in lowland tropical Latinamerica. BGMV is caused by a bipartite geminivirus that is transmitted by the whitefly, *Bemisia argentifolii / tabaci*. Red mottled beans are one of the few Andean types that are grown in low and mid-altitude areas of the region where the vector and virus are present. BGYMV is a well-established endemic problem in beans grown in the Caribbean (Cuba, Dominican Republic, Florida, Haiti and Puerto Rico), Central America (Costa Rica, Guatemala, El Salvador, Honduras and Nicaragua), Mexico (Sinaloa, Veracruz) and South America (Argentina, Brazil and Bolivia).

The objective of this work was to develop a genetic map for the cross SEL1309 x DOR476 and to undertake a QTL analysis to determine the genes controlling BGYMV and CBB resistance in these genotypes. A second objective was to find alternative markers for the SCARs that are presently in use for these diseases some of which segregate in this population.
The source of BGYMV resistance in DOR476 (pedigree DOR367 x (DOR364 x BAT1298)) is originally derived from a pyramid of resistance sources including DOR364 and DOR367 (pedigree A429 x RAO30). The A429 resistance source has been characterized to have a single recessive gene, *bgm-1*, which reduces mosaic symptoms. DOR364 is known to carry a QTL for BGYMV resistance on chromosome b04 that is derived from Porillo Sintetico, an early resistance source. Two molecular markers, both SCARs, have been used to select for BGYMV resistance. The first marker, called DOR21 is closely linked to *bgm-1*. This gene has been tagged but not mapped to a specific linkage group. Meanwhile the second SCAR marker, W12, is associated with the quantitative trait locus (QTL) for BGYMV resistance found on chromosome B04.

The source of the CBB resistance in SEL1309 (pedigree = ((BAT1579 x XAN159) x (A251 x APN18))) x (RAB489 x (A686 x G6385))) traces its CBB resistance back to XAN159 (pedigree ((G4449 x G10022) x G40020) x G4509) which was a product interspecific hybridization between common bean (*Phaseolus vulgaris* L.) and a tepary bean (*P. acutifolius* Gray) accession, G40020. XAN159 was also used to develop the most popular sources of CBB resistance for the tropics, the lines VAX3, 4, 5 and 6 that are being used for breeding at CIAT and elsewhere. A SCAR marker, SU91, has been developed that tags a major QTL for common bacterial blight resistance from XAN159 (Pedraza et al., 1997) that was tentatively mapped by Tar'an et al (2001).

Methodology

The recombinant inbred population was developed from the F2 of the cross between SEL1309 and DOR476 by single seed descent until the F5 generation. Subsequently the harvests for each of the 100 RILs were bulked until the F7 generation when they were tested in the greenhouse for CBB and BGYMV resistance. CBB was evaluated by bacterial inoculation. BGYMV resistance was evaluated by inoculation in the greenhouse with a mechanically transmissible strain. Data was collected for CBB on a 1 to 9 disease scale, where 1 = resistant and 9 = susceptible. Data for BGYMV was collected in the following categories: percent plants infected after inoculation (INF), severity on the same standard 1 to 9 scale as for above for overall symptoms (BGYMV), for leaf mosaic (MOS), for flower abortion (FAB), for pod deformation (DEF) and for dwarfing (DWF).

DNA was extracted from the RILs by the method of Afanador et al. (1998) and used for mapping experiments and for bulked segregant analysis. A total of 175 microsatellites developed in our laboratory were used to amplify the parents and the bulks of CBB or BGMV resistant or susceptible individuals. Of these, 37 were polymorphic for the parents and were amplified on all individuals in the population. In addition a total of 40 RAPD primers and 18 SCARs were amplified to generate banding patterns. The microsatellites were run on 4% polyacrylamide gels, while the RAPDs and SCARs were run on 1% agarose gels. Conditions of amplification are given in other sections of this and previous annual reports.

Results and Conclusions

A total of 160 polymorphic fragments (120 RAPDs, 5 SCARs and 37 microsatellites) were analyzed for the population. The SCARs that were polymorphic are listed in Table 1 and the parental survey and bulked segregant analysis carried out for these SCARs is shown in Figure 1. The parental survey detected size differences in the co-dominant markers SR2 and DOR21, the two markers for the bgm-1 gene, while the rest were dominant. Differences were detected between the CBB resistant and susceptible bulks that were consistent with the parent genotype for the CBB marker SU91, and for the BCMV and BGYMV markers, SW13 and SR2. In the case of the SAP6, one of the bulks amplified a band that was not present in either parent. Differences were detected between the BGYMV resistant and susceptible bulks that were consistent with the parent genotypes for the BGYMV marker SR2 and the BCMV marker SW13. The rust marker SF10 and the anthracnose marker SAB3 amplified in one of the bulks but in neither parent (data not shown).

Of the polymorphic fragments a total of 112 loci (77 RAPD, 3 SCARs, 33 microsatellites) could be placed on a framework map. The majority of microsatellites showed normal segregation and the level of heterozygosity was low (Figure 2). The resulting preliminary map covered all 11 chromosomes of the bean genome and had an average of 3.3 microsatellites and 7.0 RAPDs per chromosome. Chromosomes were identified based on the assignment of single copy microsatellites that had been previously mapped on other core mapping populations in our laboratory (DOR364 x G19833 or BAT93 x JaloEEP558) using the chromosome numbering system of Freyre et al (1998). Segregation distortion will be determined when the map is completed.

The SCAR marker SU91 tentatively identified chromosome 10. However, this identification is not complete given that we could not confirm this location with additional microsatellite markers from that chromosome. Other researchers have tentatively placed SU91 and the QTL for CBB that it tags on chromosome 8 (Miklas et al., 2001) so we will try to determine which designation

is correct. Of the other SCARs, ROC11 which tags the BCMV resistance gene, *bc-3*, could be mapped to the correct predicted location on chromosome 6, while R2, which tags the BGYMV resistance gene, *bgm-1*, could not be mapped.

Future Plans

- Identify the map location of the SCAR R2 and place the BGMV resistance gene, bgm-I, within the bean genome
- Saturate the map further with additional microsatellites.

Table 1. SCARs tested on the parents of the SEL1309 x DOR476 population.

Purpose	Marker	SEL1309	DOR476	Status
CBB	BAC6	-	-	Monomorphic
	SAP6	-	-	Monomorphic
	SU91	-	+	Polymorphic
	LG5	-	-	Monomorphic
	Phs	+(2B)	+(2B)	Monomorphic
	R7313	+(MC)	+(MC)	Monomorphic
	R4865	-	-	Monomorphic
BGMV	DOR21	+ (300)	+(270)	Polymorphic
	SR2	+(540)	+(570)	Polymorphic
	SW12	+	+	Monomorphic
BCMV	ROC11	+	-	Polymorphic
	SBD5	-	-	Monomorphic
	SW13	+/-	+	Polymorphic

Figure 1. SCARs tested on the parents and BGYMV and CBB resistant and susceptible bulks of the SEL1309 x DOR476 population.

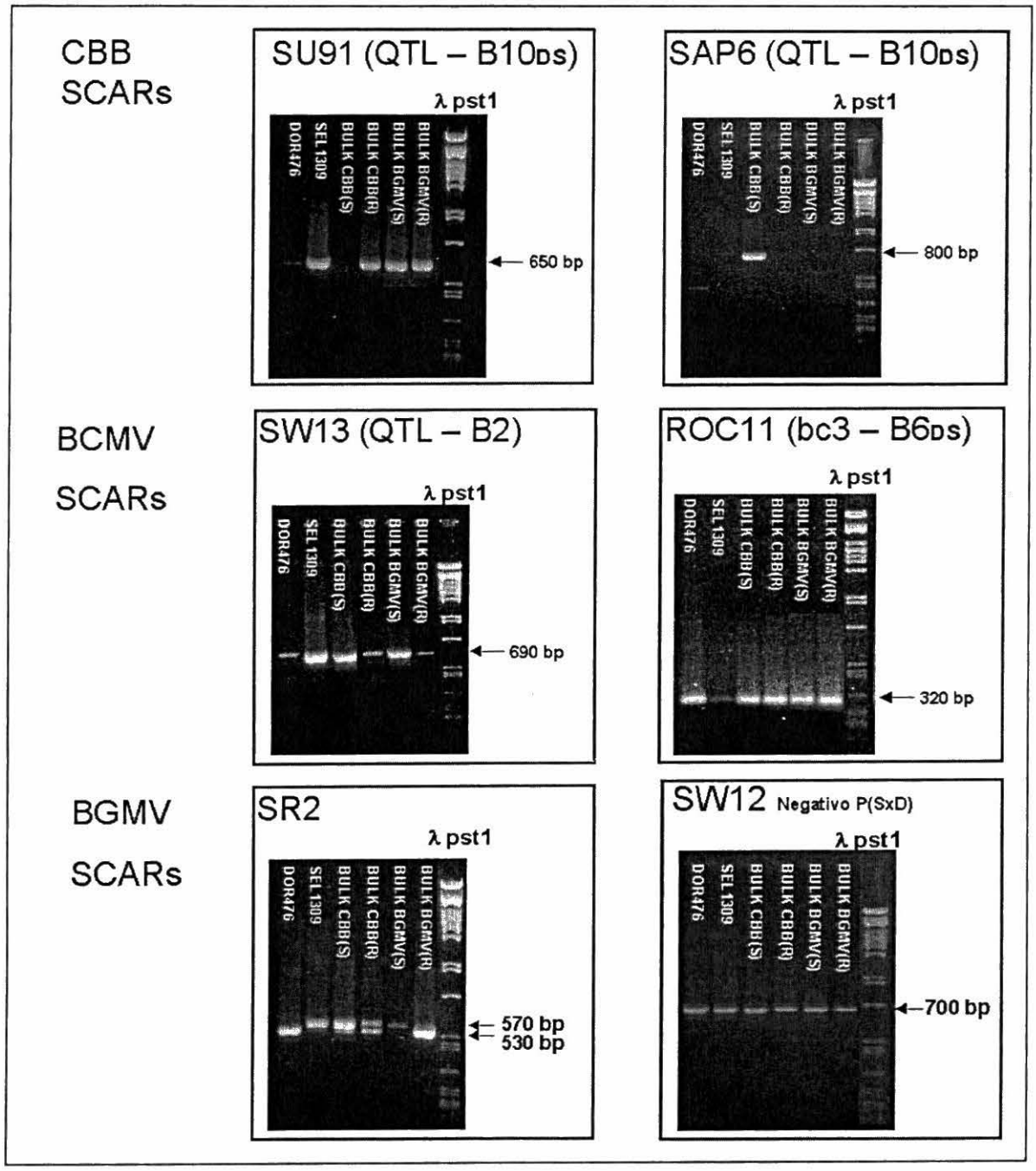
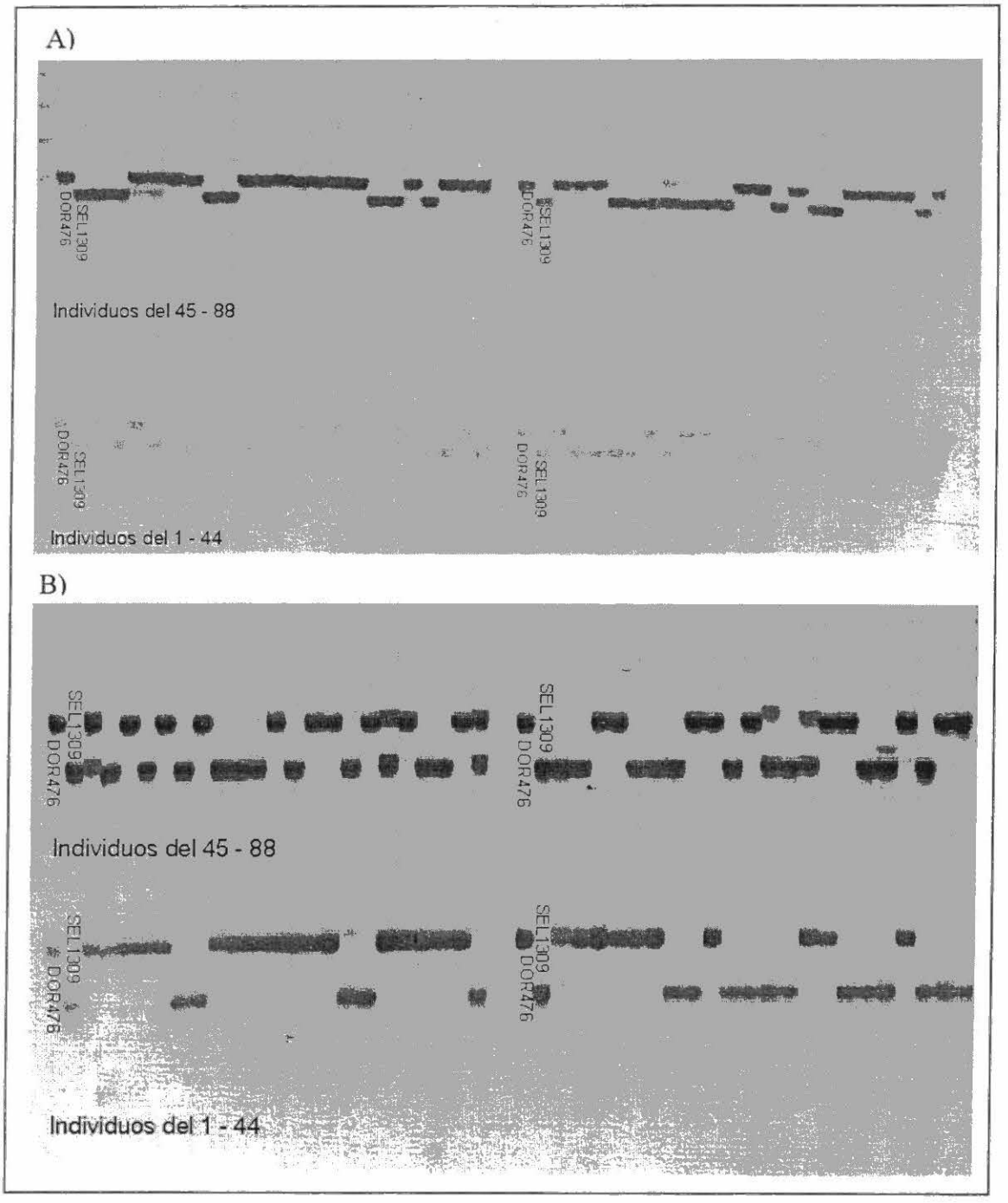


Figure 2. Microsatellite segregation in the population SEL1309 x DOR476 for two loci, (A) Clon012 and (B) Clon007.



1.2.8 Testing of a new molecular marker for BGMV resistance.

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Introduction

Large-scale marker assisted selection has been successfully carried out for a major gene conferring BGMV resistance, *bgm-1* (Beebe et al., 2002) but remains elusive for other genes that provide resistance, such as the quantitative trait locus (QTL) for BGMV resistance on chromosome 4. Although this QTL has been tagged with the W12 SCAR marker, the marker is difficult to use because of its lack of repeatability under the alkaline extraction techniques used in our laboratory. As a possible replacement for the W12 SCAR, we have identified a tightly linked microsatellite marker, J04555, that we were interested in testing for its ability to amplify DNA extracted by both the alkaline (high-throughput) and organic (high-quality) extraction techniques. Selection based on a co-dominant microsatellite marker compared to a dominant SCAR also represents a new challenge, therefore we were interested in analyzing the allelic diversity at the microsatellite locus in a range of BGMV resistant and susceptible breeding parents.

Methodology

A total of 146 varieties and advanced lines that are potential parents in the Mesoamerican and Andean breeding programs were used in this study. DNA was extracted by both the alkaline (high-throughput) and organic (high-quality) extraction techniques. The markers used were the microsatellite marker, J04555 (Yu et al., 1999) and the SCAR marker, W12 (Pedraza et al., 1998). The marker J04555 is equivalent to Pv-ctt001 (Yu et al., 2000). The genotypes ICTA Ligero, DOR364, G17341 and DOR500 were run as controls in every PCR plate and on every loading, since they have well-established BGMV reactions. DOR364 contains the W12-QTL, ICTA Ligero and DOR500 are known to carry the bgm-1 gene, and G17341 is a source of resistance. The SR2 SCAR marker (Singh et al., 2001) for the *bgm-1* gene was also amplified on the same set of parents.

Results and Discussion

In an initial experiment to compare the results between extraction techniques, both the microsatellite and the SCAR marker were amplified with DNA samples from both extraction technique for a set of 27 out of the 154 genotypes. The results were the same for both DNA samples, therefore for the remaining genotypes, both markers were amplified only with the lower quality alkaline extraction technique. Good amplification was obtained with the majority of genotypes despite the lower quality of the DNA template, however, it was noted that 43 genotypes did not amplify.

The microsatellite J04555 proved to be highly polymorphic and seven alleles (amplification products) were observed among the parents tested. Each of the genotypes used as a control had a different allele: ICTA Ligero (165 bp); DOR364 (159 bp); G17341 (157 bp); DOR500 (153bp). Of the other genotypes, a total of 10 had the ICTA Ligero allele, 32 had the DOR364 allele, 10 had the G17341 allele and 47 had the DOR500 allele. The alleles found in the small 'Mesoamerican' versus 'Andean' large seeded genotypes generally did not have different molecular weights. The fact that there was no apparent association between allele and genepool

may be because many of the 'Andean' genotypes were breeding lines with mixed ancestry that included genes from both genepools.
A total of 46 out of the 142 genotypes that were tested were positive for the W12 marker. A positive reaction was more common in those genotypes with the DOR364 or G17341 alleles of the microsatellite (84 and 70% of the time, respectively) than with the DOR500 or ICTA Ligero alleles of the microsatellite (11 and 10% of the time, respectively). This confirmed that there is probable linkage disequilibrium between the microsatellite and SCAR markers and therefore between the microsatellite and the BGYMV resistance gene underlying the QTL found on chromosome 4 where both markers are located.

Indeed, the DOR364 allele was in many of the genotypes which were positive for the W12 marker and which were bred for BGMV resistance, including A686, A800, BAT304. DICTA113, DOR476, DOR482, DOR557, DOR582, EAP9504-30B, ICA Pijao, ICTA Ostua, IPA7, MD23-24, NEB31, Negro Cotaxtla 91, Negro INIFAP, RAB609, SAM1, SAM3, Tio Canela, UPR9653-16, VAX4 and VAX5. This alleles was also present in genotypes that had been bred for BGMV resistance but which were negative for the W12 marker, such as the Carioca line A774 and the red mottled lines, RMC1, RMC3, RMC12, indicating that the microsatellite marker may be more predictive than even the SCAR. The G17341 allele was also found in a series of red mottled lines, RMC 2, 4, 5, 6, 7, 8, and 16, which were positive for W12 and were bred from pedigrees containing BGYMV resistant parents; as well as in VAX6 which was negative for the marker. The DOR500 allele was found in a series of A, EMP, FEB and RAB lines, some of which have BGYMV resistant parents in their pedigrees and other that do not. These results confirm the observation that the BGMV resistance in DOR390 and DOR500 does not come from incorporation of the W12 - J04555 chromosomal segment from DOR364 even though these are related by pedigree. It remains to be determined if the DOR500 microsatellite allele would represent a resistant allele of the W12 QTL and if there is an allelic series at the W12 QTL locus which has different grades of BGYMV resistance.

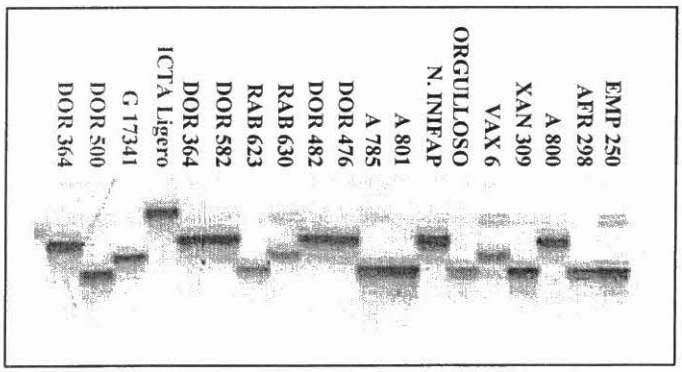

Figure 1. Sample of microsatellite alleles identified in 154 parents with the marker J04555 (Pv-ctt001).

Future Plans

- Test additional microsatellites from the region around W12 to determine if the same pattern of geneflow remains and associate microsatellite allele data with pedigree information.
- Improvements will be made in the high-throughput extraction technique given that there were individuals that did not amplify. In addition this extraction procedure DNA that

cannot be stored for a long period. Indeed we found that upon a repeat attempt to amplify the alkaline extraction DNA after a couple of weeks, it had degraded to the point of being un-amplifiable.

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1.2.9 Extension of genetic map for the cross DOR 364 x BAT 477 using microsatellites, to map genes for BNF

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Introduction

Abiotic stresses are a primary limitation on bean yields in the developing world. CIAT places high priority on developing germplasm that is tolerant to stresses including drought, low phosphorus and low soil N. CIAT participates in a project with the Catholic University of Leuven, the Center for Research on Nitrogen Fixation (CIFN), Cuernavaca, Mexico, and the Mexican national bean program of INIFAP, to develop both improved Rhizobium strains and improved bean germplasm with greater BNF capacity. One unique line, BAT 477, has been demonstrated to present superior BNF under both optimal and suboptimal conditions, including under P stress and drought stress. One object of the project is to confirm the value of QTL for BNF that were identified in greenhouse trials, to map the QTL in relation to the universal bean map, and to develop markers for Marker Assisted Selection (MAS). Maps based on locus-specific markers such as microsatellites are superior since they permit comparative mapping of traits. Furthermore, microsatellites are typically more stable and repeatable than many markers such as RAPDs, and can serve as a framework within which to map other markers such as RAPDs and AFLPs. The present exercise is designed to create such a framework that will greatly improve a RAPD map that already exists, and subsequently saturate it with AFLPs.

DNA extraction. Total DNA was extracted from the leaves of parental lines DOR 364 and BAT 477, and from the population of 132 Recombinant Inbred Lines (RILs) derived from the cross of the two parents. Young trifoliate leaves were collected in the field and in the screenhouse and subsequently macerated in liquid nitrogen. DNA was extracted from the ground leaves using the mini-prep method of Afanador et al. The DNA quality was observed by electroforesis in 0.8% agarose gels. Finally DNA was quantified in the flourometer and diluted to a concentration of 10 µl for the work with microsatellites.

Markers for map construction. Primers to amplify microsatellites were evaluated on the parental genotypes and those presenting polymorphism were then amplified on the RILs. Microsatellites were amplified by PCR under conditions that were specific for each primer pair; were separated by electrophoresis on polyacrilimide denaturing gels; and were visualized with silver staining.

A variable number of polymorphisms were detected among the parental genotypes using different random combinations of primers. AFLP were obtained by PCR and visualized on polyacrylimide gels stained with silver nitrate.

Results

Microsatellites. At present 166 microsatellite primers have been evaluated on the parental genotypes DOR 364 and BAT 477 of which approximately 21% (34 primers) have presented polymorphism . This low percentage is attributed to the close genetic relationship of these materials since both pertain to the Mesoamerican race of common bean. Among the 34 polymorphic microsatellites, 25 are co-dominant markers, which is to say, they amplify both alleles of a diallelic locus producing bands of different weight (Figure 6). The nine remaining microsatellites correspond to dominant markers, in which only one band is present from one of the two parents. Eighteen of these polymorphic microsatellites (53%) are of a genomic origin, which is to say, they amplify regions that contain both coding and non-coding sequences, while the other 16 (47%) are of genic origin and amplify coding regions. Nineteen microsatellites have been amplified and read on the 132 RILs, these corresponding to 15 co-dominant and 4 dominant markers.

AFLP markers. Six combinations of primers were evaluated on the parental genotypes revealing from 4 to 31 polymorphisms per primer combination. Combinations 4 (E-AGG + M-CAA) and 6 (E-ACA + M-CTC) produced the most bands, and can contribute to saturation of the linkage map of the RILs in the least time and work, and therefore will be employed initially with the RILs.

Linkage map. The linkage map of DOR 364 x BAT 477 contains at the moment 129 markers, of which 119 are RAPDs (Velasco, 1998), plus 10 microsatellites recently mapped on the RILs (Muñoz, 2002). The markers, with the exception of 21 that continue unlinked, are distributed in 15 linkage groups with a total distance of 655 cM. Seven of these groups could be associated with chromosomes thanks to the microsatellites that anchored them to the reference map.

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1.2.10 Marker-assisted selection for BGM resistance in common beans

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Introduction

Bean golden mosaic (BGM), which is caused by a geminivirus transmitted by the whitefly *Bemisia tabaci* (Gennadius), is a devastating disease of the common bean (*Phaseolus vulgaris* L.) in Latin America. The most important gene for conveying resistance to BGMV is the recessive gene *bgm-1*, whose SCAR marker is being used successfully in a MAS breeding scheme since 1999. Another SCAR marker (W12) appears to be linked to the QTL controlling BGMV resistance. This SCAR, which generates a 732-bp DNA fragment, was used in this study to achieve a higher level of resistance through MAS.

Materials and Methods

An alkali DNA extraction was performed as described previously, (CIAT, 2000 and 2001) and the PCR process and visualization of the *bgm-1* marker also remained the same, except that 384-well PCR microplates were used instead of the 96 wells, together with multichannel combs for loading the samples on agarose gels, in order to speed up the MAS process.

For the W12 SCAR, PCR conditions were as follows: each reaction contained 5 µl of the extracted DNA diluted 1:1 in sterile water, 0.2 mM of each dNTP, 0.2 µM of each forward and reverse primer, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase for a total volume of 25µl. The PCR profile was 35 cycles of a denaturation step at 94°C for 30 sec, an annealing step at 70°C for 30sec, and an extension step at 72°C for 1 min. PCR products were resolved in a 0.5X TBE agarose gel with ethidium bromide at a final concentration of 0.02µg/ml. The presence or absence of both SCAR markers was scored.

Results and Discussion

Two nurseries were screened for the presence of the *bgm-1* gene using DOR21 SCAR from Oct.-Dec. 2001. In total, 1241 red- and black-seeded F_5 families were planted in Darien to evaluate tolerance to low P, in Santander de Quilichao to determine resistance to ALS, and in Palmira for MAS for BGMV, where 776 plants with the *bgm-1* marker were selected and advanced to the next generation. A total of 286 F_3 elite populations for drought stress were also evaluated with DOR 21 and W12 SCARs. Results showed 204 carrying the *bgm-1* marker: 172 with the W12 SCAR and 123 with both. Four nurseries, selected on the basis of these results and seed quality, were sent to Nicaragua and Honduras. After advancing them to the F_5, the presence of *bgm-1* and W12 markers was confirmed. Tissue samples from 4 plants/line were bulked for DNA extraction and amplification of the aforementioned fragments. A total number of 552 lines (237 red seeded, 224 black seeded and 91 assorted seed color) were screened from June-Sept. 2002. Among them, 294 were found to have the *bgm-1* SCAR: 106, the W12 SCAR; and 64, both. These results were used to select nurseries that will be sent to NARS in Central America, prior confirmation of tolerance to ALS, CBB, ANT and low P.

In another trial (Jan.-Mar. 2002), 4369 individual plants from 46 F_1 multiple-cross segregant populations were screened. The 1611 red- and black-seeded lines having the *bgm-1* resistance allele were harvested and advanced. Among 127 F_3 bulks of crosses with *Rojo de Seda* for El Salvador, 103 were identified as having *bgm-1* SCAR. The high frequency of the resistance allele was explained because these populations had two resistant lines (RAB 630, obtained through MAS at CIAT, and 9824-47-1, bred in Puerto Rico) in their pedigree. Also 87 F_5 bulks of *Rojo de Seda* (backcrosses to DOR 364) were screened. The 20 identified as having the marker were advanced.

Continuing with MAS for the *bgm-1* gene, 37 segregant populations with red, black and cream-striped seeds (F_1 multiple crosses for drought stress and high Fe content) were evaluated. Among 2852 individual plants, 1761 having the *bgm-1* were harvested to continue with the MAS breeding scheme.

Conclusions

Although W12 SCAR was used for MAS purposes this year, high-throughput screening will be difficult to accomplish. On the one hand, it was sometimes difficult to amplify the fragment; on the other hand, spurious bands were seen. A new set of primers was designed (E. Gaitán pers. Comm) and will be tested in the DOR 364 x G19833 mapping population prior to large-scale screening.

Ongoing Activities

Of the 9514 plants screened for the presence of the *bgm-1* gene marker, 50% were selected for generation advancing in the breeding scheme.

Some other DNA extraction protocols are currently being assayed. Hopefully they will make it feasible to store stable DNA extracts longer than with the alkali extraction.

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CIAT (Centro Internacional de Agricultura Tropical). 2001. Annual report, Project SB-02: Assessing and utilizing agrobiodiversity through biotechnology. Cali, CO. p.

1.2.11 Diallel mating design were used to produce genetic information about the relative importance of additive, dominance and epistatic effects in different cassava traits.

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Introduction

An important aspect of the hybridizaton strategy executed last two years has been the production and planting of recombinant seed following an scheme of diallel crosses. Therefore, not only a

group of progenies with excellent agronomic performance was produced, but a genetic study without precedent for cassava was initiated. This diallelic analysis was harvested during the first semester of 2002 and produced information essential for understandin Ph.D. students: a woman from Vietnam and a man from Uganda.

The diallel trials for the North Coast (Table 1), Acid Soil Savannas (Table 2) and inter-Andean valleys (Table 3) were recently harvested and the data collected is thoroughly analyzed.

For scientific validity, a singular planting was carried out: from each crossing, 30 of the best F1 plants grown in CENICAÑA were selected to obtain at least eight stakes of excellent quality. Six of those stakes were used to plant the trials and the others were planted in nurseries to serve as seed source. The six stakes for the trials were distributed in three replicates located at two representative sites. Every F1 (with a few exceptions) from the diallel cross experiment were made up of 30 genetically different individuals conforming a full-sib family (both parents known and incommon). Each individual was represented in the trials by six plants, as mentioned above. Many of these families can provide the basis for molecular marker studies that will facilitate future work on cassava genetic improvement. It should be pointed out that in addition of the30 individual clones from each F1 cross, sister clones could also be found in the *Clonal Evaluation Trials*.

Table 1. Parents involved in the diallel experiment for the sub-humid environment of the North Coast of Colombia. The code assigned to each cross is above the diagonal[¶].

Parental Clones	CM 6754-8	CM 8027-3	SM 805-15	SM 1565-17	SM 1411- 5	SM 1219-9	SM 1657-12	SM 1665-2
Rayong 60	CM 9106	CM 9148	CM 9178	CM 9966	CM 9958	GM 266	GM 289	GM 291
CM 6754-8		CM 9921	CM 9945	CM 9907	CM 9954	GM 236	GM 237	GM 238
CM 8027-3			CM 9703	CM 9926	CM 9923	GM 246	GM 247	GM 248
SM 805-15				CM 9949	CM 9946	GM 250	GM 251	GM 252
SM1565-17					CM 9957	CM 9952	GM 280	GM 281
SM 1411-5						GM 255	GM 272	GM 273
SM 1219-9							GM 258	GM 259
SM 1657-12								GM 287

[¶] Only 30 plants were used to represent each F1 cross in the diallel study. Remaining plants from each cross were planted in an ordinary *Clonal Evaluation Trial*.

Table 2. Parents involved in the diallel experiment for the acid-soil savannas in the Eastern plains of Colombia. The code assigned to each cross is above the diagonal [¶].

Parental Clones	MTAI-8 Rayong 60	CM 7033-3	CM 4574-7	CM 6740-7	MPER-183	SM 1219-9	SM 1565-15	SM 2058-2	SM 2219-11
HMC-1	CM 8035	GM 244	GM 224	GM 234	CM 9733	GM 264	GM 277	GM 299	GM 303
MTAI-8		CM 9127	GM 226	GM 235	GM 307	GM 266	GM 279	GM 301	GM 305
CM 7033-3			GM 219	GM 227	GM 245	GM 240	-.-	GM 241	GM 243
CM 4574-7				CM 9460	GM 225	GM 220	GM 221	GM 222	GM 223
CM 6740-7					CM 9642	CM 9901	GM 229	GM 232	GM 233
MPER-183						GM 265	GM 278	GM 300	GM 304
SM 1219-9							GM 256	GM 261	GM 263
SM 1565-15								GM 275	GM 276
SM 2058- 2									GM 298

[¶] Only 30 plants were used to represent each F1 cross in the diallel study. Remaining plants from each cross were planted in an ordinary *Clonal Evaluation Trial*.

Table 3. Parents involved *in the diallel* experiment for the mid-altitude valleys of Colombia. The code assigned to each cross is above the diagonal [¶].

Parental Clones	SM 1219-9	SM 1741-1	SM 1278-2	SM 1636-24	SM 1673-10	HMC-1	MPER-183	MECU-72
CM 6740-7	CM 9901	CM 9903	GM 228	GM 230	GM 231	GM 234	CM 9642	GM 308
SM 1219-9		CM 9953	GM 254	GM 257	GM 260	GM 264	GM 265	GM 309
SM 1741-1			GM 269	GM 284	GM 292	GM 296	GM 297	GM 313
SM 1278-2				GM 267	GM 268	GM 270	GM 271	GM 310
SM 1636-24					GM 283	GM 285	GM 286	GM 311
SM 1673-10						GM 293	GM 294	GM 312
HMC-1							CM 9733	GM 314
MPER-183								GM 306

[¶] Only 30 plants were used to represent each F1 cross in the diallel study. Remaining plants from each cross were planted in an ordinary *Clonal Evaluation Trial.*

Selections for the Sub-Humid Tropical Environment

In Table 4 the averages (combined across the two locations where the trials were conducted) of the most important traits are presented.

From the breeding point of view the results from the diallel study from nine parents are equivalent to the *Clonal Evaluation Trial.* The only difference being that rather than having all the plants from a given clone planted in one-row plot, in the diallel experiment these plants were scattered in three replications at two locations. Also six rather than eight plants were used to represent each clone in the diallel. Best performing clones will also be included in *Preliminary Yield Trials.* The experiment was planted again this year.

The averages for each cross can be further consolidated to produce the averages of all the crosses involving a given parent, which are presented in Table 2.15. As it is frequently the case results from Table 5 demonstrate the difficulties in producing a perfect genotype. For instance the progenies of parent 5 (SM 1565-17) were outstanding yield wise and showed excellent reaction to trips. However, they also showed the lowest dry matter content in the entire experiment. Progenies from parent 6 (SM 1411-5) had a superior plant type (the lowest average score = 2.69), the highest dry matter content (28.42%), but low harvest index (0.51). It is clear that the results presented in Table 5 agree with those from Table 4 with the averages of each individual F1 family.

It should be remembered that results from Table 4 are averages across 30 clones making up each F1 family, and those from Table 5 grouping together the averages of all the progenies with a parent in common. The range of variation among individual clones is much larger. For instance, the highest average for yield in Table 4 was 45.68 t/ha (cross 1 x 9), but the highest yield by an individual clone was equivalent to 134 t/ha (Santo Tomás trial). When we perform the analysis of the segregation within each family (that is, among the 30 clones from each F1 family), some interesting results are expected to surface.

Selections for the Acid-Soil Savannas Environment

A second diallel study was conducted in the acid-soil savannas environment. The ten parents involved in this study were listed in Table 2. As for the diallel in the northern coast, each F1 cross

was represented by 30 clones, two environments were used, with three replications each. The trials were both planted in CORPOICA-La Libertad, but with two very contrasting soil conditions. The results of these trials are presented in Table 6 and 7, for the high- and low-fertility environments, respectively. The differences in soil fertility resulted in little development of foliar diseases in the trial with good soil fertility, but excellent disease pressure in the trial planted in soils with edaphic limitations. Likewise, productivity was very contrasting with average fresh-root yields of 28.31 and 12.10 t/ha, respectively. Two important diseases are found in the acid soil environments: bacterial blight or CBB (*Xanthomonas axonopodis* pv. *manihotis*) and the fungal super elongatio disease or SED (*Sphaceloma manihoticola*). All breeding activities for this ecological region, therefore,take particular attention to these biotic problems.

Table 4. Results of the diallel trials conducted in Pitalito y Santo Tomás (Atlántico). Each F1 cross was composed by 30 clones. Three replications per location were used.

Cross	Trips (1 - 5)	Stakes/pl. (Number)	Fresh roots (t/ha)	Harvest index (0 - 1)	Dry matter (%)	Plant Type (1 - 5)	Root Type (1 - 5)
1x2	3.03	8.98	34.93	0.54	27.45	3.18	2.89
1x3	2.70	10.58	26.51	0.43	29.47	2.97	3.21
1x4	2.27	11.23	31.45	0.45	28.78	2.95	2.92
1x5	1.63	11.87	42.29	0.57	26.45	2.66	2.78
1x6	1.75	12.29	36.51	0.47	29.10	2.64	3.01
1x7	1.91	12.13	42.35	0.55	28.14	2.71	2.82
1x8	2.72	8.68	38.14	0.55	26.27	3.35	3.18
1x9	2.93	10.55	45.68	0.56	27.00	3.11	2.79
2x3	2.40	9.26	32.82	0.54	29.05	2.84	2.86
2x4	2.63	9.88	27.69	0.47	27.05	3.13	3.18
2x5	2.04	11.66	35.48	0.55	26.64	2.59	2.97
2x6	2.23	10.67	37.98	0.53	28.26	2.80	2.78
2x7	2.28	11.40	34.76	0.52	27.64	2.68	2.94
2x8	2.86	9.08	31.63	0.52	28.19	3.29	3.11
2x9	2.68	9.38	36.25	0.52	28.12	2.99	2.85
3x4	2.47	10.50	34.22	0.49	28.57	2.82	2.94
3x5	1.65	9.87	40.99	0.59	27.07	3.00	2.84
3x6	2.22	11.47	38.90	0.49	29.23	2.57	2.92
3x7	1.95	11.24	39.37	0.54	28.59	2.76	2.89
3x8	2.61	11.05	34.77	0.49	28.36	3.00	3.08
3x9	2.59	9.49	41.16	0.59	27.74	2.99	2.94
4x5	2.04	9.40	37.29	0.56	25.29	3.23	3.09
4x6	2.21	10.88	35.59	0.51	28.38	2.74	2.95
4x7	1.73	11.20	34.01	0.46	27.93	2.97	3.15
4x8	3.12	8.44	35.53	0.53	27.74	3.45	3.10
4x9	2.93	9.27	31.49	0.51	27.20	2.99	3.20
5x6	1.64	11.95	40.98	0.52	28.00	2.69	2.83
5x7	1.36	12.38	42.59	0.54	25.66	2.71	2.98
5x8	1.97	9.48	35.96	0.57	26.81	3.19	3.02
5x9	1.94	11.32	40.65	0.58	24.74	2.82	2.90
6x7	1.76	12.76	37.49	0.46	28.07	2.76	3.01
6x8	2.44	11.11	38.58	0.51	27.93	2.82	3.08
6x9	2.29	10.62	41.28	0.56	28.40	2.51	2.74
7x8	1.94	11.48	42.87	0.54	26.36	2.74	2.79
7x9	1.58	10.64	39.48	0.52	27.98	2.69	2.99
8x9	2.57	10.25	42.70	0.55	26.89	3.04	2.83
Max.	3.12	12.76	45.68	0.59	29.47	3.45	3.21
Min.	1.36	8.44	26.51	0.43	24.74	2.51	2.74
Mean	2.25	10.62	37.23	0.52	27.63	2.90	2.96
St.Dev.	0.46	1.14	4.39	0.04	1.11	0.23	0.13

Table 5. Results of all the crosses with a common parent from the diallel trial conducted in two locations in the Sub-Humid environment (Santo Tomás y Pitalito. Atlántico).

Progenitor	Trips score (1 - 5)	Stakes per plant (Number	Fresh roots (t/ha)	Harvest Index (0 - 1)	Dry matter (%)	Plant type (1 - 5)	Root ype (1 - 5)
1=MTAI 8	2.37	).79	37.2	0.51	7.83	95	2.95
2=CM 6754-8	2.52	).04	33.9	0.52	7.80	94	2.95
3=CM 8027-3	2.32	).43	36.1	0.52	8.51	87	2.96
4=SM 805-15	2.42	).10	33.4	0.50	7.62	04	3.07
5=SM 1565-17	1.78	).99	39.5	0.56	6.33	86	2.93
6=SM 1411-5	2.07	1.47	38.4	0.51	8.42	69	2.92
7=SM 1219-9	1.81	1.65	39.1	0.52	7.54	75	2.95
8=SM 1657-12	2.53	95	37.5	0.53	7.32	11	3.03
9=SM 1665-2	2.44	).19	39.8	0.55	7.26	89	2.91
Maximum	2.53	1.65	39.8	0.56	8.51	11	3.07
Minimum	1.78	95	33.4	0.50	6.33	69	2.91
Mean	2.252	).623	37.2	0.524	7.626	899	2.961
St. Dev.	0.291	636	2.34	0.020	651	130	0.053

Looking at the best ten crosses (based on fresh-root yield) from Table 6, the parents that more frequently participated in these crosses were 7 (participating in five of those crosses) and 1 and 4 (related to four crosses each). Looking at the worst ten yielding crosses, frequent progenitors were number 5 and 8 (participating in four families each) and parent 10 (which produced three of these poor families). The average fresh-root production for the high-soil fertility trial was 28.31 t/ha, ranging from 37.51 (cross 7x10) down to 20.40 t/ha (cross 1x8). It must be remembered that these averages are coming from 30 plants in three replications (90 observations). Therefore an average of 37.51 t/ha is quite remarkable.

Results from the low-fertility trial (Table 7) are quite contrasting with the ones from the first environment. It is interesting to note how the soil fertility affected the capacity of the plants to protect themselves from the foliar diseases. It is well known that under good nutrition the plants can better react against biotic stresses, and that the contrary is also true. The two trials were less than a km away, so the differences in disease pressure can be reasonably explained by the soil fertility factor.

Some results are obvious from the summary presented in Table 6. Cross 8x9 had very little level of resistance to super-elongation disease (SED), with an average rating of 4.18. Cross 9x10 had also a high rating (4.12). One immediate conclusion is that, at least, parent 9 had very low levels of resistance to SED. On the other hand, progenitors 1 and 5 produced progenies with good reaction to the disease.

Table 6. Average of each F1-cross from the diallel study planted under high soil fertility conditions at CORPOICA-La Libertad (Villavicencio, Meta Department).

1 Cross	Plant height (cm)	lant type (1-5)	oot type (1-5)	ry matter (%)	Harvest Index.	FR yield (t/ha)
1x2	301.42	2.81	3.08	32.71	0.44	32.68
1x3	296.97	2.94	3.36	30.95	0.43	27.22
1x4	311.28	2.96	3.04	32.82	0.46	32.09
1x5	267.90	3.18	3.69	34.30	0.39	21.77
1x6	268.33	3.01	3.04	31.50	0.48	36.44
1x7	314.33	2.72	2.80	31.26	0.46	33.72
1x8	258.50	3.51	3.76	32.11	0.38	20.40
1x9	286.42	3.59	3.14	31.28	0.41	31.91
1x10	272.22	3.17	3.47	32.08	0.45	30.97
2x3	303.64	2.70	3.35	31.85	0.41	27.15
2x4	293.11	3.15	3.57	32.18	0.40	26.68
2x5	269.89	3.14	3.06	33.66	0.37	26.61
2x6	284.83	3.11	2.88	30.29	0.43	31.05
2x7	294.39	2.98	3.01	31.59	0.45	34.60
2x8	254.86	3.56	3.61	31.70	0.44	26.44
2x9	292.89	3.28	3.43	29.96	0.42	28.73
2x10	242.72	3.26	3.22	33.11	0.46	30.44
3x4	259.25	2.93	3.26	32.15	0.46	26.78
3x5	265.89	3.13	3.18	33.39	0.39	27.66
3x6	259.51	2.64	2.87	30.09	0.45	31.66
3x7	237.22	3.37	3.28	31.14	0.47	24.42
3x8	258.20	3.49	3.75	29.99	0.39	21.82
3x9	279.25	3.18	3.10	30.42	0.42	29.08
3x10	219.22	3.41	3.20	31.78	0.52	26.12
4x5	262.47	3.24	3.31	31.71	0.45	30.12
4x6	257.39	3.04	2.83	31.44	0.51	34.19
4x7	243.50	3.14	2.83	32.47	0.54	34.19
4x8	241.00	3.53	3.63	33.56	0.42	21.88
4x9	276.17	3.14	3.56	31.05	0.41	27.41
4x10	256.28	3.22	3.22	33.57	0.49	32.88
5x6	256.28	2.97	3.24	34.64	0.40	24.21
5x7	257.53	3.10	3.57	33.83	0.42	23.20
5x8	225.42	3.52	3.47	33.19	0.48	27.57
5x9	244.39	3.01	3.44	31.08	0.41	26.22
5x10	241.78	3.43	3.43	33.18	0.37	20.56
6x7	269.11	2.86	3.41	31.60	0.45	22.12
6x8	238.36	3.59	3.25	33.23	0.45	25.00
6x9	284.61	2.88	3.23	30.06	0.43	31.38
6x10	249.67	2.94	3.03	32.92	0.49	31.96
7x8	244.08	3.58	3.16	31.53	0.47	33.76
7x9	263.44	3.40	3.22	31.25	0.41	29.54
7x10	256.22	2.97	2.72	34.10	0.48	37.51
8x9	268.47	3.51	3.35	31.10	0.46	27.20
8x10	220.53	3.79	3.53	32.48	0.46	24.32
9x10	283.89	3.31	3.66	31.34	0.36	22.26
Maximum	314.33	3.79	3.76	34.64	0.54	37.51
Minimum	219.22	2.64	2.72	29.96	0.36	20.40
Mean.	265.17	3.19	3.27	32.04	0.44	28.31
St.Dev.	23.25	0.28	0.27	1.23	0.04	4.47

Table 7. Average of each F1-cross from the diallel study planted under low soil fertility conditions at CORPOICA-La Libertad (Villavicencio, Meta Department).

F1 cross	SED (1-5)	BB (1-5)	lant type (1-5)	ry matter (%)	Harvest Index (0-1)	FR yield (t/ha)
1x2	2.26	2.48	2.79	32.78	0.45	18.97
1x3	2.17	2.52	2.67	31.61	0.37	12.61
1x4	2.36	2.46	3.00	32.35	0.43	15.11
1x5	2.20	2.54	2.86	34.49	0.37	14.01
1x6	2.73	2.43	3.26	31.49	0.39	8.31
1x7	2.57	2.63	2.52	32.25	0.44	19.37
1x8	2.49	2.57	3.58	32.20	0.37	11.49
1x9	3.24	2.56	4.08	30.61	0.35	10.90
1x10	3.00	2.33	3.27	32.96	0.34	10.51
2x3	2.97	2.75	3.15	31.37	0.35	10.97
2x4	3.32	2.49	3.59	30.42	0.36	10.23
2x5	2.46	2.52	2.87	34.18	0.44	16.15
2x6	2.93	2.97	3.81	30.96	0.44	14.71
2x7	3.10	2.70	3.04	32.97	0.46	15.24
2x8	3.52	2.88	4.29	28.39	0.41	8.58
2x9	3.89	2.67	4.46	24.33	0.25	4.88
2x10	3.36	2.50	4.04	31.98	0.45	14.14
3x4	2.47	2.63	3.13	32.32	0.48	15.71
3x5	2.23	2.65	3.34	31.69	0.40	11.94
3x6	2.78	3.21	3.74	29.75	0.33	9.60
3x7	2.39	2.89	3.97	29.47	0.35	11.81
3x8	2.64	2.86	4.11	30.28	0.42	7.19
3x9	3.48	2.79	4.29	27.01	0.33	8.54
3x10	2.83	2.69	3.94	29.98	0.41	10.32
4x5	3.01	2.59	3.74	32.48	0.43	12.82
4x6	3.11	2.97	4.10	31.12	0.33	8.88
4x7	2.54	2.61	3.44	32.49	0.49	15.77
4x8	2.81	2.65	4.14	31.67	0.51	15.61
4x9	3.46	2.85	4.58	27.15	0.36	6.94
4x10	3.23	2.45	4.08	32.05	0.45	14.51
5x6	2.28	2.78	3.21	31.62	0.44	14.96
5x7	2.19	2.50	3.25	32.50	0.50	17.64
5x8	2.90	2.76	3.94	31.63	0.47	18.27
5x9	3.31	2.66	4.23	26.47	0.39	11.79
5x10	2.93	2.40	3.92	31.18	0.39	11.12
6x7	2.64	2.75	3.54	29.86	0.44	15.12
6x8	2.53	3.16	4.27	30.53	0.46	16.99
6x9	3.46	2.84	4.49	25.20	0.30	5.12
6x10	3.07	2.67	4.14	30.73	0.39	9.80
7x8	2.77	2.74	4.04	31.65	0.48	17.79
7x9	3.51	2.64	4.39	28.20	0.30	8.53
7x10	3.07	2.69	3.99	32.90	0.44	13.40
8x9	4.18	2.51	4.64	23.11	0.24	3.26
8x10	3.67	2.66	4.37	31.29	0.47	12.71
9x10	4.12	2.13	4.97	23.91	0.18	2.06
Maximum	4.18	3.21	4.97	34.49	0.51	19.37
Minimum	2.17	2.13	2.52	23.11	0.18	2.06
Mean	2.94	2.66	3.76	30.52	0.40	12.10
St.Dev.	0.52	0.20	0.59	2.65	0.07	4.15

In relation to dry matter content, parent 5 was outstanding in the high-fertility soil trial participating in seven of the 12 best crosses. Parent 10 was the second best, participating in four of the best F1 crosses for this trait. On the other hand, dry matter content under low soil fertility favored crosses from parent 1 (five F1 crosses out of the 12 best); followed by parent 4 (participating in four outstanding crosses); and parents 2, 5 and 7 (with three families each). It is possible that the relatively better performance for the progenies from parent 1, under low-soil fertility roots in its outstanding reaction to diseases. If that were the case, this would be another evidence of how adaptation to a particular environment [in this case defined by the prevalent foliar diseases] results in higher dry matter content in the roots.

Table 8. Averages of the nine F1-cross families produced by each of the ten parents evaluated in the diallel experiments conducted at CORPOICA – La Libertad (Villavicencio, Meta Department). For each parent, the first line presents the result of the low-fertility trial, and the second line for the high fertility conditions.

Progenitor	SED. (1 - 5)	CBB (1 - 5)	Plant type (1 - 5)	Root type (1 - 5)	Dry matter (%)	Harvest Index (0 - 1)	Fresh roots (t/ha)
1	2.56	2.50	3.11	3.45	32.30	0.39	13.47
CM 4574-7	-.-	-.-	3.10	3.26	32.11	0.43	29.69
2	3.09	2.66	3.56	3.68	30.82	0.40	12.65
CM 6740-7	-.-	-.-	3.11	3.25	31.89	0.42	29.38
3	2.66	2.78	3.59	3.68	30.39	0.38	10.96
CM 7033-3	-.-	-.-	3.09	3.26	31.31	0.44	26.88
4	2.92	2.63	3.76	3.61	31.34	0.43	12.84
SM 1219-9	-.-	-.-	3.15	3.25	32.33	0.46	29.58
5	2.61	2.60	3.49	3.34	31.80	0.43	14.30
SM 1565-15	-.-	-.-	3.19	3.38	33.22	0.41	25.32
6	2.84	2.86	3.84	3.68	30.14	0.39	11.50
SM 2058-2	-.-	-.-	3.00	3.09	31.75	0.45	29.78
7	2.76	2.68	3.57	3.43	31.37	0.43	14.96
SM 2219-11	-.-	-.-	3.12	3.11	32.08	0.46	30.34
8	3.06	2.75	4.15	3.69	30.08	0.43	12.43
HMC 1	-.-	-.-	3.57	3.50	32.10	0.44	25.38
9	3.63	2.63	4.46	4.25	26.22	0.30	6.89
MPER 183	-.-	-.-	3.26	3.35	30.84	0.41	28.19
10	3.25	2.50	4.08	3.76	30.78	0.39	10.95
MTAI 8	-.-	-.-	3.28	3.28	32.73	0.45	28.56

Parent 4 produced families with excellent harvest indexes in the two environments where the diallel trials were planted. For the same trait parent 10 produced good progenies in the low-fertility field and parent 7 in the high-fertility trial. Parent 9 produced five of the worst families for harvest index in the low-fertility trial, and parent 5 in the high-fertility conditions. This last observation is not surprising because parent 5 (SM 1565-15) is particularly well adapted to the savanna conditions where it shows excellent canopy development. When progenies from this clone are grown in high fertility soils the canopy development may prove to be too excessive, resulting in low harvest index.

For plant type, progenitors 1, 2, 3, 6, and 7 produced the best progenies in the high-fertility trial. In the low-fertility conditions, on the other hand, parent 1, followed by parents 2 and 5 had a superior performance based on the results of their progenies. The good level of resistance to SED found in parent 5 may have contributed to the good rating for plant type, as well.

The information from Tables 6 and 7 has been consolidated in Table 8 where the averages of the nine F1 crosses derived from each of the ten progenitors is presented, individually for each environment. Each average is based theoretically on 810 data points (nine crosses, 30 genotypes per cross, and three replications). Therefore, these figures are very solid and reliable.

For resistance to SED, parents 1 (CM 4574-7), 5 (SM 1565-15), and 3 (CM 7033-3) showed the best reaction (low score). On the other hand, parents 8 (HMC-1), 2 (CM 6740-7), 10 (MTAI 8),

and 9 (MPER 183) were mediocre based on the reaction to the disease of their progenies. In was a surprise to find out that the progenies from MTAI 8 presented good levels of reaction to the bacterial blight (CBB), with a score of 2.50 also found in the first parent (CM 4574-7). M TAI 8 was developed in Thailand (where it was named Rayong 60) and it was not known to have resistance to the disease. This situation may be due to some sort of recessive resistance in MTAI 8 (already suggested in the literature) or else, because the levels of CBB were not has high as for SED, therefore, resulting in misleading results. Progenies from SM 2058-2 presented high ratings for CBB, suggesting that this clone is very susceptible to the disease. This would also support what was concluded from the data in Table 7 where, five of the worst crosses for CBB included SM 2058-2 as one of the progenitors.

Progenitors 7 (SM 2219), 5 (SM 1656-15), and 1 (CM 4574-7) produced the progenies with highest average productivity in the low-environment trial. MPER 183, on the other hand, produced progenies with very low root productivity (almost half as much as those from the previously mentioned parents). Poor performances, in the low-fertility trial, were shown by the progenies of MTAI 8 and CM 7033-3. In the high-fertility conditions the progenies from SM 2219-11 showed the highest root-productivity, followed by those from CM 4574-7, SM 1219-9, and CM 6740-7. In this environment the progenies from SM 1565-15, HMC-1 snd CM 7033-3 yielded poorly.

Taking advantage of the huge volume of information from these diallel trials the phenotypic correlations shown in Table 9 were obtained. Very high correlations with root productivity were found for root aspect score (ρ= -0.94), harvest index (ρ= 0.88), dry matter content (ρ= 0.80), reaction to SED (ρ= -0.73), and plant type score (ρ= -0.64). Negative correlations here are due to the fact that in the scores 1= excellent and 5=very poor performance.

Table 9. Phenotypic correlations measured in the low-fertility diallel trial conducted at CORPOICA-La Libertad (Villavicencio, Meta Department). Correlations were estimated using averages for each family across the three replications.

	CBB (1-5)	Plant type (1-5)	Root type (1-5)	Fresh root yield (t/ha)	Dry matter (%)	Harvest index (0-1)
SED (1-5)	-0.10	0.77	0.77	-0.73	-0.74	-0.58
CBB (1-5)		0.20	0.01	0.07	-0.10	0.13
Plant type			0.69	-0.64	-0.73	-0.42
Root type				-0.94	-0.82	-0.83
FR yield					0.80	0.88
Dry matter						0.73

It is also worth mentioning the little association between reaction to CBB and SED. Although there seems to be a negative association (ρ= -0.10), the relationship is weak enough to suggest that it is feasible to obtain genotypes with good levels of reaction to both disease, that is that the traits are likely to be independently inherited and controlled.

Table 10. Average for the crosses (based on 30 clones) from a 9-parent diallel conducted at CIAT-PALMIRA.

Cross	Mites (1 to 5)	Plant Type (1 to 5)	Harvest Index (0 to 1)	Dry matter (%)	Fresh roots (t/ha)
1x2	3.34	3.15	0.55	35.29	50.88
1x3	3.07	3.18	0.50	35.92	36.17
1x4	3.09	3.22	0.47	36.15	46.15
1x5	3.83	3.59	0.54	36.11	36.63
1x6	3.13	2.94	0.54	36.03	46.65
1x7	3.85	3.11	0.54	34.25	41.02
1x8	2.63	2.39	0.44	36.19	56.16
1x9	3.70	3.24	0.46	37.17	34.81
2x3	3.90	3.45	0.55	35.55	40.58
2x4	3.69	3.26	0.54	33.80	37.84
2x5	3.56	3.19	0.58	36.35	45.41
2x6	3.92	3.15	0.54	35.67	43.28
2x7	4.17	3.32	0.55	35.46	44.64
2x8	2.69	2.61	0.52	34.32	61.72
2x9	3.71	2.91	0.53	34.74	64.74
3x4	4.36	3.23	0.49	34.38	41.66
3x5	3.75	3.35	0.52	36.11	31.39
3x6	3.80	3.24	0.59	35.39	41.01
3x7	4.20	3.52	0.52	34.24	34.04
3x8	3.24	3.09	0.46	34.97	45.12
3x9	4.04	2.99	0.49	34.05	48.98
4x5	3.95	3.52	0.50	36.44	36.06
4x6	3.79	2.87	0.50	36.34	44.74
4x7	4.15	4.11	0.48	33.34	29.85
4x8	2.98	2.92	0.43	35.72	49.00
4x9	3.90	3.08	0.52	34.70	61.58
5x6	3.86	3.29	0.62	37.46	51.56
5x7	4.27	3.66	0.52	34.96	37.10
5x8	2.75	3.08	0.46	35.66	50.22
5x9	4.06	3.43	0.50	33.89	46.20
6x7	4.01	3.30	0.57	35.68	42.99
6x8	2.66	2.84	0.52	35.00	44.47
6x9	3.71	2.69	0.58	35.19	57.94
7x8	3.67	3.08	0.47	34.04	47.64
7x9	4.23	3.11	0.54	33.97	47.63
8x9	2.59	2.76	0.43	33.40	52.79
Maximum	4.36	4.11	0.62	37.46	64.74
Minimum	2.59	2.39	0.43	33.34	29.85
Mean	3.62	3.16	0.52	35.22	45.24
St.Dev.	0.52	0.32	0.05	1.03	8.45

Table 11. Averages of all the progenies from each of the parents included in the diallel study conducted at CIAT-PALMIRA.

Progenitor	Mites (1 to 5)	Plant Type (1 to 5)	Harvest Index (0 to 1)	Dry matter (%)	Fresh roots (t/ha)
1= CM 6740-7	3.33	3.10	0.51	35.89	43.56
2 = SM 1219-9	3.62	3.13	0.55	35.15	48.63
3 = SM 1278-2	3.80	3.26	0.52	35.08	39.87
4 = SM 1636-24	3.74	3.28	0.49	35.11	43.36
5 = SM 1673-10	3.75	3.39	0.53	35.87	41.82
6 = SM 1741-1	3.61	3.04	0.56	35.84	46.58
7 = HMC 1	4.07	3.40	0.52	34.49	40.62
8 = MECU 72	2.90	2.85	0.47	34.91	50.89
9 = MPER 183	3.74	3.02	0.51	34.64	51.83
Maximum	4.07	3.40	0.56	35.89	51.83
Minimum	2.90	2.85	0.47	34.49	39.87
Average	3.62	3.16	0.52	35.22	45.24

Selections for the Mid-altitude valleys

As was the case for the sub-humid and acid-soil savannas environments, a dialed study was also conducted for the mid-altitude valleys. A set of nine parents was used. As in the other diallels, 30 clones represented each F1 cross. Two locations with three replications in eache were used to obtain the data. Results from the evaluation at CIAT-Palmira are presented in Tables 10 and 11, and those from AGROVELEZ-JAMUNDI are in Tables 13 and 14. The same variables are presented for the diallel studies at the two locations, with the exception of scoring for mites at CIAT-PALMIRA and for white flies at AGROVELEZ-JAMUNDI. The fact that no reliable data for white flies could be taken at CIAT-PALMIRA reflects the success of the measures taken to control this problem. In effect, white flies pressure at CIAT's experimental station in Palmira was very low during the reported period because of the interruption of cassava planting for one month every year. This measure disrupts white flies cycle and has resulted in a significant reduction of this problem in our plantings at this station.

Table 10 shows the averages for each F1 cross in the diallel at Palmira. The average fresh root yield was 45 t/ha is excellent, considering that it involves 1080 new genotypes. Averages for F1 families ranged from 29.85 to 64.74 t/ha. Best yields were observed for crosses 2x9, 2x8, and 4x9, with mean yields of 64.74, 61.72, and 61.58 t/ha, respectively. Similarly, these crosses presented the highest dry matter yields exceeding 21 t/ha. High dry matter content in the roots was observed from crosses involving parents 5 (SM 1673-10) and 6 (SM 1741-1).

Results from the diallel at Palmira were consolidated to obtain the averages for the progenies of each progenitor (Table 11). The best two parents for fresh root yield were MPER 183 and MECU 72 with average yields above 50 t/ha. This revelas the advantages of having the access to materials from the germplasm bank (both progenitors are landaraces from Peru and Ecuador, respectively). In addition, MECU 72, is not only a valuable source of resistance to white flies, but also posses good levels of resistance to mites. SM 1278-2 and HMC-1 were the worse progenitors based on the average root productivity of their progenies.

Table 12. Average for the crosses (based on 30 clones) from a 9-parent diallel conducted at AGROVELEZ-JAMUNDI.

Cross	White flies (1 to 5)	Plant Type (1 to 5)	Harvest Index (0 to 1)	Dry matter (%)	Fresh roots (t/ha)
1x2	3.07	3.54	0.40	29.95	50.43
1x3	2.56	3.31	0.42	35.02	49.12
1x4	2.71	2.89	0.42	31.60	48.35
1x5	2.94	3.08	0.40	32.93	49.29
1x6	3.09	3.06	0.45	33.30	53.45
1x7	3.18	3.21	0.47	32.62	55.94
1x8	1.73	3.19	0.38	30.34	50.71
1x9	2.97	2.92	0.38	33.69	41.26
2x3	2.87	3.18	0.50	33.50	56.47
2x4	3.38	3.34	0.35	27.95	35.46
2x5	2.65	3.23	0.46	32.38	52.17
2x6	3.83	2.99	0.46	34.15	46.20
2x7	2.99	3.12	0.41	31.00	44.95
2x8	1.61	3.48	0.41	28.61	59.26
2x9	2.67	2.69	0.42	31.27	60.18
3x4	4.13	3.04	0.44	32.81	42.96
3x5	3.37	3.08	0.42	33.85	40.26
3x6	3.75	3.29	0.41	34.51	37.60
3x7	3.46	3.15	0.46	34.00	39.27
3x8	2.06	2.97	0.39	32.89	46.92
3x9	3.11	3.15	0.39	32.48	49.91
4x5	3.00	3.08	0.37	32.49	41.54
4x6	3.60	3.22	0.38	32.92	43.76
4x7	3.47	3.26	0.38	29.74	31.38
4x8	2.00	3.12	0.35	31.20	50.07
4x9	3.67	2.84	0.43	31.04	57.41
5x6	3.48	3.13	0.52	35.15	56.92
5x7	3.21	3.05	0.44	32.66	43.09
5x8	1.69	3.18	0.36	30.44	42.48
5x9	3.24	3.08	0.37	30.30	42.58
6x7	3.57	3.23	0.48	33.55	47.80
6x8	3.02	3.34	0.37	30.28	38.42
6x9	3.73	2.81	0.44	32.41	56.67
7x8	2.05	3.35	0.41	30.88	52.76
7x9	3.45	2.84	0.46	31.44	64.42
8x9	1.82	2.88	0.34	28.89	46.87
Máximo	4.13	3.54	0.52	35.15	64.42
Mínimo	1.61	2.69	0.34	27.95	31.38
Promedio	2.98	3.12	0.41	32.01	47.95
Desv. Est.	0.66	0.19	0.04	1.80	7.59

SM 1673-10, CM 6740-7, and SM 1741-1 produced progenies with higher dry matter content based on the results from Palmira, whereas MPER 183, MECU 72, and HMC-1 generated progenies with low dry matter in their roots. HMC-1 also was characterized by progenies with higher susceptibility to mites (Table 12). The results so far analyzed from the diallel study illustrate, once amore, the difficulties in combining in one genotype desirable traits. For instance, MPER 183 has excellent levels of root productivity but very low dry matter contents. On the other hand, CM 6740-7 has high dry matter content, but its productivity was not as outstanding. HMC-1 also has high dry matter content, but low root productivity and clear susceptibility to mites.

The best crosses for dry-matter yield were 2x9 (22.49 t/ha), 4x9 (21.37 t/ha), 6x9 (20.39 t/ha), and 1x8 and 2x8 (both with 20.32 t/ha). Parent 9 is in three of these five crosses.

Results from the diallel Jamundí are presented in Tables 12 and 13. Although there was severe pressure from white flies, average fresh root yield were higher than at Palmira (47.95 versus 45.24 t/ha). Root dry-matter content in Jamundí, however, was lower than at Palmira (32.01 versus 35.22 %). Best crosses for fresh root production at Jamundí were 7x9, 2x9, and 2x8.with 64.4, 60.2, and 59.3 t/ha, respectively. Crosses 2x9 and 2x8 also had been among the highest yielding F1-cross families at Palmira.

Data from Table 13 suggest again a good performance of the progenies from MPER 183 with the highest levels of average fresh root production (52.41 t/ha), followed by SM 1219-9 (50,64 t/ha) and CM 6740-7 (49.82 t/ha). In relation to dry-matter contents, the progenies from SM 1278-2 and SM 1741-1 were the only to average above 33 %. As was observed in Palmira, the progenies from MPER 183 and MECU 72 had low dry matter contents (31.44 and 30.44 %, respectively).

SM 1741-1 proved to be susceptible to white flies (average score 3.51) and as expected, MECU 72 produced progenies with excellent reaction to this insect (average score 2.00). The progenies from CM 6740-7 and SM 1219-9 showed good levels of resistance to white flies (average scores < 3.00).

The highest dry-matter yields observed at Jamundí were from crosses 7x9 (20.25 t/ha), 5x6 (20.01 t/ha), 2x3 (18.92 t/ha), 2x9 (18.82 t/ha), and 6x9 (18.37 t/ha). Two of these five families of crosses (2x9 and 6x9), also were among the highest yielding materials at Palmira.

The problem of combining different desirable traits into one genotype is one of the difficulties frequently encountered by plant breeders. A second other limitation occurs when clones with outstanding performance in one location have a very poor one in a second location, resulting in high genotype by environment interactions. Table 14 shows the correlations for several variables measured at Palmira and Jamundí. Correlations are based on the average for each F1 cross (across three replications of each location). Fresh root at both locations showed a good correlation of 0. 68. In general all correlations were high (> 0.50) with the exception of plant type ($\rho = 0.05$) and number of commercial roots ($\rho = 0.28$). It is possible that the low correlation for plant type is a result of the two different insect pests (mites in Palmira and white flies in Jamundí) affecting the trials. The reaction to these pests has a strong effect on the plant aspect variable as well.

A last result of the diallel study is presented in Table 15, where phenotypic correlations among variables at each location are summarized.

Table 13. Averages of all the progenies from each of the parents included in the diallel study conducted at AGROVELEZ-JAMUNDI.

Progenitor	White flies (1 to 5)	Plant Type (1 to 5)	Harvest Index (0 to 1)	Dry matter (%)	Fresh roots (t/ha)
CM 6740-7	2.78	3.15	0.41	32.43	49.82
SM 1219-9	2.88	3.20	0.42	31.10	50.64
SM 1278-2	3.16	3.15	0.43	33.63	45.31
SM 1636-24	3.24	3.10	0.39	31.22	43.87
SM 1673-10	2.95	3.11	0.42	32.52	46.04
SM 1741-1	3.51	3.13	0.44	33.28	47.60
HMC 1	3.17	3.15	0.44	31.99	47.45
MECU 72	2.00	3.19	0.38	30.44	48.44
MPER 183	3.08	2.90	0.40	31.44	52.41
Máximo	3.51	3.20	0.44	33.63	52.41
Mínimo	2.00	2.90	0.38	30.44	43.87
Promedio	2.98	3.12	0.41	32.01	47.95

Table 14. Correlations for measurements taken at CIAT-PALMIRA and AGROVELEZ-JAMUNDI based on the F1-cross family averages.

Plant Type 1-5	Root type 1-5	Root color 1-5	Pulp color 1-9	commercial roots (No.)	Fresh foliage (kg/pl)	Dry matter (%)	Harvest index (0-1)	Fresh root Yield (t/ha)
0.05	0.53	0.97	0.91	0.28	0.81	0.57	0.69	0.68

Table 15. Phenotypic correlacions among variables measured in the diallel studies at AGROVELEZ-JAMUNDI (above diagonal) and CIAT-PALMIRA (below diagonal), using the averages of the 36 F1 families.

	Mites Palmira	Plant type	Root type	Root Color	Pulp Color	Commer. roots	Foliage (kg/pl)	Dry matter %	Harvest index	Fresh root Yield
White Flies	-.-	-0.15	-0.04	0.56	0.06	-0.13	-0.59	0.43	0.46	-0.20
Plant type	0.65	-.-	0.50	-0.05	-0.14	-0.32	-0.29	-0.18	-0.11	-0.27
Root type	0.13	0.51	-.-	-0.25	0.25	-0.81	-0.31	-0.26	-0.61	-0.80
Root color	0.63	0.39	0.10	-.-	-0.50	0.17	-0.30	0.10	0.43	0.10
Pulp color	-0.01	0.02	-0.24	-0.48	-.-	-0.13	-0.13	0.09	-0.17	-0.28
Comm. Roots	-0.37	-0.81	-0.57	-0.09	-0.26	-.-	0.36	0.24	0.57	0.85
Foliage kg/pta	-0.61	-0.71	-0.23	-0.49	-0.02	0.65	-.-	-0.35	-0.35	0.56
Dry matter	-0.14	-0.05	-0.27	-0.19	0.38	-0.08	-0.17	-.-	0.62	0.05
Harvest index	0.41	0.17	-0.33	0.61	-0.17	0.08	-0.63	0.20	-.-	0.48
FR Yield.	-0.39	-0.74	-0.51	-0.07	-0.22	0.92	0.74	-0.10	0.03	-.-

At Palmira yield and reaction to mites had a correlation = -0.39, whereas at Jamundí the correlation between white flies and fresh root yield was –0.20. These values were negative because a small insect damage is qualified as 1, and a severed damage with a score of 5. The correlation between yield and plant type was much higher at Palmira (ρ = -0.74) than at Jamundí (ρ = -0.27). Plant type at Jamundí was difficult to score because of the high soil fertility resulting in frequent lodging in several families. Also worth mentioning is the contrast in the correlations between harvest index and fresh root yield at Palmira (ρ = -0.03) and (ρ = 0.48).

It was not possible to establish a clear relationship between fresh root yield and dry matter content in the roots. Therefore, it should be possible to eventually generate high yielding clones with high dry-matter content in their roots. The fact that correlations are not necessarily evidence of a cause – effect relationship is supported by the meaningless correlations between root color and harvest index (ρ = 0.63 and 0.43) or between root color a reaction to insects (ρ = 0.56 and 0.63).

Conclusions

The diallel studies represet an important effort invested in producing valuable information about the genetics of agronomically important traits in cassava. They also provide a large segregation of materials that could eventually be selected as elite clones. Also, because within-family data is available the studies offer ideal populations that may be used for a more precise measurement of how a given trait segregates.

The studies have also been used by Ph.D. students from Vietnam and Uganda. Because of the large amount of information that needs to be processed the wihin-family variation has not yet been analyzed.

1.2.12 Marker-Assisted Selection (MAS) for breeding for resistance to the Cassava Mosaic Disease (CMD) at CIAT

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Introduction

The presence of cassava mosaic disease (CMD), the most important production constraint in Africa, and its absence in Latin America limits the usefulness of CIAT cassava germplasm in Africa and India. With the discovery of a dominant CMD resistance gene, *CMD2*, and 3 molecular markers tightly associated with it, it is not possible to breed for CMD resistance at CIAT. A pilot experiment was set up together with IITA in 2000, as a proof of concept of the utility of molecular markers in CMD resistance breeding. Six crosses, and reciprocals, were made between TME3 and TME9, two cassava land races from Nigeria that carry *CMD2*, and: a susceptible Nigerian land race and 2 elite cassava varieties from IITA, one tolerant and the other susceptible to CMD. We describe here findings of that experiment.

A second phase of molecular breeding for CMD resistance breeding at CIAT has also been initiated. CMD resistant progenies derived from TME3 that were obtained from IITA in 2000 were crossed to elite parents of CIAT's cassava gene pools, and to high carotene or high protein content genotypes. Seeds harvested from the crosses were germinated *in vitro* from embryo axes

in preparation for MAS and to permit sharing of the CMD resistant genotypes with collaborators in Africa and India. Thes plants will also be the basis of breeding for CMD resistance in CIAT cassava gene pools..

Methodology

The MAS crosses were made in 2000 and a seedling nursery was established at the IITA Mokwa sub-station, a low CMD pressure site in Nigeria, in 2002. The crosses were harvested December last year and re-established as a clonal observation trail at the IITA headquarters in Ibadan, a high CMD pressure area. Table 1 summarizes the families that are in the clonal observation trial. The plants were evaluated at 3, 4 and 6 months after planting for resistance to CMD.

Table 1 Crosses from TME3 and TME 9 planted in IITA for to test markers associated with *CMD2* for molecular marker-assisted selection

Family name	Female	Male	Seeds harvested	Plants in field	Total plants in field
M1	TME 3	TME 117	36	18	
M2	TME 117	TME 3	220	95	113
M5	TME 3	91934	103	49	
M6	91934	TME 3	60	12	61
M7	TME 3	30572	70	49	
M8	30572	TME 3	846	791	840
M17	TME 9	TME 117	368	309	
M18	TME 117	TME 9	174	107	416
M21	TME 9	91934	370	282	
M22	91934	TME 9	27	12	294
M23	TME 9	30572	264	214	
M24	30572	TME 9	700	552	766
				Grand Total	2490

DNA was isolated from 1-2g of young leaves and dried for 24h in an oven at 48°C. The dried leaves were ground using a power drill and washed sand. DNA isolation was from 200mg leaf tissue using a miniprep version of the Dellaporta (1983) protocol. Molecular marker analysis was at CIAT. A single dilution, 10X, was employed for all samples. The samples were analyzed with the SSR marker NS158, the closest marker found to date to *CMD2*. PCR analysis and acrylamide gel analyses were carried out as described by Akano et al. 2002. Gel image was captured by scanning and transferred to a Microsoft Excel file for the inclusion of resistance data and interpretation.

A large number of crosses were made between CMD resistant progenies, introduced from IITA to CIAT, and elite parents of CIAT cassava genepools, high beta-carotene varieties and high protein and dry matter content wild *Manihot* accesions and inter-specific hybrids (Table2). To permit for sharing of this invaluable germplasm with collaborators in India and Sub Saharan Africa and still

being able to keep a copy here for breeding purposes, the seeds are being germinated from embryo axes. Once germinated, the plantlets will be multiplied, molecular-assisted selection (MAS) will be performed using the marker NS158, and CMD resistant ghenotypes will be shipped to collaborators.

Results

DNA isolation using dried leaves, a power drill and the mini Dellaporta protocol allowed for the processing of 130-150 samples daily. Yield of DNA was between 10-20ug/ 200mg of leaves, which provides enough DNA for more than 200 PCR reactions. This DNA also stores very well and can be used at again at a later time. For more routine MAS work, DNA extraction using 96 well plates and TE (Tris-HCL 10Mm (pH8.0), and EDTA (1mM) pH 8.0) currently being used for bean MAS at CIAT will be tested (CIAT 2001).

Molecular marker analysis, using the NS158 SSR marker that is tightly associated to *CMD2*, of the crosses revealed the marker to be an excellent prediction tool for CMD resistance in some crosses but not in others (Figure 1 and Table 2). Scrutiny of the data, reveals that in crosses where the marker is a good prediction tool, "recombinants", genotypes that are disease susceptible but carry the NS158 marker allele associated with resistance and vice-versa, were predominantly those with the marker allele associated with resistance but are "resistant" from phenotypic data (Table2). The second class is made up of disease susceptible varieties, roughly one quarter of the total number, that carry a presumably resistance allele. The first class of recombinants underscores a fundamental problem in the field evaluation of disease resistance, i.e uneven pathogen pressure. The crosses employed to test the MAS concept have been in the field for only 6 months and uneven disease pressure can lead to susceptible genotypes being erroneously scored as resistant.

Table 2. Summary of the molecular marker analysis of the crosses and recombinants.

Cross	No. genotypes	Recomb. (R)	Recomb. (S)	% total R.
TME3xTME117 (+reciprocals)	110	31	0	28.1
TME3xTMS91934 (+reciprocals)	61	15	0	24.6
TME3x TMS30572 (+reciprocals)	815	40	4	5.3
TME9xTMS91934 (+reciprocals)	223	44	4	22.5
TME9xTMS30572 (+reciprocals)	733	32	3	4.7
TME9xTME117(+reciprocals)	395	93	4	23.5

In an earlier study where a similar set of crosses had been in a field in Uganda for 2 years under heavy disease pressure, and had been pruned back after a year, there where no recombinants (CIAT 2001). Pruning increases CMD disease symptoms in susceptible varieties. This strengthens the case for MAS in CMD resistance breeding even where segregating populations can be evaluated in the field.

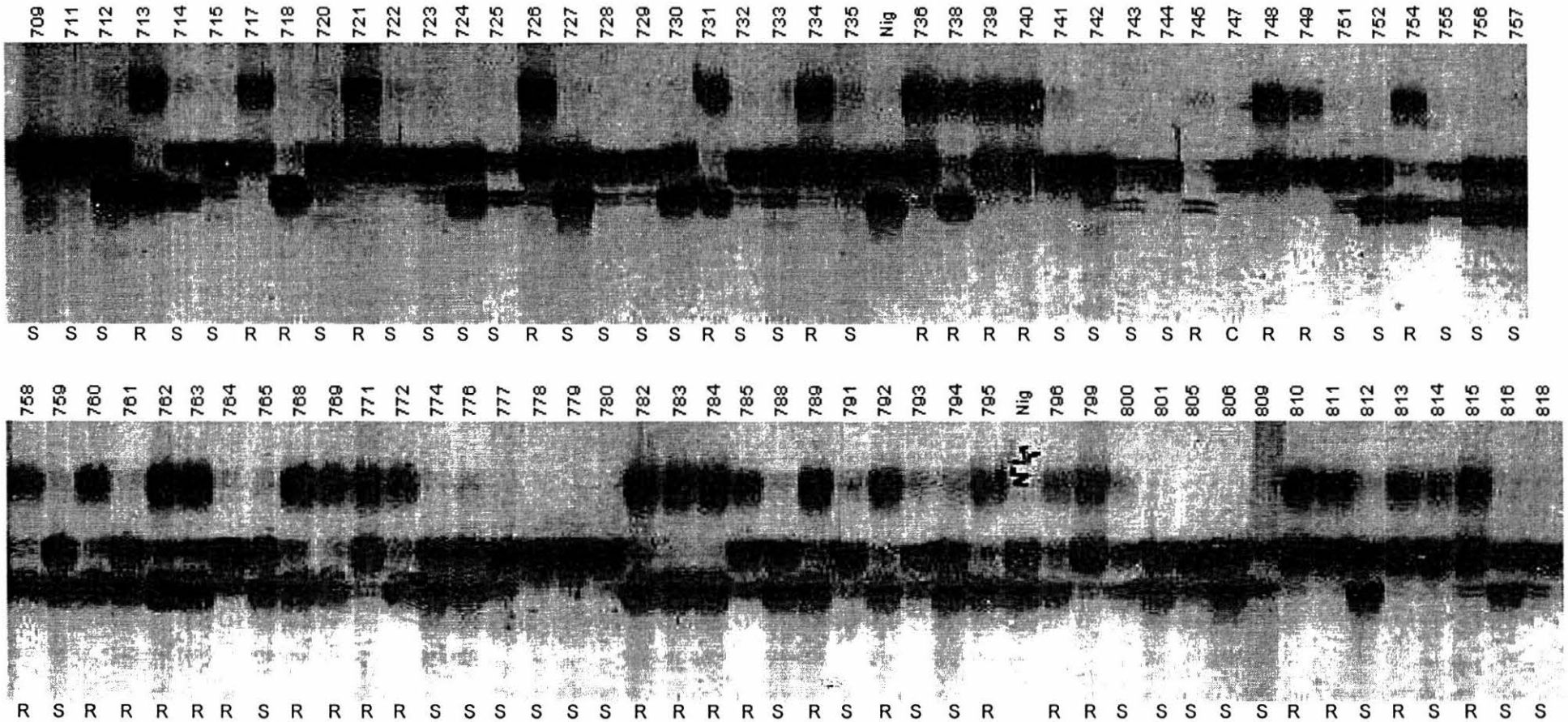

Figure 1. **Silver stained gel polyacrylamide gel showing PCR analysis of SSR marker NS158 in the cross TME3 x TMS30572 and field resistance data of resistant (R and susceptible (S) genotypes. The topmost alleles is associated with resistance. Three recombinant genotypes can be observed (in red).**

The second class of recombinants reveals a problem of the molecular marker being used. A revision of the allele sizes for marker NS158 of the genotypes TME3, TME9, TME117, and TMS91934 revealed that the CMD resistant genotypes (TME3 and TME9) and the susceptible genotypes (TME117 and TM91924) have the same allele size of allele for the allele that is associated with resistance. Although NS158 is tightly linked to CMD2, less than 1 cM, it has the same allele size for certain resistant and susceptible genotypes The proportion of the susceptible "recombinants", roughly one quarter of the total, also agree with the hypothesis of a confounding allele from the susceptible parent. This highlights the need to develop a marker that is truly unique to CMD2. Efforts are underway to clone, by positional cloning, *CMD2* and to develop an allele specific PCR fragment for use as a sequence characterized amplified region (SCAR) marker.

More than 7000 seeds were obtained from crosses between the CMD donor parents and elite parents of CIAT's cassava gene pools (Table 3). These seeds are being germinated *in vitro* and MASwill be employed to select resistant genotypes for shipment to collaborators in India and Africa.

Table 3. List of seeds obtained this year from genetic crosses between CMD donor parents and parents at CIAT for multiple purposes

Mother	Father	Fuente	Purpose 1	Purpose2	No. of Seeds
CMD donor parents x elite parents of agro-ecology zone one (tropical lowlands)					
CG 1141- 1	C 413	GY200122	Z01	ACMD	10
CM 3306- 4	C 4	GY200122	Z01	ACMD	56
CM 3306- 4	C 18	GY200122	Z01	ACMD	11
CM 3306- 4	C 33	GY200122	Z01	ACMD	30
CM 3306- 4	C 39	GY200122	Z01	ACMD	8
CM 3306- 4	C 243	GY200122	Z01	ACMD	5
CM 3306- 4	C 413	GY200122	Z01	ACMD	24
CM 6754- 8	C 33	GY200122	Z01	ACMD	1
SM 1411- 5	C 33	GY200122	Z01	ACMD	1
C 4	MTAI 8	GY200122	ACMD	Z01	470
C 33	CM 3306- 4	GY200122	ACMD	Z01	34
C 33	CM 6754- 8	GY200122	ACMD	Z01	18
C 33	MTAI 8	GY200122	ACMD	Z01	9
C 39	CM 3306- 4	GY200122	ACMD	Z01	19
C 127	MTAI 8	GY200122	ACMD	Z01	54
C 243	MTAI 8	GY200122	ACMD	Z01	8
C 413	MTAI 8	GY200122	ACMD	Z01	6
MTAI 8	C 4	GY200122	Z01	ACMD	28
MTAI 8	C 18	GY200122	Z01	ACMD	6
MTAI 8	C 33	GY200122	Z01	ACMD	32
MTAI 8	C 39	GY200122	Z01	ACMD	35
MTAI 8	C 243	GY200122	Z01	ACMD	44
MTAI 8	C 413	GY200122	Z01	ACMD	33
					943

CMD donor parents x elite parents of agro-ecology zone 2 (acid savannahs)

CM 523- 7	C 4	GY200122	Z02	ACMD	11
CM 523- 7	C 33	GY200122	Z02	ACMD	138
CM 523- 7	C 39	GY200122	Z02	ACMD	79
CM 523- 7	C 243	GY200122	Z02	ACMD	26
CM 4574- 7	C 18	GY200122	Z02	ACMD	1
SM 909- 25	C 4	GY200122	Z02	ACMD	95
SM 909- 25	C 18	GY200122	Z02	ACMD	4
SM 909- 25	C 33	GY200122	Z02	ACMD	58
SM 909- 25	C 39	GY200122	Z02	ACMD	37
SM 909- 25	C 413	GY200122	Z02	ACMD	16
SM 1219- 9	C 243	GY200122	Z02	ACMD	3
C 4	CM 523- 7	GY200122	ACMD	Z02	16
C 4	CM 4574- 7	GY200122	ACMD	Z02	8
C 4	SM 909- 25	GY200122	ACMD	Z02	18
C 4	SM 1219- 9	GY200122	ACMD	Z02	1
C 18	CM 4574- 7	GY200122	ACMD	Z02	2
C 33	CM 523- 7	GY200122	ACMD	Z02	3
C 33	CM 4574- 7	GY200122	ACMD	Z02	31
C 33	SM 909- 25	GY200122	ACMD	Z02	7
C 39	CM 4574- 7	GY200122	ACMD	Z02	9
C 39	SM 1219- 9	GY200122	ACMD	Z02	2
C 243	CM 4574- 7	GY200122	ACMD	Z02	2
C 243	SM 1219- 9	GY200122	ACMD	Z02	26
					596

CMD donor parents x elite parents of agro-ecology zone 4 (mid-altitude Andean)

CM 7951- 5	C 4	GY200122	Z04	ACMD	44
CM 7951- 5	C 18	GY200122	Z04	ACMD	13
CM 7951- 5	C 33	GY200122	Z04	ACMD	82
CM 7951- 5	C 39	GY200122	Z04	ACMD	27
CM 7951- 5	C 243	GY200122	Z04	ACMD	22
CM 7951- 5	C 413	GY200122	Z04	ACMD	8
SM 1741- 1	C 4	GY200122	Z04	ACMD	61
SM 1741- 1	C 18	GY200122	Z04	ACMD	8
SM 1741- 1	C 33	GY200122	Z04	ACMD	133
SM 1741- 1	C 39	GY200122	Z04	ACMD	28
SM 1741- 1	C 413	GY200122	Z04	ACMD	26
C 4	AM 244- 31	GY200122	ACMD	Z04	6
C 4	SM 1741- 1	GY200122	ACMD	Z04	5
C 4	MCOL 1734	GY200122	ACMD	YRT	135
C 4	MCOL 2206	GY200122	ACMD	YRT	195
C 18	SM 1741- 1	GY200122	ACMD	Z04	2
C 33	SM 1741- 1	GY200122	ACMD	Z04	31
C 39	SM 1741- 1	GY200122	ACMD	Z04	4
C 127	SM 1741- 1	GY200122	ACMD	Z04	6
C 127	MCOL 1734	GY200122	ACMD	YRT	28
C 243	SM 1741- 1	GY200122	ACMD	Z04	5
					869

CMD donor parents x wild species (high protein content and CGM resistance)

OW 183- 4	C 127	GY200122	ALW	ACMD	28
C 4	CW 66- 60	GY200122	ACMD	CGM	2
C 4	CW 66- 73	GY200122	ACMD	CGM	12
C 4	CW 67- 42	GY200122	ACMD	CGM	3
C 4	OW 280- 1	GY200122	ACMD	PTN	13
C4	OW230-3	GY200123	ACMD	PTN	159
C4	OW 231- 4	GY200122	ACMD	PTN	183
C 243	OW 280- 1	GY200122	ACMD	PTN	12
					412

CMD donor parents x high beta carotene *content varieties*

C 243	MCOL 1734	GY200122	ACMD	YRT	2
C 243	MCOL 2206	GY200122	ACMD	YRT	13
C 18	MCOL 2056	GY200122	ACMD	YRT	4
C 33	MCOL 2056	GY200122	ACMD	YRT	19
C 33	MCOL 2206	GY200122	ACMD	YRT	16
C 127	MCOL 2206	GY200122	ACMD	YRT	47
MBRA 1A	C 18	GY200122	YRT	ACMD	4
MBRA 1A	C 39	GY200122	YRT	ACMD	10
MCOL 1734	C 4	GY200122	YRT	ACMD	54
MCOL 1734	C 18	GY200122	YRT	ACMD	5
MCOL 1734	C 33	GY200122	YRT	ACMD	48
MCOL 1734	C 127	GY200122	YRT	ACMD	37
MCOL 2206	C 4	GY200122	YRT	ACMD	7
MCOL 2206	C 18	GY200122	YRT	ACMD	22
MCOL 2206	C 127	GY200122	YRT	ACMD	8
MMAL 66	C 18	GY200122	YRT	ACMD	30
MTAI 2	C 18	GY200122	YRT	ACMD	13
Total					**338**

Open pollinated seeds of CMD donor parents

C 4	GY200122	ACMD	4200
C 18	GY200122	ACMD	20
C 127	GY200122	ACMD	100
C 243	GY200122	ACMD	50
		Total:	**4370**
		Grand Total:	**7527**

Future perspectives

- The need to develop a marker that is truly unique to CMD2 to eliminate confounding effects of alleles from susceptible genotypes having the same size with the allele associated with CMD resistance
- MAS of the new crosses

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1.2.13 Mining the Primary and Secondary Gene Pool: Protein and Dry Matter Yield Genes from Wild *Manihot* Species

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Introduction

The advanced back cross QTL (AB-QTL) identification and introgression of favorable alleles of gene for high protein and dry matter content, pest resistance and starch quality in cassava is in its second year. During the first year more than 1,200 accessions of wild species and inter-specific hybrids representing 7 wild *Manihot* species were evaluated for the above traits and genotypes with high protein and dry matter content, excellent resistance to white flies and very high amylopectin content were identified (CIAT 2001). The best genotypes were selected for second year evaluation of six plants (clonal observation or single row trial, SRT) and also planted in the hybridization block for genetic crosses to elite CIAT parents. Genetic crosses between the selected wild accessions and CIAT elite parents will provide F_1 families to initiate the AB-QTL scheme. This year we report on the SRT trial, 6 plants as against 1 last year, of the wild accessions and also on genetic crosses made.

A major problem this year was the high incidence of frog skin disease (FSD) in the clonal observation trial, more than 70%, which has lowered considerably dry matter content and affected protein content in an unknown way. Some genotypes turned out with an exceptionally high amount of protein, while others showed a significant reduction compared to last year's result. FSD infected materials that showed high values for the above traits have been re-planted in Santander the Quilichao pending a virus clean-up of these genotypes by tissue culture and thermotherapy. Nevertheless, seeds obtained from crosses to the selected wild accessions are supposedly virus free and have been planted in the seedling nursery for transfer to the field and trait evaluation at harvest.

Methodology

Accessions of inter-specific hybrids and wild *Manihot* species with high protein and dry matter content, good resistance to white flies, and high amylopectin content starch identified from last year's evaluation were established at CIAT Palmira this year as a clonal observation trial. A selection index program developed by the cassava breeding unit (CIAT annual report 2000) was used to select the best 12 genotypes for protein content, dry matter content, and white fly resistance and the best 4 genotypes low amylose content, a total of 145 wild accessions and 343

inter-specific hybrids for genetic crosses. Due to very poor germination of a majority of the wild species planted directly in the fields from woody stakes, a principal problem with wild species, it was necessary to plant these accessions again in bags in the green house. The stakes were treated with growth hormones to aid germination and transferred to the field after 2 months in the green house. For some materials, the problems with poor germination continued and open pollinated seeds obtained last year from these genotypes had to be planted to obtain information on the trait of interest.

At 10 months after planting, all six plants were harvested and measured for fresh root yield, dry matter content, foliage weight, harvest index, culinary quality, starch content/quality, and frog skin disease according to standard CIAT procedures. Dried roots from genotypes that had high protein content last year were sent for total protein determination at the CIAT analytical service lab. Seeds from the genetic crosses were also collected and germinated in a seedling nursery in preparation for transfer to the field. Due to the lateness in establishing in the field some of the wild accessions, particularly the high protein content genotypes, mature seeds could not be harvested before the end of the hybridization season, the immature seeds were therefore harvested and germinated from embryo axes (see activity 6 for more details).

Results

The second year evaluation of inter-specific hybrids and wild accessions selected for high protein and dry matter content, and other traits of interest confirmed the stability of the trait value across years (Table 1). However, the experiment was greatly inhibited by the difficulty of establishing the wild accessions from woody stakes.

Table 1. Crude protein percentage of dry root scored over 2 years in wild *Manihot* accessions with high protein content. Data from 2002 is based on 6 plants.

Genotype	Mother	%Protein 2001	%Protein 2002	FSD
OW 284- 1	TST XXX- 77	7.00		Infected
OW 131- 2	TST XXX- 2	7.13	9.48	Infected
OW 134- 1	TST XXX- 8	7.26		Infected
OW 136- 3	TST XXX- 13	8.22		Infected
OW 230- 2	FLA 441- 5	9.20		Infected
OW230-3	FLA 441- 5	10.50	9.63	
OW230-4	FLA 441- 5	10.34	6.99	
OW 230- 5	FLA 441- 5	9.14		Infected
OW 231- 3	FLA 444- 7	7.16	5.24	Infected
OW 231- 4	FLA 444- 7	11.00	7.69	Infected
OW 231- 6	FLA 444- 7	8.27		
OW 280- 1	TST XXX- 51	7.24	7.28	Infected
OW181-2	FLA 423- 6	5.89		Infected
OW132-2	TST XXX- 3	11.71	9.48	

Note: Missing values are due to no storage roots, a effect of severe FSD infection

The inter-specific hybrids fared much better, an observation worthy of mention is a genotype, CW67-30, from an inter-specific hybrid family between cassava and its progenitor, *M. esculenta* sub species *flabellifolia* that had a fresh root yield yield of 114 t/ha or 39t/ha dry matter yield (Table 2). The genotype CW67-30 showed very vigorous growth and profuse production of foliage, it has been planted again with more replications to examine if the extraordinary yield will be repeated. Also planted again are the above best 25 inter-specific hybrids.

A large number of crosses were made between wild accessions and inter-specific hybrids having high protein and dry matter content, waxy starch, and pest resistance and more than 2000 seeds in total were obtained (Table3). Good size populations exist for the different traits and for the identification of F_1 genotypes for the AB-QTL scheme. The seeds have been planted at CIAT Palmira and they will be evaluated for the relevant traits at 9-10 months after planting.

Table 2. Dry matter yield and yield components of the best 25 inter-specific hybrids from 4 families having the same *M. esculenta* sub spp *fabellifolia* accession as male parent

Clone	Mother	Father	Harvest index	% Dry matter	Yield t/ha	Dry matter yield (t/ha)	Starch	Taste
CW 67-30	MFLA 437- 007(3)	MCOL 2215	0.46	34.08	114.42	39.00	1.00	4.00
CW 66-10	MFLA 437- 007(3)	CM 2766- 5	0.33	38.40	49.00	18.82	2.00	2.00
CW 67-33	MFLA 437- 007(3)	MCOL 2215	0.36	36.73	44.92	16.50	5.00	5.00
CW 65- 75	MFLA 437- 007(6)	CG 501-16	0.50	23.66	61.60	14.58	1.00	4.00
CW 67-24	MFLA 437- 007(3)	MCOL 2215	0.50	33.76	40.92	13.81	2.00	5.00
CW 67-116	MFLA 437- 007(6)	MCOL 2215	0.49	32.57	40.58	13.22	3.00	5.00
CW 66-28	MFLA 437- 007(3)	CM 2766- 5	0.45	34.62	36.00	12.46	3.00	3.00
CW 67- 40	MFLA 437- 007(6)	MCOL 2215	0.43	31.71	36.83	11.68	3.00	3.00
CW 66-76	MFLA 437- 007(3)	CM 2766- 5	0.36	35.40	29.83	10.56	1.00	4.00
CW 67-124	MFLA 437- 007(6)	MCOL 2215	0.62	33.48	28.00	9.38	2.00	5.00
CW 67-55	MFLA 437- 007(6)	MCOL 2215	0.27	36.31	25.75	9.35	1.00	2.00
CW 67-152	MFLA 437- 007(6)	MCOL 2215	0.51	36.00	25.83	9.30	2.00	3.00
CW 66-61	MFLA 437- 007(3)	CM 2766- 5	0.28	35.37	26.17	9.25	3.00	5.00
CW 67-136	MFLA 437- 007(6)	MCOL 2215	0.25	34.71	26.40	9.16	3.00	4.00
CW 67-18	MFLA 437- 007(3)	MCOL 2215	0.24	34.90	26.20	9.14	1.00	3.00
CW 67-77	MFLA 437- 007(6)	MCOL 2215	0.21	32.14	26.33	8.46	4.00	5.00
CW 67-98	MFLA 437- 007(6)	MCOL 2215	0.35	29.70	27.17	8.07	3.00	5.00
CW 64-7	MFLA 437- 007(6)	CG 487-2	0.22	29.70	27.17	8.07	3.00	5.00
CW 65-79	MFLA 437- 007(6)	CG 501-16	0.20	33.72	23.92	8.06	1.00	4.00
CW 66- 35	MFLA 437- 007(3)	CM 2766- 5	0.31	35.87	21.50	7.71	1.00	3.00
CW 67-129	MFLA 437- 007(6)	MCOL 2215	0.71	35.87	21.33	7.65	3.00	5.00
CW 67-121	MFLA 437- 007(6)	MCOL 2215	0.30	28.22	25.25	7.12	3.00	3.00
CW 66-21	MFLA 437- 007(3)	CM 2766- 5	0.24	33.64	21.00	7.06	3.00	3.00
CW 67-44	MFLA 437- 007(6)	MCOL 2215	0.40	31.13	22.60	7.04	1.00	4.00
CW 67-126	MFLA 437- 007(6)	MCOL 2215	0.62	31.13	22.00	6.85	3.00	5.00
CW 66-49	MFLA 437- 007(3)	CM 2766– 5	0.43	20.03	27.00	5.41	3.00	4.00

	Statistics of best 25 inter-specific hybrids evaluated							
Maximum			0.71	38.40	114.42	39.00	1.00	3.00
Minimum			0.20	20.03	21.00	5.41	5.00	5.00
Average			0.39	32.80	33.76	11.07	2.35	3.96
Standard Dev.			0.14	4.06	19.16	6.54	1.09	1.00
	Statistics of 343 inter-specific hybrids evaluated							
Maximum			0.71	55.88	114.20	39.00	1.00	5.00
Minimum			0.01	16.30	5.80	1.19	5.00	2.00
Average			0.16	27.59	9.66	2.70	2.80	4.10
Standard Dev.			0.12	7.17	9.90	3.46	1.37	1.00

Table 3. Summary of sexual seeds obtained from crosses between inter-specific hybrids and wild *Manihot* accessions high in protein, dry matter content, and waxy starch.

	Family	Mother	Father	Purpose of cross		No. of seeds
	Protein					
175	CW 179	OW 132- 2	MTAI 8	PTN	Z01	21
178	CW 185	OW 180- 1	MTAI 8	PTN	Z01	9
186	CW 204	OW 231- 3	AM 244- 31	PTN		14
187	CW 205	OW 231- 3	MTAI 8	PTN	Z01	11
188	CW 206	OW 280- 1	AM.244- 31	PTN		64
189	CW 207	OW 280- 1	MTAI 8	PTN	Z01	291
190	CW 207	MTAI 8	OW 280- 1	Z01	PTN	16
						426
	Protein and yellow roots					
221	CW 73	CM 1585- 13	OW 284- 1	YRT	PT-MS	15
222	CW 177	OW 132- 2	CM 1585- 13	PTN	YRT	128
223	CW 184	OW 180- 1	MCOL 1734	PTN	YRT	13
224	CW 186	OW 181- 2	CM 1585- 13	PTN	YRT	13
225	CW 188	OW 181- 2	MCOL 1734	PTN	YRT	23
226	CW 207	OW 280- 1	CM 1585- 13	PTN	YRT	215
227	CW 212	OW 284- 1	MCOL 1734	PT-MS	YRT	13
228	CW 251	MCOL 1734	OW 189- 1	YRT	PT-MS	18
229	CW 256	MCOL 1734	OW 280- 1	YRT	PTN	13
						451
	Dry matter and yellow roots					
21	CW 69	CM 1585- 13	CW 30- 29	YRT	DMC	6
22	CW 69	CW 30- 29	CM 1585- 13	DMC	YRT	6
23	CW 70	CM 1585- 13	OW 234- 2	YRT	DMC	58
24	CW 71	CM 1585- 13	OW 240- 8	YRT	DMC	19
25	CW 72	CM 1585- 13	OW 280- 2	YRT	DMC	1
26	CW 100	CW 30- 29	MCOL 1734	DMC	YRT	3
27	CW 100	MCOL 1734	CW 30- 29	YRT	DMC	4
28	CW 101	CW 30- 31	CM 1585- 13	DMC	YRT	9
29	CW 115	CW 30- 73	CM 1585- 13	DMC	YRT	1
30	CW 127	CW 30- 73	MCOL 1734	DMC	YRT	1
31	CW 127	MCOL 1734	CW 30- 73	YRT	DMC	46
32	CW 147	CW 47- 3	MCOL 1734	DMC	YRT	3
33	CW 147	MCOL 1734	CW 47- 3	YRT	DMC	11
34	CW 153	CW 48- 1	MCOL 1734	DMC	YRT	54
35	CW 154	MCOL 1734	CW 48- 1	YRT	DMC	66
36	CW 155	CW 56- 5	CM 1585- 13	DMC	YRT	23

37	CW 166	CW 56- 5	MCOL 1734	DMC	YRT	38
38	CW 181	OW 146- 1	MCOL 1734	DMC	YRT	11
39	CW 211	OW 280- 2	MCOL 1734	DMC	YRT	11
40	CW 249	MCOL 1734	CW 28- 38	YRT	DMC	34
41	CW 250	MCOL 1734	CW 30- 65	YRT	DMC	44
42	CW 252	MCOL 1734	OW 234- 2	YRT	DMC	16
43	CW 253	MCOL 1734	OW 240- 6	YRT	DMC	113
44	CW 254	MCOL 1734	OW 240- 8	YRT	DMC	16
45	CW 255	MCOL 1734	OW 269- 4	YRT	DMC	71
						665
	Dry matter					
46	CW 82	CM 7951- 5	OW 240- 8	Z02	DMC	22
66	CW 111	CW 30- 31	MTAI 8	DMC	Z01	16
82	CW 128	CW 30- 73	MTAI 8	DMC	Z01	4
90	CW 137	CW 30- 87	MTAI 8	DMC	Z01	4
107	CW 167	CW 56- 5	MTAI 8	DMC	Z01	168
108	CW 169	CW 60- 7	SM 1036- 8	DMC		5
109	CW 170	CW 60- 7	MTAI 8	DMC	Z01	27
112	CW 180	OW 146- 1	AM 244- 31	DMC		70
118	CW 197	OW 213- 4	MTAI 8	DMC	Z01	71
121	CW 216	SM 1219- 9	CW 48- 1	Z02	DMC	11
122	CW 221	SM 1460- 1	CW 30- 65	Z02	DMC	3
123	CW 222	SM 1460- 1	CW 48- 1	Z02	DMC	19
124	CW 228	SM 1460- 1	OW 240- 8	Z01	DMC	6
125	CW 262	MTAI 8	OW 234- 2	Z01	DMC	83
126	CW 263	MTAI 8	OW 240- 6	Z01	DMC	4
127	CW 264	MTAI 8	OW 240- 8	Z01	DMC	33
						546
	Waxy starch					
2	CW 143	CW 39- 2	MTAI 8	ALW	Z01	5
5	CW 191	OW 183- 4	MCOL 1734	ALW	YRT	1
						6
Grand total						2094

Future Perspectives

- The evaluate the inter-specific hybrids generated for the target traits
- Continue making crosses

References

CIAT, (2000). Annual Report Project SB2, Assessing and Utilizing Agrobiodiversity through Biotechnology, CIAT, Cali, Colombia, pp 239-241.

1.2.14 Mining the Primary and Secondary Gene Pool: Resistance Genes for Resistance to Green Mites from *Manihot esculenta* sub species *flabellifolia*

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CIAT
Funding: CIAT core funds

Introduction

The cassava green mites (*Mononychellus tenajoa*) is a biotic stress of cassava that becomes prominent during periods of prolonged dry periods. In East Africa, overlapping outbreaks of CMD and CGM during the dry season tend to result in very heavy losses and a severe reduction in farm profits (Legg et al. 1998). January this year at CIAT Palmira was particularly dry and, not surprisingly, a very heavy incidence of mites was recorded on the station. The CIAT cassava entomology group conducted a thorough evaluation of cassava plants in the field and while most plants had damage ratings of 4 on the CIAT scale of 1-5, where 1 is no symptoms, and 5 is severe leaf damage and stunted growth, 4 inter-specific hybrid families from the wild *Manihot* accession MFLA 437- 007 showed an almost equal number of susceptible(score of 3-4) and resistant (score of 1-2) genotypes.

The very high level of resistance found in the inter-specific hybrids and the almost equal number of susceptible and resistant genotypes, which suggests a simple mode of inheritance of the resistance gene(s), makes deployment of this source of mite resistance very attractive. Bulk segregant anlysis (BSA) using 500 SSR markers was quickly used to identify molecular markers associated with resistance. At the same time, highly resistant hybrids were crossed to CMD resistant parents, to combine CMD and CGM resistance for Africa, and also to elite cassava parents at CIAT.

Methodology

A clonal observation trial of 6 plants per genotype of inter-specific hybrids between the cassava varieties CG487-2, CG501-16, MCol2215, and CM2766-5 and the *M. esculenta* sup spp *flabellifolia* accession MFLA 437- 007, designated CW68, CW65, CW67, and CW66 respectively were planted at CIAT Palmira August last year. They were evaluated for resistance to mites during a very heavy mite infestation January this year. A high level of resistance was observed in about half of the inter-scpefic hybrids. It was thought desirable to transfer this high level of resistance to elite parents of the cassava gene pools. Genetic crosses where therefore made to elite parents of the cassava gene pool.

Following the interesting distribution of resistant genotypes observed in the families, the family CW67 was chosen for bulk segregant analysis of CGM resistance. Ten resistant and ten susceptible genotypes were used for molecular analysis. DNA was isolated from 1-2g of young leaves and dried for 24h in an oven at 48°C. The dried leaves were ground using a power drill and washed sand and DNA was isolated from 200mg leaf tissue using a miniprep version of the Dellaporta (1983) protocol. DNA from the cassava parent Mcol2215 was also isolated for inclusion in the analysis. The wild parent no longer exists it was eliminated from the field in 2000 during an eradication of the wild *Manihot bank*, many of which were contaminated with

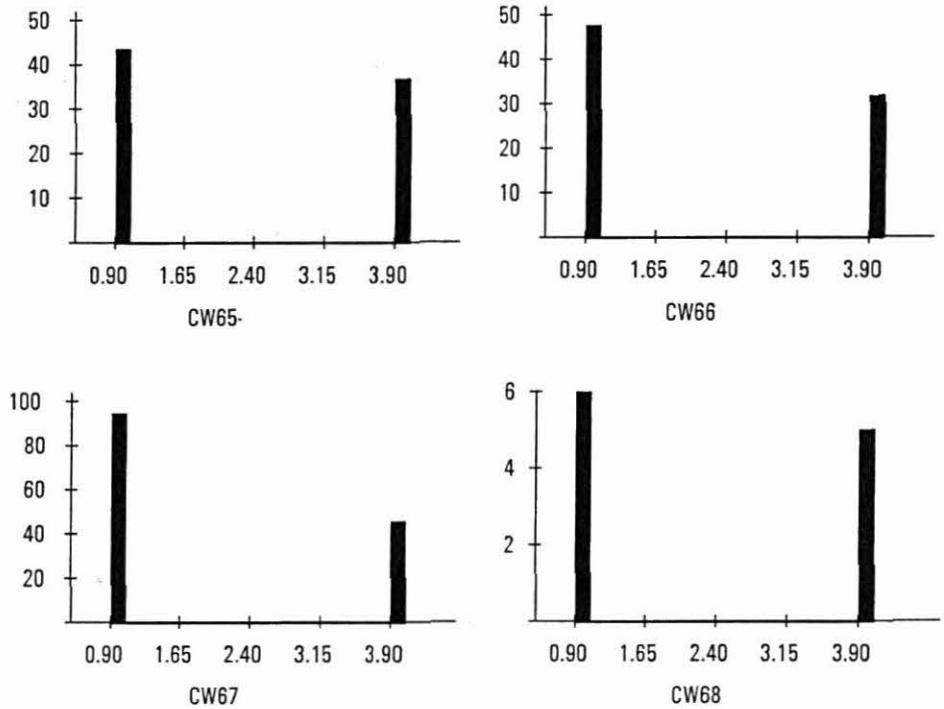
frog skin disease, the crosses were made in 1995. DNA from the bulks and parent was genotyped with the 500 available cassava SSR markers. Markers that were polymorphic in the bulks were analyzed in individual genotypes that make up the bulks.

Results

Resistance response to CGM in the families CW65, 66, 67 and 68 was qualitative, i.e., all 6 plants of resistant genotypes showed no visible symptom, while all plants of susceptible genotypes were always heavily infected. The percentage of resistant to susceptible plants was about the same (Figure 1).

A chi square of the ratio of resistant to susceptible plants was not significantly different from a 1:1 ratio at a probability level of 0.05 for CW65, 66, and 68. This fits the expected segregation ratio for a single dominant gene heterozygous in the wild accession. BSA revealed an allele of the SSR marker, SSRY330, is present in the resistant parent and in the resistant bulk but absent in the susceptible bulk and the susceptible parent (Figure 2).

Figure 1. Distribution of response to CGM in 4 inter-specific hybrids derived from a single accession of *M. esculenta* sup spp *flabellifolia* MFLA 437- 007 as father.



The polymorphism was confirmed when the individuals of the bulks were screened with the SSR marker although 3 and 1 recombinant could be observed in the resistant and susceptible bulk respectively (Figure 2). The SSR marker is currently being analyzed in all individuals of CW67, as well as those of the other three families.

Genetic crosses have been made between inter-specific hybrids having high CGM resistance and elite parents of CIAT gene pools, a total of 832 seeds were obtained (Table3). This cross can be loosely described as a back cross and it is the second step of the AB-QTL scheme. Seeds obtained have been planted at CIAT Palmira and they will be evaluated for the relevant traits at 9-10 months after planting.

Future Perspectives

Analyze the marker SSRY330 in the entire individuals of all 4 families namely, CW65, CW66, CW67, and CW68
Second year evaluation of the inter-specific hybrids in Santander the Quilichao

References

Legg, J.P.; Whyte, J. Khizzah, B. and Ogwang, J. (1998). CGM/CMD interaction studies. In: Project 6. Integrating Management of Cassava Pests and Diseases 1998. Annual Report Project Report. International Institute of Tropical Agriculture, Ibadan, Nigeria pp: 8-9

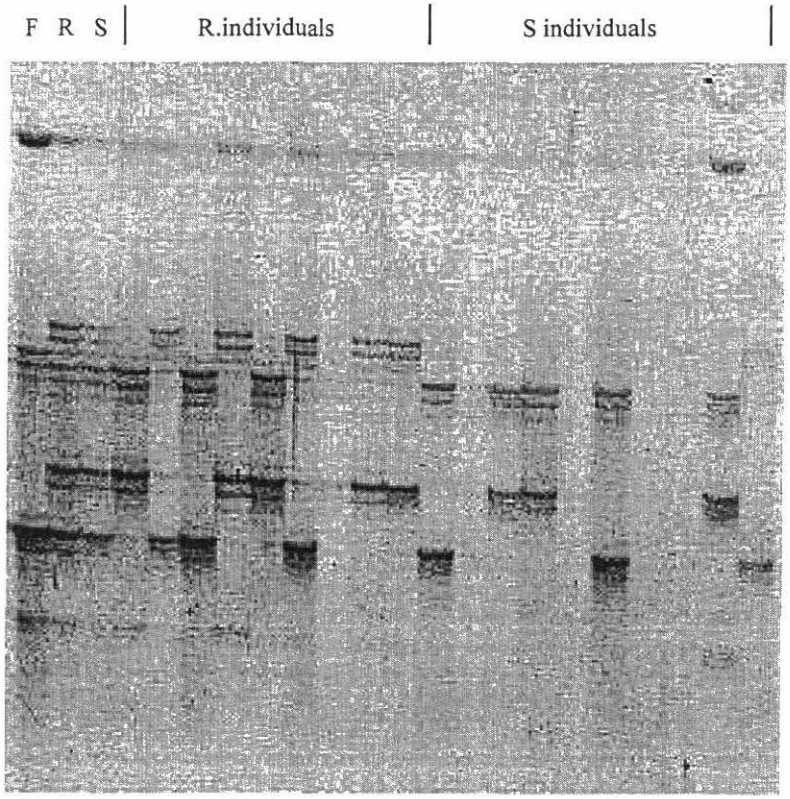


Figure 2. Silver stained polycrylamide gel of bulk segregant analysis (BSA) of CGM resistance in the CW67 family, the topmost fragment segregates with resistance. Three and one recombinants respectively can be observed in the CGM resistant and susceptible varieties. F stands for father, the male parent (MFLA 437- 007), while R and S stands for the resistant and susceptible bulk respectively.

Table3. Seeds obtained from crosses between the inter-specific hybrids with high levels of resistance to CGM and elite parents of cassava gene pools at CIAT.

Family	Mother	Father	No. of seeds
CW 75	CM 3306- 4	CW 66- 60	8
CW 76	CM 3306- 4	CW 68- 3	12
CW 77	CM 7951- 5	CW 65- 77	7
CW 78	CM 7951- 5	CW 66- 19	4
CW 79	CM 7951- 5	CW 66- 62	2
CW 80	CM 7951- 5	CW 67- 42	5
CW 81	CM 7951- 5	CW 67- 98	4
CW 213	SM 805- 15	CW 67- 39	1
CW 214	SM 805- 15	CW 67- 87	11
CW 215	SM 909- 25	CW 66- 60	10
CW 217	SM 1219- 9	CW 65- 77	23
CW 218	SM 1219- 9	CW 66- 73	24
CW 219	SM 1219- 9	CW 66- 74	4
CW 220	SM 1219- 9	CW 67- 123	15
CW 223	SM 1460- 1	CW 66- 19	18
CW 224	SM 1460- 1	CW 66- 60	22
CW 225	SM 1460- 1	CW 66- 62	42
CW 226	SM 1460- 1	CW 66- 73	16
CW 227	SM 1460- 1	CW 68- 3	3
CW 229	SM 1511- 6	CW 67- 87	35
CW 230	SM 1565- 15	CW 66- 19	5
CW 231	SM 1565- 15	CW 66- 60	27
CW 232	SM 1665- 2	CW 66- 19	31
CW 233	SM 1665- 2	CW 66- 60	4
CW 234	SM 1665- 2	CW 66- 74	49
CW 235	SM 1665- 2	CW 67- 87	129
CW 236	SM 1669- 5	CW 66- 19	58
CW 237	SM 1669- 5	CW 66- 60	12
CW 238	SM 1669- 5	CW 66- 62	2
CW 239	SM 1669- 5	CW 66- 73	7
CW 240	SM 1669- 5	CW 66- 74	36
CW 242	SM 1669- 7	CW 67- 87	13
CW 241	SM 1669- 5	CW 67- 123	8
CW 243	SM 1741- 1	CW 66- 19	9
CW 244	SM 1741- 1	CW 66- 60	18
CW 245	SM 1741- 1	CW 66- 62	3
CW 246	SM 1741- 1	CW 67- 91	12
CW 247	SM 1778- 45	CW 66- 19	4
CW 248	SM 1778- 45	CW 67- 45	4
CW 257	MTAI 8	CW 65- 77	33
CW 258	MTAI 8	CW 66- 60	31
CW 259	MTAI 8	CW 66- 73	59
CW 260	MTAI 8	CW 66- 74	6
CW 261	MTAI 8	CW 67- 123	5
			832

1.2.15 QTL Mapping of Cyanogenic Potential (CNP) in Cassava

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Funding: BIOEARN, SAREC,Stockholm, Sweden

Introduction

Cassava produces cyanogenic glucosides which is 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. Conventional breeding for low cyanogenic potential (CNP) is fraught with the confounding effect of the environment and developmental stage at which CNP is measured. A project has been initiated in Uganda to map the genes controlling cyanogenic potential (CNP) in cassava with funding from the Swedish project for Biotechnology and Biosafety Research Network in East Africa, (BIOEARN). The identification of molecular marker associated with CNP will increase the efficiency and the cost-effectiveness of breeding low CNP cassava varieties for Uganda. The BIOEARN project is being conducted as a joint project between the MBL, Swedish University of Agricultural Sciences (SLU), Uppsala, NARO, Namulonge, and International Centre for Tropical Agriculture (CIAT). Activities in the project so far include the development of mapping populations and their establishment at National Agricultural Research Organization (NARO), Namulonge. In addition, facilities for simple sequence repeat (SSR) genotyping of the mapping population have been made available at the MBL. A meeting of partners under the project was planned for mid-may to

a. Resolve bottlenecks in the molecular marker activity
b. Appraise progress made so far and write a short report
c. Make recommendations on future activities

Methodology

Two key requirements for the genetic mapping of CNP are segregating populations with a simple pedigree and easily assayed molecular markers on a genome wide basis. For cassava two kinds of markers are currently available on a genome-wide basis: simple sequence repeat (SSR) markers and restriction fragment length polymorphism (RFLP) markers. The SSR are highly informative and polymerase-chain-reaction (PCR)-based markers making them most appropriate for genetic mapping. The RFLP markers are also informative, but tedious to use, they are therefore being converted to PCR-based markers known as single strand conformation polymorphism (SSCP) markers.

To implement the SSR marker technology at the MBL for genetic mapping of CNP, 8 SSR markers from the Cassava MapPairs were amplified by PCR in 7 parental genotypes, 2 grand parental and 5 parental, of some of the F_2 mapping progenies using a Perkin Elmer 9700 PCR machine. The PCR reaction was electrophoresed on 6% polyacrylamide gels and visualized by silver staining.

The original mapping population for the study was several F_2 families derived from selfing F_1 progeny from the Gomani x Mbundumali cross. Initial *in vitro* establishment of the sexual seeds

from embryo axes was conducted at the tissue culture facility of the Namulonge station using 30 seeds from the F_2 family GMM13. Poor growth of the majority of the cultures was observed. It was then decided to continue establishment in pots filled with sterile soils. An issue raised with the use of the Gomani x Mbundumali F_2s is their poor adaptation to Uganda, particularly adaptation to the devastating cassava mosaic disease (CMD). It was therefore decided to create back-up F_2 families generated from local varieties. A very bitter local variety known as Tongolo was crossed to two CMD resistant varieties, SS4 and TME4. The F_1 progenies were planted directly in the field March 12 and are now 10 weeks old.

Results

The parental survey of parents of the mapping population with more than 500 SSR markers have begun. Results obtained so far reveal a good level of polymorphisms in the parents (Fig1). A summary of families and number of plants expected are shown in Table Not all families will be established due to their small sizes.

Table 1. Summary of the total number of seeds, viable seeds and expected plants from Gomani x Mbundumali F_2 families

Code	Total No. of seeds	Total No. of viable seeds	Plants expected (80% germination)
GMM3	333	221	160
GMM96	266	173	138
GMM66	151	121	96

Table 2 summarizes the F1 and reciprocals of the Tongolo crosses currently growing in the field at NARO, Namulonge:

Table2. Summary of the total number of plants from the Tongolo x SS4 and Tongolo x TME4 and their reciprocals.

Code	Cross	Number of plants
To be determined	Tongolo x SS4	170
To be determined	SS4 x Tongolo	73
To be determined	Tongolo x TME4	102
To be determined	TME4 x Tongolo	37

It is necessary to keep the field weed free and watered during the oncoming dry season that begins in June. The plants also will greatly benefit from some fertilizer application to ensure that woody stakes can be obtained by December when they will be cloned. At the moment, the crosses have not been given a code, neither have the individual plants been labelled, it is necessary that this be done within the next two months to avoid a mix-up later in the experiments.

Future Perspectives

- MAS Parental survey of Mbundumali, Gomani, Tongolo, SS4, TME 4 and selected progenies using SSR and SSCP markers. To be included are 5 parents and 7 progenies (a total of 12 samples) to be analyzed with all available markers
- Harvest of two roots from the new CNP crosses at 7 months after planting (MAP), evaluation for CNP using the picrate method and generation of F2 families for mapping

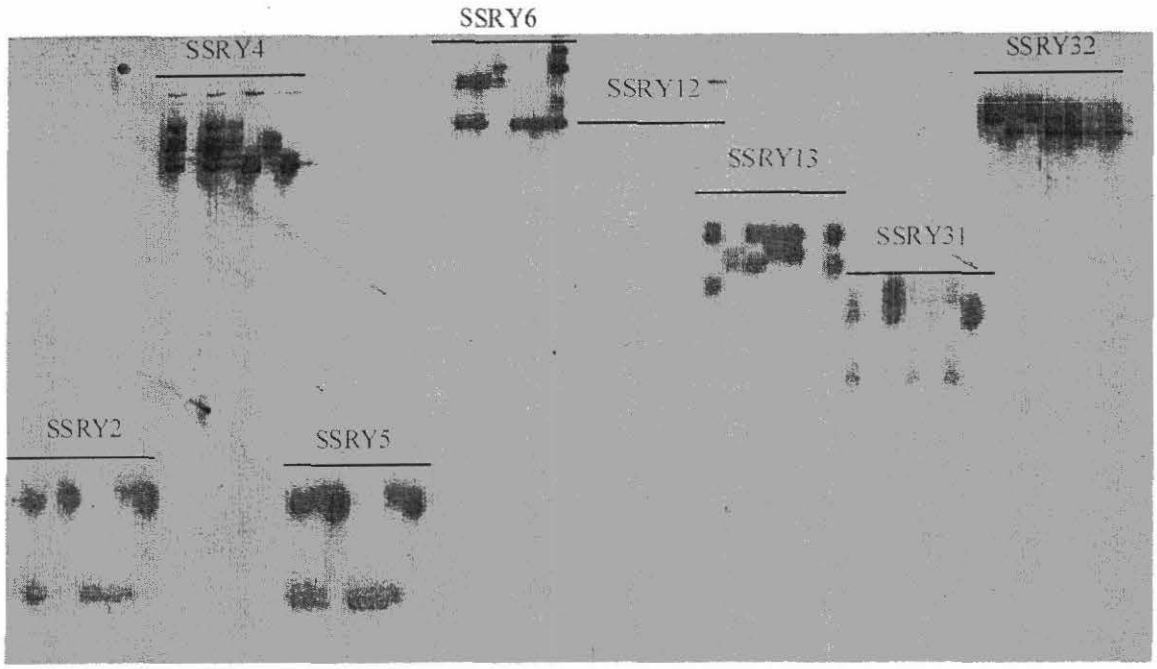

Fig 1. Polyacrylamide silver stained gel of 8 SSR markers and parental genotypes of the CNP mapping F2 populations. Orders of the parents are: Gomani, Mbundumali, GMM46, GMM66, GMM88, GMM91, and GMM96.

1.2.16 Gene Tagging of Beta-Carotene Content in Cassava

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SB-2
Funding: CIAT

Introduction

Occurrence of vitamin A deficiency and other nutritional problems overlaps with areas where cassava is an important staple food. Efforts have therefore been invested to realize the potential of cassava to improve the vitamin A consumption of people living in the tropical belt, where it is a predominant crop. Both roots and leaves of cassava contain considerable amounts of vitamin A precursors (β-carotene), 0.102 to 1.040 mg/100 g fresh roots and 12.05 to 96.42 mg/100 g fresh leaf weight (CIAT 2001). Several studies have highlighted the possibility of increasing available content of beta-carotene in both leaves and roots (Iglesias et al 1997, CIAT 2002, this report). Furthermore, the high β- carotene content is not often combined with high dry matter yield, resistance to pests and diseases and acceptable root quality. Crosses between selected sources of cassava with high nutritional quality and adapted local will need to be made.

Given the long growth cycle of cassava, improvement of a trait that is expressed only late in the growth cycle of the crop, such as β carotene, will benefit from marker-assisted selection. The inheritance of β- carotene in cassava has been demonstrated to be controlled by 2 genes (Iglesias et al. 1997). It is therefore a relatively simple trait to identify markers for. Molecular markers

associated with β-carotene will allow for its selection early in the breeding cycle. We describe here discovery of a S_1 cross from the cassava land race Mcol72 with a wide spectrum of segregation for beta-carotene from pure white to pink color. This family is appropriate for the development of markers for beta-carotene, the precursor of vitamin A.

Methodology

As part of an initiative to develop cassava populations tolerant to inbreeding, 14 genotypes commonly used as parents in the CIAT breeding were selfed and the seeds established at the experimental station of CENICAñA last year. This year, a clonal observation experiment, a single replication of 10 plants in a row per genotype, was set up at CIAT Palmira. At 10 months after planting the experiment was harvested. It was observed that in the S_1 family of 38 plants from the Colombian land race MCol72, a wide segregation was observed in root color from white to pink. The roots were scored qualitatively using the usual CIAT scale of 1 (white) to 8 (pink); pictures were also taken of a cross section of the root. Two genotypes with the deepest pink coloration were selected for quantitative determination of carotenes in the roots using high performance liquid chromatography. The parental genotype MCol72 has a root color that is normally described as cream colored or 4 on the CIAT scale.

The discovery of a wide segregation for root color in an S_1 cross from a cream colored variety provides an ideal population for bulk segregant analysis (BSA) of β carotene content and to identify markers associated with genes controlling the above trait. For DNA isolation, 1-2g of young leaves were harvested from all 38 genotypes into small paper envelopes and dried for 24h in an oven at 48°C. The dried leaves were ground using a power drill and washed sand and DNA was isolated from 200mg using a miniprep version of the Dellaporta (1983) protocol. Two bulks of 6-10 DNA samples from genotypes with pure white and pink roots respectively were created. DNA from the bulks and parent will be genotyped with the 500 cassava SSR markers; markers polymorphic in the bulks will be employed to analyze the entire population. Markers associated with yellow/pink color will be determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the computer package Q-GENE 2.30B (Nelson, 1997). The amount of phenotypic variance explained by each marker will be considered significant if the probability of observing an R^2 value is less than 0.005.

Results

The S_1 cross showed a wide spectrum of segregation for root color (Figure 1).

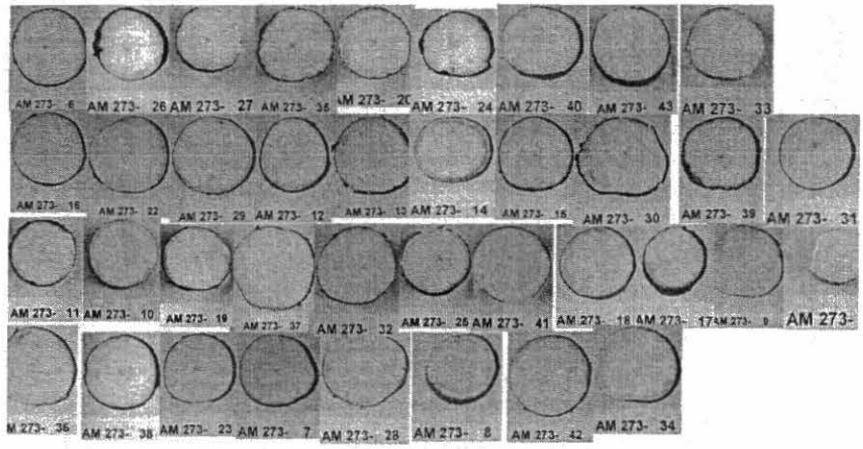

Figure 1. Cross section of root parenchyma of 38 genotypes of the S1 family derived from MCol72. A wide spectrum of variation in color can be seen from pure white to pink. Also to be noted is the pattern of deposition of beta-carotene.

The evaluation of two genotypes with pink roots revealed total beta-carotene content of 1.69mg/100g fresh weight (AM273-23) and 1.38100g fresh weight (AM273-7) respectively. The value obtained from AM273-23 is the highest to date from the characterization of the CIAT germplasm bank for beta-carotene. These results reveal the potential to increase beta-carotene in the roots. A series of experiments have therefore been planned to:
Generate a larger S_1 family from Mcol72 for gene tagging of beta-carotene.
Generate additional S_1 families from other cream, yellow and pink varieties and tag the genes involved

Determine if alleles of genes that control beta-carotene content from different varieties are complementary by making crosses between genotypes that carry them.
Crosses planned can be observed in Table 1.

Table 1. Summary of crosses established at CIAT Palmira this year to identify favorable alleles of genes controlling beta-carotene content.

	Genotype	No. of plants
Cream or yellow varieties		
1	CM 507-34	10
2	CM 996- 6	10
3	SM 526- 3	10
4	MCOL 144	10
5	MCOL 721	10
6	MCOL 1530	10
7	MCOL 1721	10
8	MCOL 2435	10
9	MCR 54	10
10	MGUA 29	10
11	MPER 572	10
Deep yellow or pink varieties		
1	MCR87	10
2	MBRA337	10
3	COL 2199	10
4	MCOL 2318	10
5	MPER297	10

References

Iglesias, C., Mayer J., Chávez A.L., Calle, F., 1997. Genetic potential and stability of carotene content in cassava roots. Euphytica 94:367-373.

1.2.17 Development of Populations Tolerant to Inbreeding Depression in Cassava

Nelson Morante, Teresa Sanchez, Hernan Ceballos, Martin Fregene
SB-2
Funding: CIAT

Introduction

Cassava is an allogamous tetraploid that accumulates a significant genetic load that is released on inbreeding. The average yield of selfed lines was observed to be about half the average yield of parental genotypes and the degree of inbreeding varied greatly amongst different genotypes (Kawano et al. 1978). Inbreeding depression primarily affects the general growth and vigor of the plant, reflected directly in lower root yield, and therefore no inbreeding is carried out at any stage of traditional cassava breeding. Cassava breeders in general strive to select progenitors and make crosses that mazimize heterozygosity and minimize inbreeding

But inbreeding is desirable for cassava for the reduction of the genetic load currently carried by many elite clones which in turn permit the use of valuable breeding schemes such as back-cross breeding. But more importantly inbreeding is important because it eliminates the confounding effect of dominance process and maximizes the additive gene variance on selection (Ceballos et al 2002). Inbred lines also have the advantage that they can be shipped and stored as botanical seed, facilitating the exchange of germplasm which at the moment can only be done via expensive tissue culture shipments. A selection program was therefore set-up for tolerance to inbreeding in 1999, at the moment more than 300 S_1 lines have been produced from 5 elite clones and S_2 lines were produced this year (see net section of this report). We describe here evaluation of the S_1 families

Methodology

Fourteen cassava genotypes were chosen for the development of populations tolerant to inbreeding. The genotypes were chosen due to their good general combining ability performance for yield, dry matter yield or root quality. They include the following lines: MCOl22, CM523-7, MCOL1684, MBRA12, MCOL2060, MVEN77, MCOL1522, MTAI1, MPAN51, MECU169, MCOL1468, MCOL72, CM849-1, HMC1. More than 300 pollinations were made per genotype and between 30-150 seeds were obtained per genotype. The seeds were planted at the CENICAñA experimental station October 2000.
At 10 months after planting, harvested August 2001, all plants were harvested and measured for fresh root yield, dry matter content, foliage weight, harvest index, culinary quality, starch content/quality, and frog skin disease according to standard CIAT procedures. Six families were selected to continue the process of inbreeding based on their average yields and flowering. The families were also planted in an observational trial, six plants in single row replication, in October 2001 at CIAT Palmira and harvested in May 2002. The traits described above were evaluated for all plants.

Results

Inbreeding depression was severe in many of the genotypes, particularly in MBRA12, MCOL1684, MCOL1468 and MCOL1522; inbreeding depression of more than 70%, vigor was very poor and sufficient stakes could not be obtained for further experiments and were therefore eliminated. Six families showed better tolerance to inbreeding depression as observed from their yield data (Table 1), highlighting the great differences in cassava to inbreeding depression. Maximum yield of these families were on an average of 18 tons/ha of fresh root with the exception of family AM312 that had 35t/ha, this family also had the largest standard deviation. Average root yield was less than half of the average yield of the parents which is in agreement with earlier observations of inbreeding depression in cassava. The 2002 experiment, the clonal observation experiment agreed quite closely with data from the seedling trial (data not shown). Distribution of dry matter content and harvest index from the clonal observation revealed normal distribution of the traits in all families.

Table 1. Summary of fresh root yield from a seedling trial of S_1 families conducted a the CENICAñA experimental station in 2001

Family name	Parent	Maximum fresh root yield t/ha	Minimum fresh root yield t/ha	Average root yield t/ha	Standard deviation
AM244	MCOL1505	18.5	0.00	7.58	5.46
AM266	HMC-1	18.5	0.00	8.31	4.17
AM273	MCOL72	14.7	0.00	7.0	4.38
AM277	MTAI1	18.0	0.00	6.64	5.96
AM278	MVEN77	15.0	0.00	3.86	5.38
AM312	CM849-1	35.5	0.00	6.04	6.04

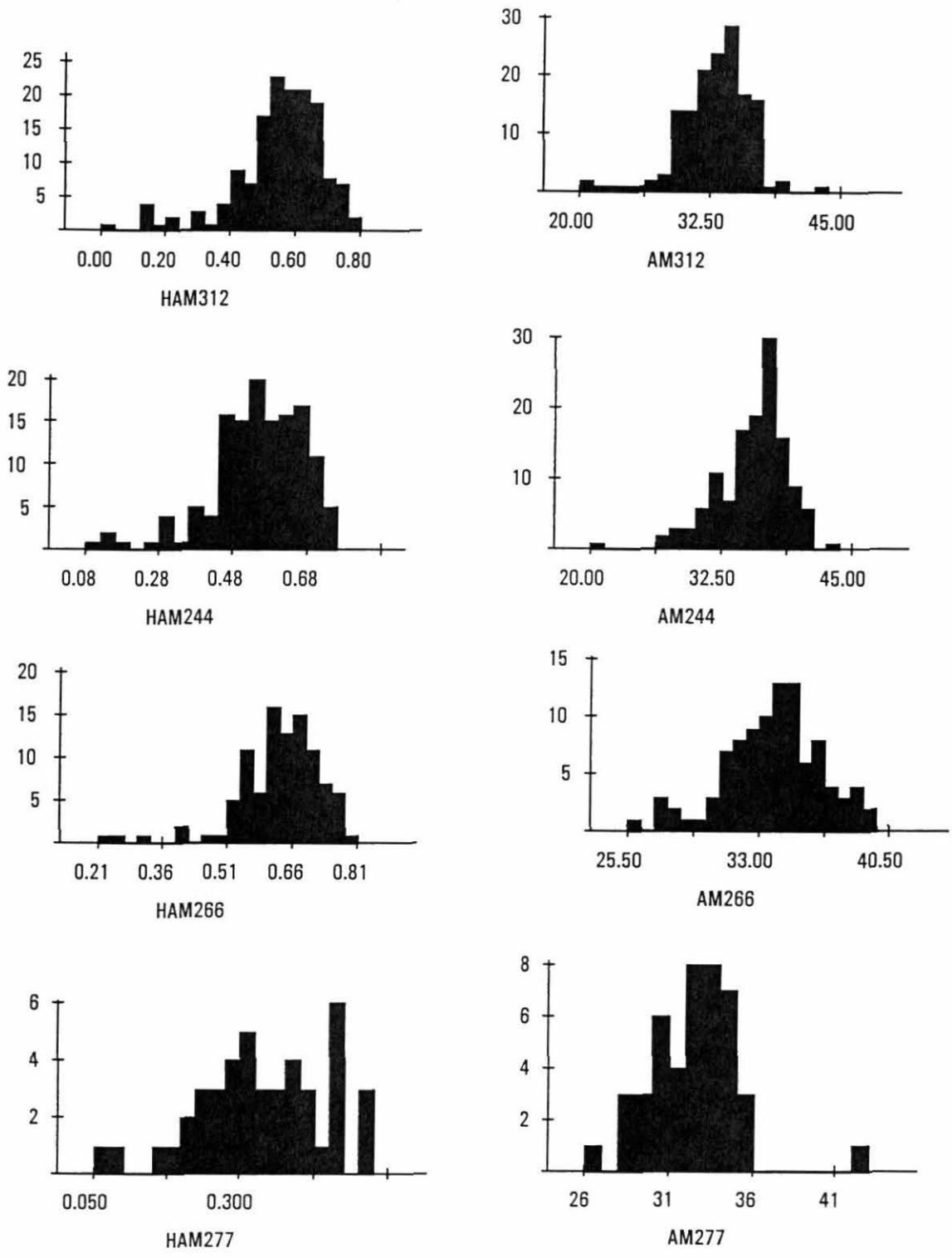

Figure 1. Distribution of harvest index (prefix H-) and dry matter content in 4 S_1 families

Table 1 Best 25 genotypes for dry matter yield and their yield components in 6 S_1 families

Genotype	Parent	FY t/ha	HI	% DMC	DMY t/ha	Starch	Taste
AM 266- 108	HMC 1	98.00	0.66	33.58	32.91	1	1
AM 266- 103	HMC 1	91.20	0.69	35.73	32.59	1	1
AM 312- 18	CM 849- 1	78.25	0.56	37.32	29.20	2	3
AM 266- 21	HMC 1	77.88	0.63	38.68	30.12	1	1
AM 244- 79	MCOL 1505	75.17	0.54	37.14	27.92	2	3
AM 312- 15	CM 849- 1	72.75	0.65	35.57	25.88	2	2
AM 266- 58	HMC 1	72.08	0.53	33.34	24.03	1	2
AM 266- 24	HMC 1	71.63	0.68	37.66	26.97	1	1
AM 312- 103	CM 849- 1	68.17	0.63	37.14	25.32	2	5
AM 266- 18	HMC 1	66.80	0.60	36.40	24.31	1	1
AM 312- 32	CM 849- 1	66.17	0.62	37.48	24.80	1	2
AM 266- 113	HMC 1	65.83	0.64	34.84	22.93	1	1
AM 312- 130	CM 849- 1	62.83	0.67	34.08	21.41	3	4
AM 312- 92	CM 849- 1	60.42	0.54	36.53	22.07	3	5
AM 266- 82	HMC 1	60.00	0.64	39.47	23.68	1	1
AM 244- 39	MCOL 1505	58.75	0.69	40.03	23.52	2	2
AM 312- 49	CM 849- 1	58.38	0.73	32.10	18.74	3	3
AM 266- 87	HMC 1	58.20	0.71	35.55	20.69	1	1
AM 312- 138	CM 849- 1	58.17	0.58	30.55	17.77	2	5
AM 266- 31	HMC 1	58.13	0.62	37.00	21.51	1	1
AM 244- 53	MCOL 1505	58.10	0.58	33.76	19.62	1	3
AM 244- 129	MCOL 1505	58.00	0.71	31.64	18.35	2	2
AM 277- 29	MTAI 1	57.83	0.45	34.40	19.89	4	4
AM 266- 19	HMC 1	57.50	0.76	33.51	19.27	2	2
AM 266- 17	HMC 1	57.33	0.61	36.68	21.03	1	2
		Statistics of best 25 inter-specific hybrids evaluated					
Maximum		98.00	0.76	40.03	32.91	1.00	1.00
Minimum		57.33	20.03	30.55	17.77	4.00	5.00
Average		66.70	0.63	35.61	23.78	1.68	2.32
Standard Dev.		10.90	0.07	2.41	4.29	0.85	1.38
		Statistics of 343 inter-specific hybrids evaluated					
Maximum		98.00	0.80	43.69	32.90	1.00	1.00
Minimum		0.50	0.03	18.69	0.00	5.00	5.00
Average		27.37	0.53	33.42	9.45	1.96	2.78
Standard Dev.		16.68	0.15	3.63	6.14	1.08	1.35

FY: Fresh yield; DMY: Dry matter yield; HI: Harvest Index; DMC: Dry matter content

Table 2 Best 25 genotypes for dry matter content in 6 S_1 families

Genotype	Parent	FY t/ha	HI	% DMC	DMY t/ha	Starch	Taste
AM 312- 140	CM 849- 1	32.75	0.56	43.69	14.31	3	5
AM 244- 95	MCOL 1505	34.75	0.68	42.93	14.92	1	2
AM 277- 44	MTAI 1	37.08	0.32	42.07	15.60	5	5
AM 244- 146	MCOL 1505	37.20	0.51	41.01	15.25	1	2
AM 244- 82	MCOL 1505	25.75	0.63	40.84	10.52	1	2
AM 244- 164	MCOL 1505	36.42	0.58	40.73	14.83	1	1
AM 244- 81	MCOL 1505	45.17	0.46	40.22	18.16	1	2
AM 244- 101	MCOL 1505	39.00	0.50	40.08	15.63	1	2
AM 244- 39	MCOL 1505	58.75	0.69	40.03	23.52	2	2
AM 244- 33	MCOL 1505	52.25	0.64	39.68	20.73	1	1
AM 312- 17	CM 849- 1	54.50	0.65	39.62	21.59	2	5
AM 244- 133	MCOL 1505	22.75	0.59	39.61	9.01	1	1
AM 266- 82	HMC 1	60.00	0.64	39.47	23.68	1	1
AM 244- 62	MCOL 1505	21.92	0.46	39.47	8.65	1	2
AM 244- 154	MCOL 1505	48.08	0.52	39.43	18.96	1	2
AM 312- 33	CM 849- 1	42.42	0.66	39.33	16.68	1	2
AM 244- 75	MCOL 1505	49.67	0.62	39.21	19.48	2	2
AM 244- 56	MCOL 1505	50.00	0.46	39.16	19.58	1	2
AM 244- 96	MCOL 1505	44.40	0.58	39.12	17.37	5	5
AM 266- 92	HMC 1	39.50	0.70	39.02	15.41	1	1
AM 244- 98	MCOL 1505	28.17	0.62	38.95	10.97	1	2
AM 244- 88	MCOL 1505	30.08	0.50	38.84	11.68	1	3
AM 266- 21	HMC 1	77.88	0.63	38.68	30.12	1	1
AM 266- 64	HMC 1	35.33	0.61	38.65	13.66	1	2
AM 244- 124	MCOL 1505	29.92	0.58	38.64	11.56	1	1
		Statistics of best 25 inter-specific hybrids evaluated					
Maximum		77.88	0.70	43.69	30.12	1.00	1.00
Minimum		21.92	0.32	38.64	8.65	5.00	5.00
Average		41.35	0.57	39.94	16.48	1.52	2.24
Standard Dev.		13.09	0.09	1.31	5.05	1.16	1.33
		Statistics of 343 inter-specific hybrids evaluated					
Maximum		98.00	0.80	43.69	32.90	1.00	1.00
Minimum		0.50	0.03	18.69	0.00	5.00	5.00
Average		27.37	0.53	33.42	9.45	1.96	2.78
Standard Dev.		16.68	0.15	3.63	6.14	1.08	1.35

FY: Fresh yield; DMY: Dry matter yield; HI: Harvest Index; DMC: Dry matter content

Two families, AM266 and AM312 account for 83% of the best 25 genotypes for fresh yield in the clonal observation trial (Table 2). A genotype from AM266 had maximum yield of 98 t/ha, this same family produced the genotype with the highest yield in the seedling trial albeit different genotypes, underscoring the big error that can occur when evaluation is based on a single plant and the effects of inter-genotypic competition. The family AM312 accounted for 67% of the best 25 genotypes for dry matter content. The trend observed in the data from the second year evaluation that individuals from certain S_1 families tend to dominate the group of best genotypes for certain traits highlights the important role additive genes play in the expression of these traits.

Future Perspectives

- Establishment and evaluation of S_2 progenies

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

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Introduction

Cassava, which is a staple crop for millions of people in the tropics, represents the basis for food security in several marginal regions. Studies performed by CIAT scientists in the past, however, showed that farmers' yields usually range from 10-12 t/ha in Colombia; whereas experimental yields have reached as high as 66 t/ha/yr. This is mainly due to diseases such as cassava bacterial blight (CBB), anthracnose, Phytophthora root rot, Fusarium root rot, African cassava mosaic and cassava frogskin.

CBB is a major disease caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). The most suitable and practical approach for controlling CBB is through host resistance. The development and release of resistant germplasm have been limited in the past for lack of knowledge on the genes involved in resistance and defense, the inheritance of important traits and the interaction of resistance genes with the environment.

The development of genomics and bioinformatics tools is increasing our knowledge of plant genome structure, organization and gene function. An understanding of the factors leading to incompatibility in the cassava–pathogens interaction will help clarify the rapidly expanding

knowledge of host resistance/susceptibility. Our goal is to identify those important factors that contribute to incompatibility in cassava to *Xam.*

Materials and Methods

Plant material, inoculations and RNA extraction. Two resistant (M Bra 685 and SG 107-35) and a susceptible (M Col 1522) cassava cultivar were inoculated with strains CIO 151 and CIO 46, respectively. Four-week old plants were inoculated by stem puncture with 10^8 ufc/ml. Stem tissues were collected at 6, 12, 24, 48, 72 h, 7 and 15 days postinoculation (pi). The controls were healthy noninoculated plants and plants inoculated with sterile water (mock-inoculated).

The tissue was ground in liquid nitrogen, and total RNA was isolated according to the Proteinase K method (Hall et al., 1978 and Rocha, 1995).

Construction of cDNA libraries. Six cDNA libraries were constructed as follows: Suppression subtractive hybridization (SSH; Diatchenko et al., 1996) was used to generate three cDNA libraries, enriched for sequences expressed specifically in infected stems of cv. M Bra 685, M Col 1522 and SG 107-35 at 6, 12, 24, 48 and 72 h, 7 and 15 days pi. cDNA from healthy and mock-inoculated plants (from tissue collected at the same time points as the infected tissue) was used as the driver to remove common sequences. Three libraries (corresponding to three inoculated cassava cultivars) were constructed using the same protocol, but no hybridizations were carried out.

cDNA microarray technology. Microarray experiments utilized arrays prepared in the Project SB-02 (Assessing and utilizing agrobiodiversity through biotechnology) laboratory. The slides were polysine coated (Erie Scientific Co.). The PCR product from each clone was microarrayed onto glass slides.

After the spotting, slides were snap dried on the thermocycler at 95°C for 10 sec, UV cross-linked at 650 μJ, and dried at 80°C for 1 h. Microarray probing was done as per Hedge et al. (2000) with some modifications. Slides were then scanned at CIAT facilities and analyzed using ArrayPro software. The entire experiment was repeated to ensure accurate gene expression profiles. In order to assess spot variations within slides, the spots on each slide were printed 4 times.

Data analyses. Spot densities from scanned slides were quantified by using Array-Pro software (media Cybernetics). Local background was calculated with the local-ring technique. Scans for the two fluorescent probes were normalized following the protocol proposed by (http://afgc.stanford.edu/~finkel/talk.htm).

Results and Discussion

Three cassava cultivars were selected to construct the cDNA libraries. In the susceptible cv. M Col 1522, symptoms were observed very rapidly pi with the highly aggressive *Xam* strain, CIO 46. In the highly resistant SG 07-35, an incompatible interaction was observed when challenged with strain CIO-151. The M Bra 685 Cv. also showed a high level of resistance when inoculated with CIO-151 even though some symptoms were observed at 7 or 15 days pi.

Six cDNA libraries were constructed for each cultivar: three subtracted and three nonsubtracted. A total of 1920 clones were obtained from the six libraries as follows: 384 clones for each library of M Bra685, subtracted and not subtracted; 384 clones for each subtracted library made of SG

107-35 and M Col 1522; 288 clones of the nonsubtracted library made from SG 107-35; and 96 clones from the nonsubtracted library of M Col 1522.

In a first experiment, the three nonsubtracted libraries were spotted. Microarrays containing 768 clones were constructed. Each glass slide contained two copies of the entire array, each of which consisted of 2 subarrays with 10 rows and 21 columns. Slides were hybridized with cDNA from healthy tissue (Cy3) and with cDNA from inoculated tissue collected 48 h pi (Cy5) (Figure 1). A very low signal was detected for the Cy-3 labeling in the hybridizations. Efforts are now being made to troubleshoot this problem and analyze data for all time points. However, an analysis was made in order to identify clones that were significantly expressed in the inoculated tissue. Figure 2 shows that most of the genes have greater intensities at Cy-3. Differential expression in the inoculated tissue compared with the healthy control. Of the 94 spots, however, only 5 showed the differential expression in both replicates within the array and were sequenced. This low number of differentially expressed genes after inoculation can be due in part to the high background in blocks 1 and 2 of the array (Figure 1). There is also a need to perform more replicates of the hybridization, as well as a hybridization using Cy-3 to label RNA extracted from infected material and Cy-5 for RNA extracted from healthy tissue (a reverse hybridization experiment).

The five clones sequenced showed the following homologies when compared to the EST and nonredundant databases of GenBank using the Blastn and Blastx algorithms, respectively. Two sequences showed no significant homologies to sequences in the GenBank, one sequence showed homology to an *Arabidopsis thaliana* protein with an unknown function, one sequence was homologous to a *Phytopthora infestans*-challenged leaf of a *Solanum tuberosum* cDNA clone (e value = 3e-7), and the fifth clone was homologous to an expansin protein (e value = 3e-88). Expansins are a group of extracellular proteins that directly modify the mechanical properties of plant cell walls, resulting in a turgor-driven cell extension. Their role in defense has been reported (Lee et al., 2001). Oligosaccharides released from cell walls are also potent elicitors of plant defense.

Figure 1. **Cassava cDNA microarray. cDNAs from the *Xam*-inoculated cassava cv. M Bra 685, M Col 1522 and SG 197-35. cDNAs were spotted in a 4 x 4 format using a 4-pin head. The image is a two-color overlay obtained with green–control tissue (fluorescently labeled Cy-3) and red–treated tissue (fluorescently labeled Cy-5) co-hybridized with a single microarray.**

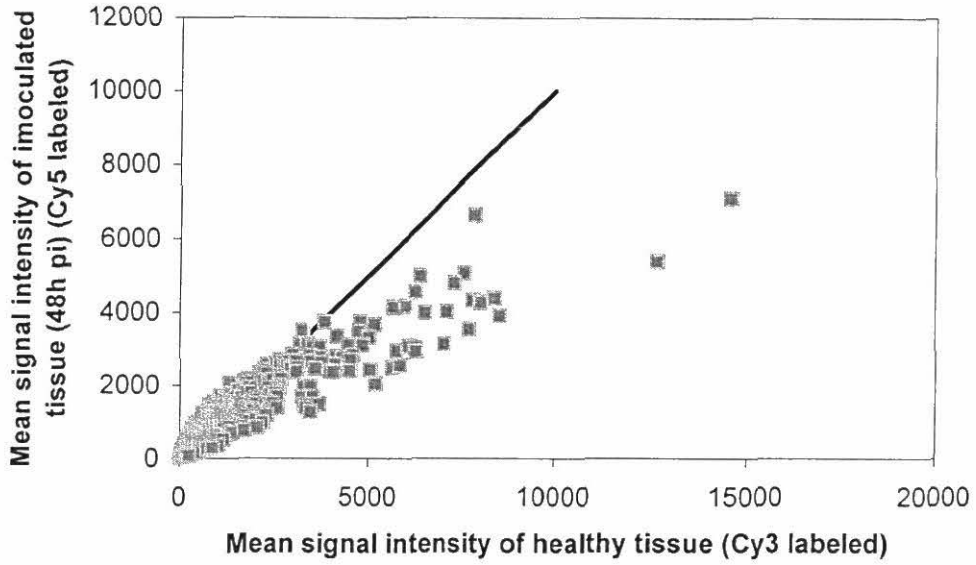

Figure 2. Scatter plot comparing the spot intensities in hybridizations with probes from healthy tissue (Cy-3 labeled, X axis) and inoculated tissue collected 48 h pi (Cy-5 labeled, Y axis). Data from images of both Cy-3 and Cy-5 were plotted as the signal intensity after normalization.

Future Activities

- Hybridize arrays with RNA extracted from tissue collected at different pi time points
- Confirm the differential expression of the characterized clones by a RT-PCR or a Northern blot analysis

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1.2.19 Identification of QTLs for yield and yield components in rice: Populations derived from backcrosses between wild species and cultivated rice

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Introduction

In rice, twenty-one wild species and two cultivated species (*O.sativa* and *O. glaberrima*) represent wide genetic variability for rice breeding programs. It has been suggested that the *Oryza* wild species represent a potential source of new alleles for improving yield, quality, and stress resistance of cultivated rice (Xiao et al. 1998). However, limited use of this variability has been made. Barriers still exist in effectively utilizing genes from wild species. Advanced backcross populations and molecular mapping techniques are good alternatives for introgression of these genes and to detect these new alleles in segregating populations.

This report focuses on progress made in identifying quantitative trait loci (QTLs) associated with yield increase in a double haploid population derived from a *Oryza glaberrima* x O.*sativa* cross.

Materials and methods

Experiments were set up under greenhouse, and field conditions, as well as in the anther culture and the CIAT Biotechnology laboratories. Methods described by Tanksley and Nelson. 1996, and Lentini et al 1997 were used to develop the double-haploid lines (DHs) from a BC3F1 population, as shown in Figure1.

An improved rice cultivar Caiapo was used as the recurrent parent whilst *Oryza glaberrima* (IRRI, accesion #103544; Jones, 1996) was used as the donor parent. Very high sterility was found in each generation. The BC_3F_1 population was processed by anther culture (Lentini *et al.* 1997) to obtain the DH BC_3F_1 population (900 DHs were generated) but only 312 DHs were selected for field testing and molecular tests (Figure 1).

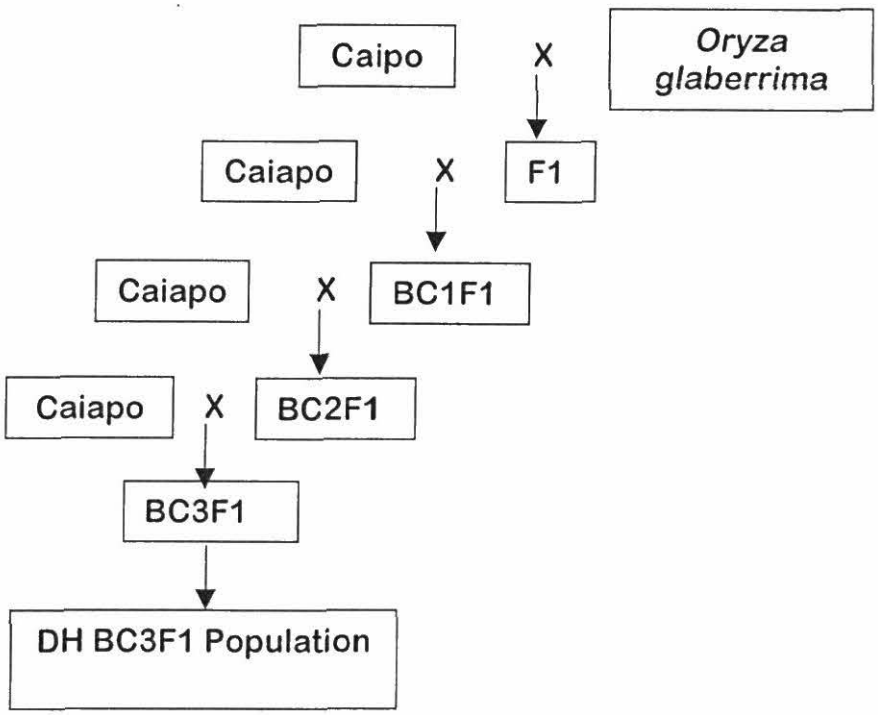

Figure 1. Generation of DH BC3F1 population, derived from Caiapo and *Oryza glaberrima* parentals.

Plants from each DH were planted in the greenhouse. Young leaves (0.2gr) were collected for DNA extraction by the Dellaporta method (McCouch et al. 1988) and subsequent molecular assays were performed.

From 209 SSRs screened in the parents Caiapo and *O. glaberrima* reported by Temnykh *et al.*, 2000 (Figure2), 100 SSRs were selected for PCR standarization and screening of the 312 DHs. (Figure 3). The 312 DHs were yield tested and phenotyped under irrigated conditions. A completely randomized block design with three reps was used, and two-row plots, 5-meter long and 30x30 spacing were planted.

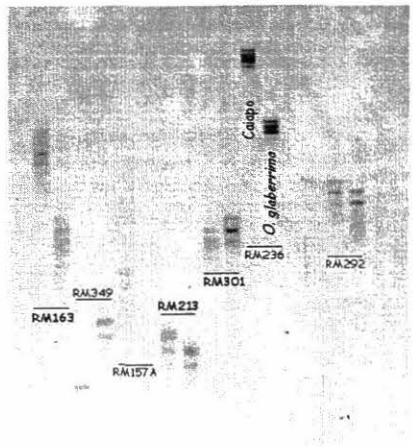

Figure 2. Screening of SSR in the parents Caiapo and *Oryza Glaberrima*. Polyacrilamide 6% gel, 3M Urea, Rel 19:1, 0.5X TBE. 110W/cm2, 2Hours

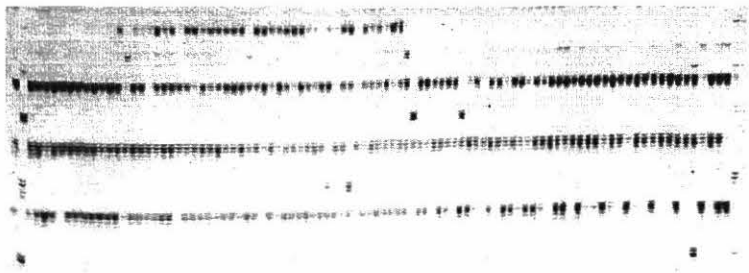

Figure 3. Screening of RM234 rice microsatellite in the 312 individuals of the DH BC3F1 progeny. Caiapo and *O. glaberrima* were placed at the beginning and at the end of each plot of 92 individuals. Gels were loaded four times to complete the whole population. Polyacrilamide 6% gel, 3M Urea, relat 19:1, 0.5X TBE. 110W/cm2, 2 hours.

Statistical Analysis

A normality test ("S" statistical test of Bowman and Sheriton, 1975) was carried out for each quantitative trait (Lynch, M. and Walsh, B. 1998). The order of the microsatellites in the molecular map was defined by the Cornell published molecular rice map (Temnykh, *et al.* 2000). Chi squared test ($\propto = 0.05$) was used to calculate the segregation percent of each marker and to define deviations of the Mendelian proportions.

A linear regression with 1000 permutations was used to identify association between molecular markers and traits by QTL Cartographer program 1.16 (Basten, et al. 2002).

Results and discussion

Plant height, number of panicles per plant, panicle length, 1000 grain weight and % sterility were taken on five plants/ rep in each DH line (Table 1) whilst grain yield was taken on a plot basis. Forty eight percent of the 209 SSRs were polymorphic and the molecular characterization of the 312 DHs showed that all of them were homozygous either for Caiapo or O.*glaberrima.* Introgression of O.*glaberrima* alleles occurred throughout the whole genome. Positive transgressive segregation was observed in all traits measured, except for panicle number (Figure 4).

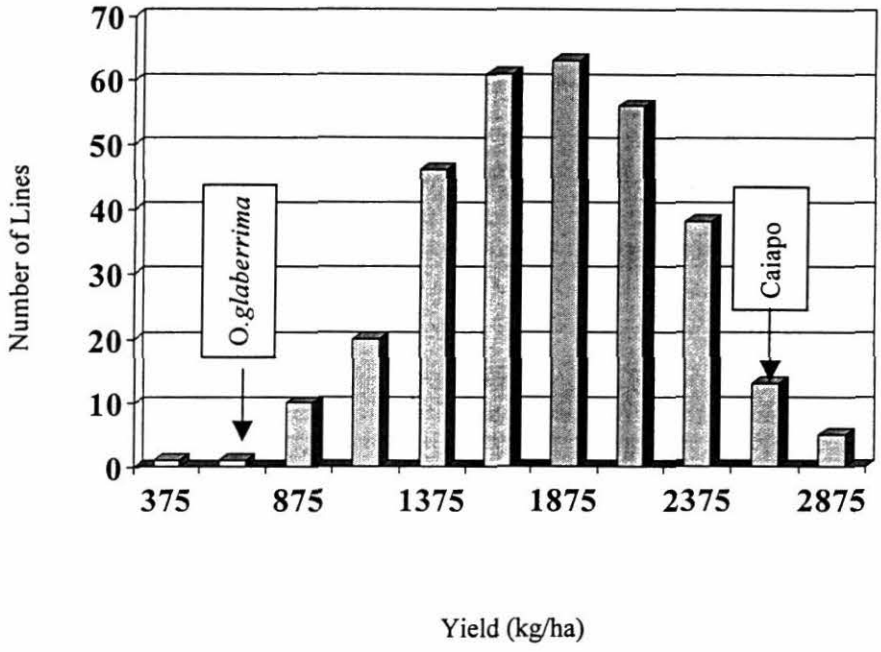

Figure 4. Frecuency distribution of grain yield (kg/ha) in 312 DHs derived from BC3F1 population from the Caipo / *Oryza glaberrima* cross.

Based on the 100 microsatellites from the RF- Cornell framework map screened on 312 DH lines, 70 putative linkages were identified for yield, and yield components. However, only 3 of these were confirmed by the 1000 permutations analysis. Results using QTLcartographer software, indicated associations of RM127 marker on chromosome 4 with plant height, RM283 marker on chromosome 1 with panicle sterility, and RM292 marker on chromosome 1 with grain yield. Data suggested that 50% of the putative linkage (35) with a positive effect on the trait of interest was due to *O. glaberrima* alleles. Results obtained for grain yield on chromosomes 1 and 5 showed similar associations to results from Moncada *et al.* 2001 and Ishimaru *et al.* 2001; other associated markers in this study were observed in different studies (Castaño, C. 2002).

Main achievements

Laboratory bottlenecks that affect the process of molecular characterization and statistical analysis were identified and preventive measures were put in place to improve the efficiency of the overall process.

Table 1. Yield and plant traits measured in some doubled-haploid lines derived from the cross Caiapo/O.glaberrima. Relative value compared to Caiapo (%)

PEDIGREE	YIELD kg/ha	*YIELD*	PLANT *HEIGHT*	*NUMBER OF* PANICLES	PANICLE LENGTH	PERCENT OF STERILITY	WEIGHT OF 1000 SEEDS
CT16330(1)-CA-2-M	2896	108		115	101	128	
CT16333(1)-CA-15-M	2809	105	107	165	101	190	
CT16338-CA-7-M	2775	103	102	111		138	102
CT16315(1)-CA-11-M	2771	103		135		124	
CT16346-CA-17-M	2771	103		131		195	
CT16342-CA-5-M	2721	102	106	146		314	
CT16342-CA-6-M	2715	101	105	146		286	100
CT16338(1)-CA-19-M	2714	101	101	108		147	102
Caiapo	**2679**	**100**	**100**	**100**	**100**	**100**	**100**
Oryza glaberrima	466	17	70	269	77	176	68

On-going activities

Standardize and evaluate more SSRs markers in non-saturated regions on the map of the DH BC3F1 population to confirm the 67 putative linkages and to identify new associations.
Complete the statistical analysis of other populations from the project, like the BC2F2 population derived from BG90-2 / *Oryza rufipogon* and the BC3F2 population derived from Lemont/ *Oryza Barthii*.

Initiate the analysis of agronomic and molecular data from different generations obtained of the BG90-2 / *O. rufipogon* cross, which were sowed in the same location, to determine the environmental effect and to look for gene introgression from the wild species in these different populations.

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

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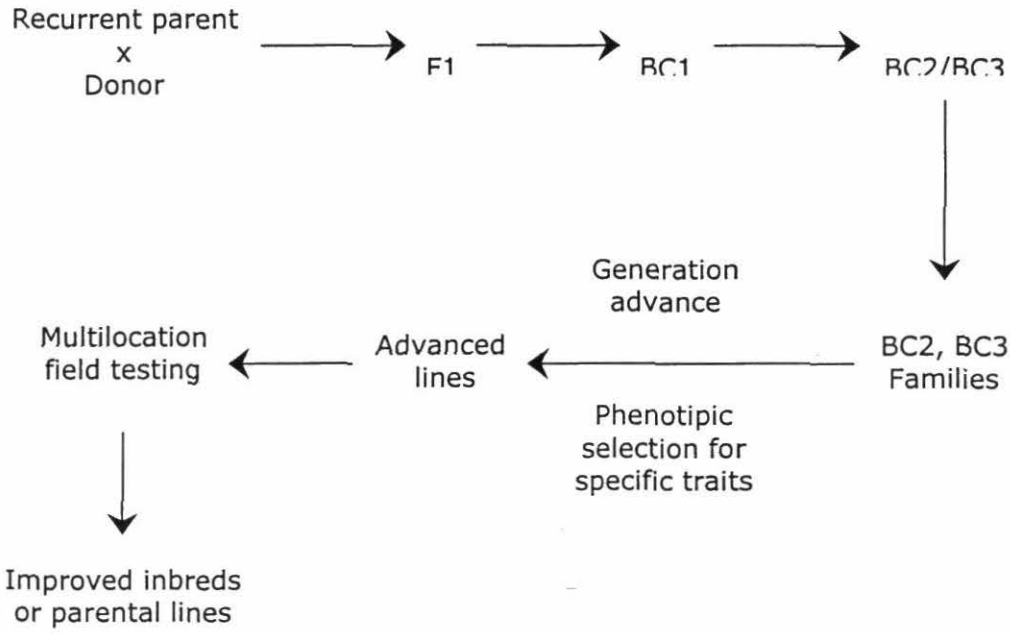
Introduction

In spite of the great impact made in rice production in Latin America (LAC) there is a need to increase it in a sustainable way. New alleles can provide genetic variability for crop enhancement. The Oryza wild species represent a potential source of new alleles for improving the yield, quality and stress resistance of cultivated rice. The purpose of this paper is to provide increasing evidence that certain regions in *O. rufipogon* and *O. glaberrima* harbor genes of interest for the genetic improvement of cultivated rice.

Materials and Methods

Breeding scheme. Improved varieties (Bg90-2, Oryzica 3 and Caipo) were used as recurrent parents whilst *O. rufipogon* (IRGC105491), and O. *glaberrima* (IRGC103544) were used as donors in an interspecific-backcross breeding scheme shown below.



Replicated yield trials. Twenty eight lines derived from the cross Bg90-2/*O. rufipogon* were planted in replicated yield trials in six locations under irrigated conditions in Colombia. Transplanting was done in CIAT whilst direct seeding was done elsewhere. A completely randomized design with three reps was used. Data on main agronomic traits, including grain yield, was taken. A two-way analysis of variance was used for the analysis of grain yield, whilst a GEBEI package that implements appropriate clustering and ordination procedures was used in a preliminary analysis of the GxE data.

Evaluation for tolerance to biotic stresses. Advanced breeding lines from the cross Oryzica 3/*O. rufipogon* were field tested in a "hot spot" for reaction to three diseases in Tolima, Colombia, using the Standard Evaluation System for Rice and three reps.
Doubled-haploid lines derived from the cross Caiapo/*O. glaberrima* were evaluated in a "hot spot"area in Meta, Colombia, for tolerance to the Rice Stripe Necrosis Virus.

Selection for grain quality. Advanced breeding lines were evaluated for grain length and translucency in the CIAT Quality Lab.

Results

Grain yield. Data are presented in Figure 1. Statistical analysis showed no significant yield difference in grain yield between Bg90-2 and its progenies in each location.

Although none of the interspecific lines was excellent in all locations, a few lines performed better than Bg90-2 suggesting that there was a good genetic variability present in this group of lines. A few lines performed as well as Fedearroz 50, the highest yielding variety planted by farmers in Colombia.

However, the GxE interaction was very high (75%), suggesting that the performance of genotypes was dependent on the climatic/soil conditions found in each location (data not shown).

Tolerance to biotic stresses. Some fungal diseases, particularly Rhizoctonia sp., Sarocladium and Helminthosporium sp., considered of minor importance in the past are now causing yield losses in several areas in LAC. Most commercial varieties grown are susceptible. Results from field tests suggest that lines derived from the cross Oryzica 3/*O. rufipogon* showed a good tolerance level to these diseases (Table 1).

The fungus *Polymyxa graminis* transmits the rice stripe necrosis virus (RSNV), disease first reported in Ivory Coast in 1977; it was reported in Colombia in 1991 and now is found in Panama and Brazil. All commercial varieties are susceptible to RSNV (Table 2); however, high level of tolerance was found in *O. glaberrima.* Our results show that tolerance to RSNV has been successfully transferred to improved varieties.

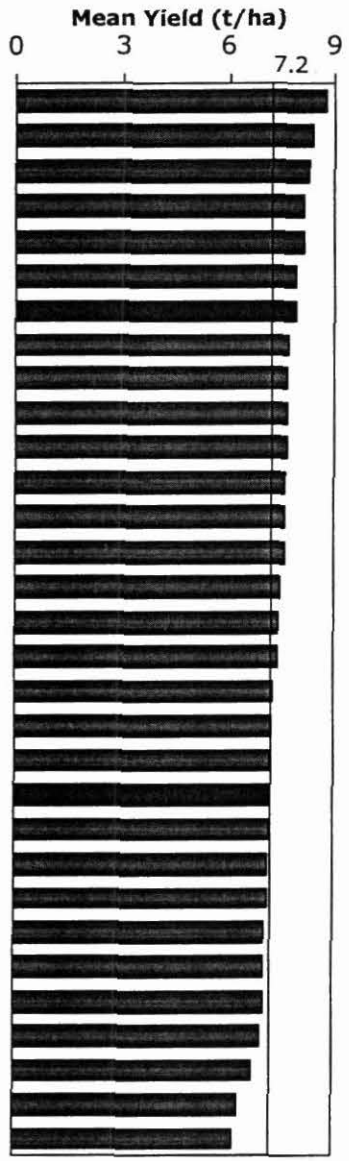
Table 1. Tolerance to several diseases of advanced lines from the cross Oryzica 3/*O. rufipogon* under field conditions in Saldaña, Tolima. Fedearroz 2002.

Pedigree	Rhizoc.1/	Sarocl. 1/	Helm. 1/	Helm 2/
CT14524-2-M-2-M	**3**	**3**	**5**	**15**
CT14524-2-M-3-3	**3**	**3**	**5**	**15**
CT14529-12-M-1-2	**3**	**1-5**	**5**	**0**
CT14529-12-M-2-3	**3**	**5**	**7**	**30**
CT14529-18-M-3-M*	**3**	**3-5**	**3**	**0**
CT14529-18-M-4-M*	**3**	**1-5**	**3**	**20**
CT14534-12-M-1-3	**5**	**3**	**7**	**0**
CT14534-12-M-3-4*	**3**	**1**	**1**	**0**
CT14534-12-M-4-1	**5**	**3**	**1**	**0**
CT14537-8-M-4-M	**3**	**1**	**1**	**0**
CT14537-9-M-4-1*	**3**	**5**	**3**	**0**
CT14537-21-M-6-3	**3**	**3**	**3**	**0**
CT14539-31-M-1-1*	**3**	**5**	**3**	**0**
CT14539-34-M-4-M-2*	**3**	**3**	**3**	**0**
Oryzica 3 (Check)	**7-9**	**5-7**	**1-3**	**0-20**
CT14524-3-M-2-2	**7-9**	**7**	**5-7**	**40**

1/ Scale 1-9. IRRI Standard Evaluation System
2/ % Panicle neck infection
• Lines selected by Fedearroz

Figure 1. Performance[1/] of breeding lines derived from Bg90-2/*O. rufipogon* in farmers' fields in six locations, 2002

Line	Location[2/]					
	1	2	3	4	5	6
CT13941-27-M-11-6-M-M	1.13	0.96	1.00	–	--	–
CT13941-27-M-15-2-M-M	0.98	1.03	1.00	–	--	–
CT13941-11-M-25-5-M-M	1.07	1.21	1.17	1.20	0.96	1.52
CT13958-13-M-17-5-M-M	1.05	1.02	1.26	1.24	0.98	1.40
CT13941-11-M-25-1-M-M	1.04	1.20	1.18	1.31	0.83	1.51
CT13946-26-M-5-3-M-M	1.09	1.11	1.05	0.94	1.01	1.40
Fedearroz 50	0.97	1.10	1.05	1.52	1.00	1.15
CT13941-27-M-11-5-M-M	0.98	0.87	0.89	–	--	–
CT13941-11-M-25-4-M-M	0.98	1.05	1.04	1.37	0.91	1.30
CT13958-13-M-7-5-M-M	1.11	1.07	1.06	0.87	0.99	1.18
CT13956-29-M-29-2-M-M	1.08	1.14	1.01	1.04	0.93	1.27
CT13946-26-M-5-6-M-M	1.06	0.89	1.11	1.34	0.82	1.25
CT13958-13-M-2-3-M-M	1.06	1.18	1.16	1.19	0.82	1.02
CT13956-29-M-14-1-M-M	1.02	1.12	1.10	1.23	0.74	1.38
CT13959-3-M-10-5-M-M	1.13	1.03	0.99	0.94	1.02	1.16
CT13956-29-M-8-3-M-M	1.02	0.80	1.08	0.94	0.94	1.24
CT13958-12-M-1-7-M-M	1.13	0.85	1.01	0.81	1.02	1.35
CT13941-27-M-15-3-M-M	–	--	--	1.53	1.04	1.16
CT13958-13-M-26-5-M-M	1.07	1.11	0.94	1.09	0.87	1.01
CT13958-13-M-2-1-M-M	1.04	1.12	1.04	0.76	0.85	1.07
Bg90-2	1.00	1.00	1.00	1.00	1.00	1.00
CT13958-13-M-2-4-M-M	1.05	1.13	1.02	1.06	0.80	0.93
CT13958-13-M-26-4-M-M	1.05	1.00	1.03	0.94	0.83	1.13
CT13958-13-M-33-1-M-M	1.11	1.01	0.93	0.94	0.80	1.22
CT13976-7-M-14-1-M-M	1.03	0.98	0.93	0.82	0.97	1.03
CT13941-27-M-19-1-M-M	0.96	1.00	0.98	1.21	0.76	1.13
CT13959-3-M-10-4-M-M	1.05	0.79	0.96	0.97	0.93	1.16
CT13956-29-M-25-7-M-M	0.99	0.94	1.10	0.77	0.76	1.27
Local checks	0.95	0.60	0.87	1.14	0.78	1.16
CT13941-27-M-5-4-M-M	–	--	--	1.27	0.89	1.07
CT13941-27-M-4-1-M-M	–	--	--	1.08	0.80	–



1/ Relative value compared to BG90-2

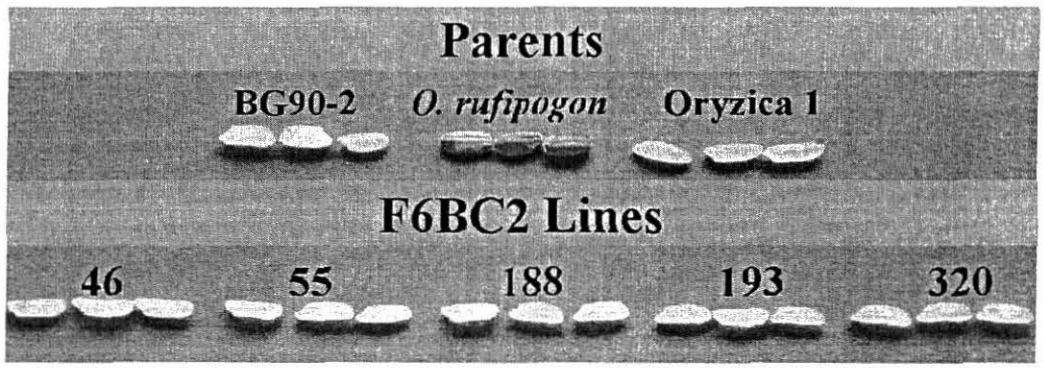
2/ Location 1 = Aceituno 2 = Ciat 3 = Montería
4 = Jamundí 5 = Saldaña 6 = Villavicencio

Table 2. Tolerance to Rice Stripe Necrosis Virus in doubled-haploid lines derived from the cross Caiapo/*O. glaberrima* under field conditions. Meta, Fedearroz 2002.

Pedigree	%Diseased Plants
1. CT16322-CA-6	2.4
2. CT16323-CA-3	5.1
3. CT16311(2)-CA-3	5.1
4. CT16318-CA-3	5.8
5. CT16308-CA-3	6.5
6. CT16322-CA-7	7.0
7. CT16313-CA-16	7.3
O. glaberrima (ACC #103544)	2.0
CG-20 (*O. glaberrima* A)	2.4
Cimarrón (Check)	40.0
O. Caribe 8 (Check)	14.0

Grain quality. Development of high yielding varieties with tolerance to biotic stresses and excellent grain quality is the main breeding objective of national rice programs in LAC. Both parents, Bg90-2 and *O. rufipogon*, posses poor grain quality. However, positive transgressive segregation for superior grain quality was observed in the BC2F2 generation which led to the selection of advanced lines with long and slender, translucent grains (Figure 2).

Figure 2. Advanced lines with excellent grain quality were derived from the cross Bg90-2/*O. rufipogon*



Discussion

It has been shown (Xiao et al, 1998; Moncada et al, 2001) the Oryza wild species represent a potential source of new alleles for improving the yield, quality and stress resistance of cultivated

rice. Our results from evaluations under greenhouse and farmer's fields confirm that *O. rufipogon* and *O. glaberrima* possess alleles with positive effects on yield, stress resistance and grain quality. Molecular markers are being used to map the QTL in various crosses and near isogenic lines are being developed for further use in breeding programs.

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1.2.21 Selecting apomictic genotypes of *Brachiaria* using the N14 *SCAR* marker

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Introduction

Apomixis is a trait of great potential impact as a plant breeding tool in agriculture because even highly heterozygous genotypes breed true through seed, retaining their vigor. The common mode of reproducing commercial *Brachiaria* is by facultative apomixis. A SCAR marker derived from RAPD N14, closely linked to the apomixis gene (6cM) (Rocha et al., 1997), was chosen for MAS purposes due to its stability and easy handling in PCR processes, which allow high-throughput screening.

Materials and Methods

Young leaf tissue was collected and dried at 40-50°C for 18-20 h. Tissue grinding was performed in a Mixer Mill MM-2 (Retsch, Inc). DNA was extracted from 30-50mg of tissue as follows: 600 µl of 200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5% SDS and 1% β-mercaptoethanol were mixed for 10 sec. Then 200 µl of 5 M cold potassium acetate was added, and the tubes were held on ice for 10 min. After 10 min centrifugation at 14000 rpm, the DNA was precipitated overnight at –20°C, by adding 400 µl of cold isopropanol to 500 µl of the supernatant. The DNA was pelleted by centrifugation at 14000 rpm for 10 min, washed with 70% ethanol, dried for 3-5 min in a Speedvac and dissolved in 50 µl of 10 mM Tris-HCl pH 8.0. Then 5 µl of the extracted DNA was diluted in 45 µl of sterile water.

PCR reactions were carried out in a final volume of 25 µl as follows: 5 µl of diluted DNA was added to a mix containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 µM of each forward and reverse primer, and one unit of *Taq* polymerase. The PCR profile included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of a

denaturation step at 94°C for 30 sec, an annealing step at 55°C for 30 sec, and an extension step at 72°C for 1 min. PCR products were resolved in a 0.5X TBE agarose gel with ethidium bromide at a final concentration of 0.02 µg/ml. The presence or absence of the SCAR linked to the apomixis gene was scored.

Results and Discussion

The presence or absence of the N14 marker was scored in 121 maternal genotypes BR00NO. The plants having the N14 marker were also identified as apomictic according to the progeny test conducted in the field. The rest of the plant material (not showing the N14 marker) showed sexual behavior in the field. The concurrence of the results from both lab and field tests shows the potential use of this marker for indirect selection of apomictic or sexual genotypes in order to improve *Brachiaria* breeding.

A set of 375 hybrid clones obtained by crossing 40 sexual genotypes as female parents with the genotype CIAT 16320 (*B. brizantha*) were also evaluated for the presence of the N14 SCAR. When the SCAR was run, the presence/absence ratio was approximately 1:1, with 190 plants showing the DNA fragment. These results will be compared with the progeny test to be conducted in field. After evaluating these materials for resistance to *Rhizoctonia,* the sexual clones identified will continue in the breeding scheme.

Ongoing Activities

Given that tissue grinding is still one of the bottlenecks in speeding up MAS breeding in *Brachiaria*, efforts are being made to overcome this situation. Two 96-microtubes are being adapted to the Retsch Matrix Mill MM-2 racks, which will make it possible to grind 192 samples at a time, by adding stainless steel beads (3-mm diam.) inside each microtube. We have observed that differences in tissue hardness among samples result in uneven grinding of the tissue; thus we are attempting to make changes in the sample collection to prevent this problem.

References

Rocha et al., 1997.

1.2.22 Identification of molecular markers associated with gene(s) conferring tolerance to aluminum toxicity in a *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrid population

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[1]SB2 ; [2] CAMBIA; [3]IP-5; [4] IP-5-IP1-PE-2

Introduction

Aluminum (Al) toxicity is one of the most important constraints to crop production on acid soils (Rao et al., 1993). Signalgrass (*B. decumbens* Stapf cv Basilisk) is one of the most widely sown forage grass in the tropics, with 26.4 million ha in Brazil alone. Unlike most food crops, it is directly derived from a wild apomitic germplasm accession that is highly resistant to Al and well adapted to infertile acid soils (Sanchez and Salinas 1981; Paulino et al., 1987). Ruzigrass (*Brachiaria ruzziziensis* Germain and Evrard cv Common), a closely related species of the same

agamic complex, is less Al resistant (Miles and do Valle 1996). Previous experiments have established significant differences in aluminum (Al) tolerance between *B. decumbens* (resistant) and *B. ruziziensis* (sensitive) (Wenzl et al., 2001). The main objective of this work is to identify microsatellite (SSR) markers associated with the gene or (genes) that confer aluminum tolerance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* cross.

Materials and Methods

Plant material: A parent *B. decumbens* 606 (tolerant) and *B. ruziziensis* 44-2 (sensitive) were used to generate hybrid population of 280 individuals.

DNA Extraction: DNA was isolated using the protocol described by Edwards et al., (1991) and modified by Quintero et al., (2000). DNA was quantified on a DYNA QUANT 200 fluorometer (Hoffer Scientific Instruments, San Francisco CA). Quantified DNA was diluted to a final concentration of 5 ng/ul. Then 25 ng of DNA was used for PCR.

Microsatellites: The isolation of microsatellites and the methodology for PCR amplification and evaluation of polymorphism have been described previously by Gaitan et al., (2000). We are using silver staining to visualize the allelic segregation of the markers.

Results and Discussion

We started the evaluation of the segregating F1 population (280 individuals) using 39 Simple Sequences Repeat SSR that showed polymorphism (Table 1 and Figure 1), to find markers associated to aluminum resistance for mapping and ultimately cloning the resistant genes.

Table 1 Polymorphic SSRs for evaluation of the segregating 44-2 x 606 cross.

SSR	Size (bp)	SSR	Size (bp)	SSR	Size (bp)	SSR	Size (bp)	SSR	Size (bp)
GM1	190-210	GM22	190-230	GM49	190-215	GM97	160-210	GM117	200
GM2	200-230	GM24	140-200	GM58	190-260	GM98	240-300	GM118	200
GM3	190-210	GM28	190-220	GM71	125-130	GM99	130-190	GM122	125-150
GM6	120-150	GM33	220-260	GM75	155-180	GM105	200-250		
GM7	150	GM36	230-240	GM79	90-145	GM106	180-200		
GM8	125-140	GM37	210-250	GM80	160-180	GM107	210-300		
GM11	175-185	GM40	200-290	GM88	90-130	GM108	130-180		
GM12	135-145	GM44	80-125	GM90	90-140	GM109	130-155		
GM18	140-180	GM46	180-240	GM91	160-180	GM116	190-230		

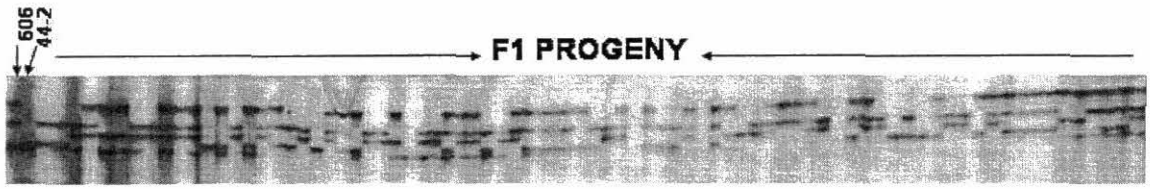

Fig 1 SSR 24 EVALUATED IN F1 OF 44-2 X 606 CROSS

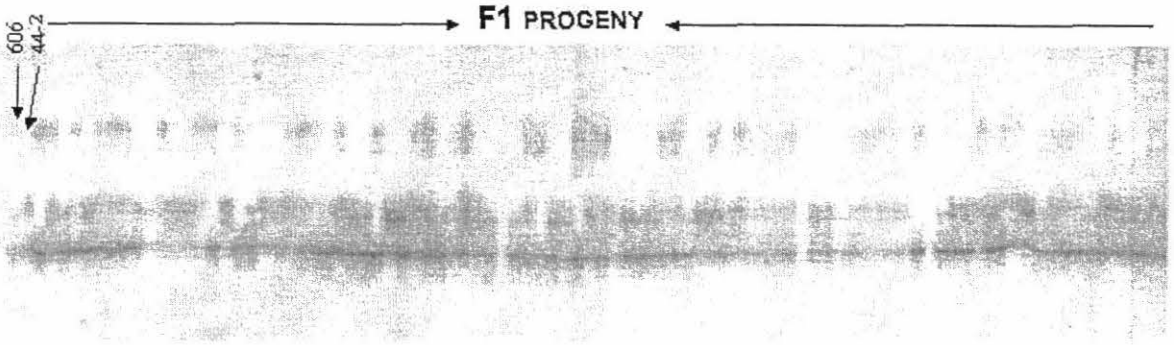

Fig 1 SSR 8 EVALUATED IN F1 OF 44-2 X 606 CROSS

Linkage analysis: The Segregation of Single Dose Restriction Fragments (SDRF) will be determined by departure from the hypothesis of 1:1 segregating ratio by the Chi-square test. The data matrixes obtained for presence or absence of bands will be analyzed with MAPMAKER v3.0b for PC Lander et al. (1987).

Future Perspectives

- Establishment and evaluation of S_2 progenies

-Evaluate both parents to look for more polymorphisms.
-Finish the evaluation of the SSR in the F1 population.
-Analysis of the data to construct a linkage map for Aluminum resistance.

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

Main Achievements

- Genomic resources (principally cDNA libraries and expressed sequence tags) have been developed for common bean roots and nodules under abiotic stress, concentrating on genes expressed during phosphorous deficiency. Other abiotic stress tolerance genes are likely to be found in these sequences and cDNA libraries.

- Using a candidate gene approach, locate a genetic region in common bean (*Phaseolus vulgaris*) that correlates well with resistance to angular leaf spot was located. The region contains a cluster of Resistance Gene Analogs (RGA) of the TIR-NBS-LRR type. Sequencing of a BAC clone (about 80 kb long) covering part of the cluster has been completed.

- New SSR markers were used to increase the saturation of the cassava molecular genetic map using PCR-based markers. Out of the total of 157 SSR markers, the genome loci corresponding to144 of them or 92% were successfully amplified.. In all, 59 out of these 144 markers, or 38%, were polymorphic and therefore used in screening the mapping population. 20% of these polymorphic SSR markers formed allelic bridges. From the present work, 52 of these new SSR markers were placed on the existing molecular genetic framework map of cassava with 47 of them being uniformly distributed over 16 of the existing 18 linkage groups. Also, 5 of these were linked to a group of markers that are yet to be assigned to any of the existing linkage groups.

- Expressed Sequence Tags for cassava starch were generated. A total of 10, 368 clones were isolated from CM523-7 from MPer183. A total of 3770 unique clones' sequences were obtained. A number of interesting homologies were obtained between some of these sequences and starch biosynthesis related genes in the Genbank.

- Differentially expressed sequences were isolated in resistant *Brachiaria* plants infested with spittelbug.

1.3.1 Analysis of cDNA libraries from common bean roots under abiotic stress

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Background

Expressed sequence tags (ESTs) have proven useful for gene discovery for many crops. In common bean, there is a need to isolate cDNA clones from various tissues and begin generating EST sequence data for the crop. For this it will be necessary to construct multiple cDNA libraries that are of high quality and representative for genes expressed in the tissues sampled. There is an EST working group in the Phaseomics consortium and EST sequence generation is one of the principal objectives of this group of scientists. However, to date, there have been very few ESTs generated (a total of 35 are found in Genbank as of July 2002). At CIAT we are very interested in generating some of the cDNA libraries for this effort using as starting material RNA extracted from a) priority tissues where genes for agronomic and quality traits can be expressed (roots, leaves, pods, seeds); b) tissues affected by conditions of induction that are important to production of beans by small farmers in the tropics (disease or insect resistance, low fertility, drought or heat stress tolerance); and c) genotypes that have been developed or tested at CIAT that are leading varieties in Latin America or Africa and which have advantages in terms of the traits described above.

In addition to the information generated on gene expression by EST sequencing, the sequence information can also be used to design molecular markers that tag expressed genes either as single nucleotide polymorphism (SNP) of sequence tagged sites (STS) markers. A certain percentage of EST sequences have also been observed to contain simple sequence repeats (SSRs) that can be used to design microsatellite markers.
The objectives of this research were to 1) develop two cDNA libraries from common bean roots that were stressed or unstressed for phosphorous deficiency; 2) begin EST sequencing and gene discovery using these libraries; and 3) estimate the frequency of microsatellites in the EST sequences and use them for marker generation.

Methodology

Tissues and RNA extraction: Plants of the genotype DOR364 were grown under phosphorous sufficiency or deficiency for 5 days as follows: seeds were sterilized with bleach, germinated and grown in a nutrient solution with and without phosphorous. For both treatments, the seeds were placed about 3 cm from the top of rolled-up germination paper, which was wrapped in foil, and the bottom kept in the nutrient solution which was always replaced as used. Tissues were collected from tap roots and from lateral and basal roots of plants grown under both the optimum phosphorous and stressed conditions. Total RNA was extracted using a Trizol extraction technique. The RNA was quantified with spectrophotometrically at Abs $_{260nm}$ and size selected to be in the range of 1 to 3 kb in length. Poly-A messenger RNA was selected with a Dynabead protocol (Dynal).

Libraries: Two cDNA libraries were constructed from the messenger RNA that was isolated as described above. The procedure followed was based on the UniZAP – cDNA synthesis kit (Stratagene). mRNA from both treatments was primed in the first strand synthesis with a hybrid oligo dT linker-primer that contains an *Xho*I restriction site and was transcribed using StrataScript reverse transcriptase and 5-methyl dCTP. Following ds cDNA synthesis, EcoRI adapters were ligated to the blunt ends and the sample was digested with *Xho*I. The result was cDNA with an EcoRI sticky end on one side and an *Xho*I sticky end on the other. This size-fractionated cDNA was precipitated and directionally ligated in the UniZAP XR vector arms. The lambda library was packaged in Gigapack III Gold extracts, and plated into *E. coli* cell line XL-1-Blue MRF' for *In vivo* excision. The cells were plated on Q-plates with Carbenicillin (100 mg/L) and colonies were picked and arrayed into 96-well plates. A total of 36,000 bacterial colonies were picked from the excised library by a Q-bot robot. Automatic blue-white selection (with standard parameters as set on the Q-bot) was used to find the insert-containing clones. All the clones were stored in 384-well plate format glycerol stocks that were copied once into working and master copies of the library that were stored at -80 C. Each of the libraries was spotted onto gridded Hybond N+ membrane filters with six fields each with a 96 position double-replicate 4 x 4 pattern. The low phosphorous library was named Pv_DEe, while the high phosphorous library was named Pv_DEd.

Clone sequencing: As a preliminary analysis of the library quality, 1440 clones were miniprepped by a modified alkaline lysis method (CUGI laboratory protocols, 2002). and sequenced from the 5'side of the clone using either Sp6 or M13 Reverse primers. The clones were sequenced from the 5' end using the T3 primer, and then from the 3' end with the T7 primer. All sequences were searched for vector segments to check for insert integrity. The sequences were scored with PHRED quality values. The successful sequences contained a minimum of 100 continuous high quality bases, with a PHRED value of 20 or above and were trimmed to remove vector, adaptor, *E. coli* and Poly A sequences.

Results and Discussion

Two root cDNA libraries were successfully constructed from poly A mRNA that was captured from 4 ug of total RNA isolated from high and low phosphorous treated roots of the genotype DOR364. Preliminary analysis of the clones for each library showed that the average insert size was around 1.5 - 1.8 kb, which is about normal for most cDNA libraries that are well represented. The 18,000 clones that were picked for each library could fit on two filters each. In future hybridization experiments, clones could be identified by their position in the double-replicate pattern found at each grid axis in the address system.

The cDNA libraries are interesting because they represent genes that are turned on as an early response to optimum or low phosphorous in young plants. The stage of phosphorous deficiency response is considered early since the roots were harvested when they had started to develop initiating lateral roots but before adventitious rooting starts. At the time of the basal and tap root sampling, there was not much difference in shoot growth given that the seed reserves are known to last for about 7 to 9 days, and only after that does slower growth set in with the low P plants. Therefore, symptoms of phosphorous deficiency were not seen in the plants harvested for the RNA extraction. The phosphorous levels were 1 mM for high P, and none added for low P (note that the low phosphorous treatment was not actually quite zero since the tap water used for this had about 0.5 uM phosphorous). Although P content of leaves was not measured in these plants, previous work with plants that were grown under similar conditions to these had shoot P concentration after 5 days of 172 umol/gdw for high P and 155 um/gdw for no P (Bonser et al 1996).

We selected the genotype DOR364 to use for these libraries for various reasons. First, DOR364 is a tropically adapted; red seeded variety grown throughout Central America, from the Mesoamerican genepool and as such is an important variety to understand. This was important to the present research given that the genes discovered in this variety could be important for adaptation of this variety to farmer's conditions. Secondly, DOR364 is also the parent of the CIAT mapping population, DOR364 x G19833, and therefore any genes that would be of interest from the sequencing portion of the project could be mapped by conversion to markers for linkage analysis in the mapping population.

We have started the EST sequence analysis of the two libraries and have obtained a total 1911 sequences so far (Table 1). Of these raw sequences, 1414 were high quality, giving a success rate of 74%. The individual success rate was better for the Pv_DEd library (80%) than for the Pv_DEe library (69%). The average number of bases per trim sequence was also higher in the Pv_DEd library (533 nt) than in the Pv_DEe library (489 nt). The sequencing success rate was about standard for EST libraries at Clemson with stringent rules for what constitutes successful sequences. Also the sequencing so far confirms good insert size and also that there are no chimerics in the library. The sequences represent identifiable 5' and 3' ESTs since they came from a directionally cloned cDNA library and were sequenced with either T7 or T3 primers. All the sequences have been BLAST searched against each other to check for sequence redundancy (redundant clones consist in 90% identity over half the nucleotides in the sequence). The vast majority of the sequences were unique, and when compared to all plant proteins in the Genbank database identified significant homologies (EXP<1e-6).

The results of the EST sequencing also gave us a good idea of which simple sequence repeats are common in cDNA sequences, where these are located and how frequently they occurred in the library. The large majority of the repeats occurred in the 5'untranslated region (UTR) upstream of the start codon for the open reading frame (ORF) where these could be identified. Another proportion of the SSRs occurred in the 3' UTR, while fewer were found within the ORF. The total percentage of ESTs that might be expected to contain potential SSRs based on this study is approximately 5.6 %.

The present work brings to a total of 4182 the number of ESTs for beans generated here at CIAT (3086 of high quality), including sequences from leaf and root tissues. More will be generated this year through collaborations with several sequencing labs. After screening for microsatellites, a total of 540 EST sequences have been annotated and submitted to Genbank so far. This collection of new gene sequences for common bean represents many more individual sequences than are currently in the Genbank public database for all *Phaseolus* species together.

Future plans

- Develop microsatellite primers from the simple sequence repeat containing ESTs
- Annotate and submit sequences to Genbank
- Map ESTs with SNP (single nucleotide polymorphism) based assays, especially as simple procedure such as dense chips, become available.
- Future libraries will be made from adventitious roots of symptomatic phosphorous deficient and sufficient plants probably of the genotype G19833

References

Bonser et al (1996) New Phytol 132:281-288.
CUGI's Laboratory Protocols, 2002

Table 1. Benchmarks for root cDNA sequencing project.

Process	Library		Total
	PV_DEd High P.	PV_DEe Low P.	
Total Number of Sequenced EST's	862	1049	1911
Number of Submission Successful Sequences	690	724	1414
Success Rate	80%	69%	74%
Average Number of Bases per Trim Sequence	533	489	-
Average Number of High Quality Bases per Trim Sequence	340	338	-
Number of Sequences with match to Genbank [1]	574	622	1196
Number of clones with microsatellites[2]	88	109	197
Number of primer pairs[3]	52	55	107

1/ Genbank searches were done to the non-redundant protein database; and homology threshold was <1e-6)
2/ Microsatellites identified with SSRIT tool
3/ Primer pairs to be designed for non-redundant sequences containing a minimum of 6 di-nucleotide or 5 tri-nucleotide repeats.

1.3.2 Genomic resources for the study of biological nitrogen fixation in the common bean genotype BAT477

MW Blair[1], LC Muñoz[1], MC Giraldo[1] S Gonzalez-Rizzo[2], JJ Drevon[2]
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Introduction

Common bean grown in the tropics is often produced on poor soils or marginal land that is deficient in phosphorous and other essential nutrients. Low phosphorous can adversely affect symbiotic biological nitrogen fixation (SNF). The association of low nitrogen fixation with low phosphorous use availability is a double blow to crop productivity, which often affects small farmers who do not have access to commercial fertilizers. Some genotypes, with the best example being the CIAT line BAT477, can fix nitrogen despite lower amounts of available phosphorous. The mechanism by which this genotype is more efficient is not fully understood, although progress has been made in understanding some of the physiological responses that underlie this response (Vadez and Drevon, 2001).

In this research we were interested in exploring the genetic control of good SNF potential under low phosphorous conditions by comparing nodule-expressed genes under both phosphorous sufficient and deficient conditions. The specific objective was to initiate the construction of three cDNA libraries for genes expressed in mature nodule cortex tissues in three genotypes that were inoculated with *Rhizobium tropici*, CIAT strain 899 and grown under phosphorous stress conditions: BAT477 (phosphorous efficient), DOR364 (phosphorous inefficient) and Line 115 (even higher phosphorous efficient). We were also interested in analyzing a series of candidate genes that had been isolated from nodule cortex under stress conditions. The nodular cortex is thought to control the effective function of the nodule by controlling oxygen permeability and nutrient transport, which is thought to change with nutrient deficiency, salinity or drought stress. This research was part of a project entitled "Candidate Genes for Tolerance of Symbiotic Nitrogen Fixation (SNF) to Phosphorus Deficiency in Common Bean (*Phaseolus vulgaris* L.)" that was approved by the French science program called the Agropolis platform.

Methodology

Genotypes and growth conditions: Three genotypes were used for cDNA library construction: BAT477, DOR364 and line 115, one of the F5:9 progeny from the cross of these two parents. Seeds of each genotype were germinated in petri dishes where they were inoculated with strain CIAT899 of Rhizobium *tropici* at four days after germination. The seeds were then transferred to nutrient solutions (Drevon et al. 1988) and grown under two phosphorous treatments applied as KH2PO4 : limiting phosphorous: 75µmoles per plant and sufficient phosphorous: 250 µ moles per plant.

RNA extraction: At approximately three weeks after planting, 150 nodules (of 3 mm in diameter or larger) were collected, the inner cortex and bacteroids were removed by dissection with a razor blade and a blunt ended needle. The dissected nodular cortex was ground immediately in liquid nitrogen and the resulting powder was stored at -80°C. RNAeasy extraction kits (Quiagen) were used to isolate total RNA and oligo dT coupled Dynabeads (Dynal inc.) were used to isolate poly-A messenger RNA. CDNA library construction was carried out with Stratagene's "UniZap-cDNA synthesis kit. Size fractionation was done with sephadex columns (GIBCO-BRL).
Sequencing of candidate genes: A series of 70 clones representing nodule-expressed genes from soybean, medicago, common bean and other legumes were mini-prepped and sequenced to confirm insert size in preparation for use in screening the common bean nodule cortex library. Of these, 59 were clones from a differential display experiment carried out in INRA (Hassan et al., 2000). Standard sequencing techniques were carried out with dye terminator chemistry on an ABI 377 automated sequencing machine (Applied Biosystems).

Results and Discussion

In both genotypes sufficient RNA of high quality was obtained to conduct RNA hybridization experiments, with total yields of 5 ug for Line 115 and 3.5 ug of BAT477 and DOR364 in both high and low P treatments. RNA quality was determined spectrophotometrically and on 1% agarose gels. cDNA synthesis proceeded well for Line 115 RNA but after fractionation yields of cDNA were at a low concentration of 50ng/ul and since the protocol recommended higher concentrations this was not used for library construction. With BAT 477 RNA there was sufficient cDNA concentration for the ligation to the vector and for packaging. We have not yet sequenced any of the clones from these libraries but plan to start in the upcoming year.

In the meantime we have started to sequence clones that would be of interest for screening the resulting libraries. The most likely candidate genes were aquoporins (from *Vigna mungo*,

Medicago truncatula and *Phaseolus vulgaris*), carbonic anhydrase (from *P. vulgaris* and *Glycine max*) and phosphate transporters (from *G. max*). In addition we identified two common bean genes from the set of differential display clones with the following homologies: a) to a putative senescence associated protein from *Pisum sativa* and b) to a Ca-dependent protein kinase from *Arabidopsis thaliana*. We have also tried to use the RNA extractions in preliminary macroarray hybridization experiments, using filters prepared for EST clones from phosphorous-starved, arabidopsis roots and from mycorhizae-colonized medicago roots (kindly provided by P. Nacry and by H. Kuester).
Under this project, two CIAT scientists and one researcher from UNAM (Universidad Nacional Autonoma de Mexico) participated in a laboratory exchange program with INRA – Institut National de la Recherche Agronomique) - ENSA - UMR Sols et Environement. The project will continue through the year 2003.

Future plans

- Finish construction of cDNA libraries, attempt to subtract libraries made for high and low phosphorous and begin sequencing nodule specific genes.
- Test differential mRNA samples in gene expression experiments with Medicago or Soybean microarrays.

References

Drevon JJ et al (2001). Journal of Biotechnology 91 257-268.

Vadez V and JJ Drevon (2001). Agronomie 21 691-699.

1.3.3 Microsatellites isolated from common bean small insert and cDNA libraries

MW Blair[1], MC Muñoz[1], MC Giraldo[1], HF Buendia[1], M Atkins[2], D Main[2], J Tompkins[2]
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Introduction

Various techniques exist for discovering new microsatellite markers from anonymous genomic sequence. All of these techniques rely on the availability of DNA libraries. Microsatellites have been developed using both small-insert clones 1-kb) and cDNA sequences for a wide range of plant species. In this study, we were interested in developing common bean microsatellites from small-insert and cDNA libraries that were constructed and screened last year for simple sequence repeat. The new set of gene-based and genomic microsatellites will be useful for mapping and tagging projects in common bean.

Methodology

Libraries: Four libraries were screened for microsatellites. The largest library was a leaf cDNA library from the genotype G19833 consisting of 64,128 clones called Pv_GEa. In addition, three small-insert genomic libraries from the genotype DOR364 were screened. These were based on

three restriction enzymes: (1) *Rsa*I library (19,584 clones) 2) *Alu*I library (18,432 clones); and 3) *Hae*III library (19,968 clones). These libraries are described in more detail in the 2000 Annual Report.

Clone selection and sequencing: Clones were selected from the cDNA and small-insert library filters with repeat containing oligonucleotides as described in last year's annual report. Putative simple sequence repeat-containing clones were picked from their appropriate position in 384-well plate format glycerol stocks and sequenced on an automated ABI377 sequencer. The positive clones were sequenced initially from one end of the insert. In the case of the cDNA library, the 5'side of the clone was sequenced using either Sp6 or M13 Reverse primer and the 3' end was sequenced with either the T7 or M13 primers. In the case of the small insert libraries the clones were sequenced with either T7 or T3 high temperature primers. Any cDNA clone that did not contain an SSR in the 5'end was sequenced again from the opposite 3' end with T7 or M13 Forward primers. Small insert clones were only sequenced from the opposite direction if the insert was larger than 500 bp which was rare.

Preliminary Testing and Parental Survey: The new microsatellite primer pairs were tested with a rapid PCR protocol of 3 min 92 C, followed by 34 cycles of 15 sec denaturing 92 C, 15 sec annealing 47 C and 30 sec extension 72 C. Amplification was done on a panel of 18 genotypes as described previously (CIAT, SB2 Annual Report, 2000). The PCR products were run on 4% polyacrylamide (29:1) gels for 1 hour at 130 constant volts.

Results and Discussion

A total of 2710 sequences were obtained for this project of which 2271 were from cDNA clones and 439 were from small insert clones (Table 1). The cDNA clones that were sequenced included a "random set" that had to been selected to contain simple sequence repeats and a "selected set" that had been. A total of 768 individual 5' sequences were obtained for the random set, while a total of 1503 individual 5' (927 reads) and 3' (576 reads) sequences were obtained for the selected set. These raw sequences were processed with the UNIX-based programs, Crossmatch and Phred (P. Green and B. Ewing) to remove vector and adaptor sequences and to determine sequence quality. Phred determines confidence values for each base call in a sequence based on the peak height of that base in the electropherogram of that sequence. Phred values equal to or above 20 indicated high read quality, values between 16 and 19 were acceptable but not ideal and values below 15 were considered poor. A total of 1672 successful sequences were obtained from the cDNA library.

After sequence trimming, the program Sequencher (v. 4.1.2 Macintosh, Gene Codes Co.) was used to compare similar sequences. All clones were checked against all other clones from the library to remove redundancies between clones. Any pair of clones containing a similarity of 90% or greater in 50% of the total bases sequences were considered redundant and only the better quality sequence was selected for further analysis and for primer design. The sequences were also searched for vector segments to check for insert integrity.

After redundancy checks, the sequences were screened for simple sequence repeats using the SSRIT tool. Meanwhile, among the clones selected by hybridization, a total of 421 cDNA sequences (28% of the total) and 110 small insert sequences (25% of the total) were found to have simple sequence repeats, reflecting a relatively low efficiency of the hybridization based screening to detect SSR positive clones. By comparison, in the random set of cDNA clones there were 80 (10.4% of the total). It was notable that for the cDNA sequences, simple sequence

repeats were more frequent in the 5'end sequences (354 positive or 38% of the total) than in the 3' end sequences (67 positive or 11.6% of the total). This suggests that simple sequence repeats are more common in the 5' untranslated region of the cDNA or initial part of the open reading frame than in 3' untranslated regions of the cDNA This observation was corroborated by EST sequencing in additional libraries that we have analyzed at CIAT.
In the case of the cDNA clones that contained microsatellites, the sequences were analyzed with the program ORF finder (http://www.ncbi.nlm.nih.gov) so as to identify the longest uninterrupted open-reading frame and the probable 5' or 3' untranslated regions of the cDNA. We were interested in knowing the location of the simple sequence repeat relative to these parts of the individual cDNA. This was important since many of the microsatellites, especially those based on di-nucleotide repeats, were located in the untranslated regions of the cDNA and not in the open reading frame. After running the ORF finder, the positive sequences were searched for homology to the non-redundant Genbank database using the program BLAST (http://www.ncbi.nlm.nih.gov). A large number of the cDNA clones identified homologous genes in this database, in the MIPS *Arabidopsis thaliana* database, or in Swissprot (Table 2). The ORF finder program was not used in the case of small-insert genomic clones, as these were assumed to be mostly from non-coding regions of the genome.

After the sequence processing, the software program Primer 3.0 ((http://www-genome.wi.mit.edu/cgi-bin/primer/primer3) was used to analyze the regions flanking the simple sequence repeat to design primers for the microsatellite markers. A total of 341 primers pairs were designed for cDNA sequences and 77 for small insert sequences. However for an initial ordering of microsatellite markers only the di- or tri-nucleotide containing motifs were ordered, including 238 primer pairs for cDNA clones and 59 for small insert clones. Tetra and penta-nucleotide motifs will be ordered separately at a later date.
All the primers were designed in regions of high quality sequence (Phred score above 15) that had balanced amounts of GC / AT content and that fulfilled the following design parameters: non-complementary ends, melting temperature (T_m) of 60 C, primer length at least 20 nucleotides long. The average product size was between 100 and 200 base pairs, however the product size for the cDNA clones was kept slightly smaller than for the small insert clones given the risk of crossing intron-exon boundaries in the cDNA sequence. For the small insert clones, no effort was made to keep the product size especially small given that these were genomic sequences. Many of the primer pairs designed for cDNA sequences were anchored to the start codon of the open reading frame where these could be identified. More than one primer pair was designed for several cDNA and small insert clones which had multiple simple sequence repeats.

So far we have tested 191 primer pairs (132 from the cDNA sequences and 59 from the small insert sequences) for amplification ability and potential polymorphism on a set of parents discussed elsewhere in the annual report. Of the cDNA-based microsatellites, 51% presented polymorphisms, 40% were monomorphic, 1% showed multi-copy amplification, and 8% did not amplify. Of the microsatellites based on small-insert clone sequences, 44% were polymorphic, 44% were monomorphic and 12% did not amplify. The level of polymorphism appeared to be related to the number of repetitions in the simple sequence repeat more than the specific motif that the simple sequence repeat had. Microsatellite markers designed for simple sequence repeats of more than ten repetitions were much more likely to be polymorphic than those designed for simple sequence repeats with less than seven repetitions.

Future plans

- Sequence a random set of the small insert library clones to determine common motifs.

Test the new microsatellites on additional parental surveys and assemble the information into BeanGenes and Oracle databases that we are maintaining at CIAT.

Polymorphic microsatellites will be mapped on either the DOR364 x G19833 or BAT93 x JaloEEP558 core populations or on additional mapping populations that the lab is working on.
Sequences will be submitted to the EST database at Genbank. The sequenced cDNA clones represent the first substantial number of EST sequences in beans. So far, 540 of the 2271 EST sequences described here have been uploaded to Genbank. These ESTs are high quality sequences that contain no SSRs. This effort has increased the number of ESTs for *Phaseolus* in the ESTdb at Genbank from 35 entries earlier this year to 575 entries now.
EST sequences will be used to develop SNP (single nucleotide polymorphism) based assays, especially as simple procedure such as dense chips, become available.

Table 1. Benchmarks for microsatellite development project and sequencing of cDNA and small insert libraries for this purpose.

Process	CDNA			Small Insert	Total
	Random set	SSR set	Total		
Number of sequenced	768	1503	2271	439	2710
Number successful sequences	745	927	1672	--	--
Percentage success	97%	62%	74%	--	--
Number of sequences with SSR	80	421	501	110	611
Number of designed primer pairs	--	341	341	77	418
Number of ordered primer pairs	--	238	238	59	297
Number of polymorphic markers	--	67	67	26	93
Number of monomorphic markers	--	53	53	26	79
Number of non-amplifying markers	--	11	11	7	18

Table 2. Sequence homologies of common bean leaf cDNA library clones.

Criteria	Random set	SSR selected set	Total
Number of Non Redundant Sequences (nr)	745	782	1527
Number of Sequences with match to MIPS *Arabidopsis thaliana*:	-	644	-
Number of Sequences with match to Swissprot:	240	342	582
Number of Sequences with match to Genbank	170[1]	545[2]	715

1/ Genbank nr protein database. (EXP <1e-6)
2/ Genbank Plant database. (EXP <1e-12)

1.3.4 Testing of cDNA and small insert derived microsatellites

MW Blair, HF Buendia, MC Giraldo
SB-2 Project

Introduction

The objective of this study was to test the new microsatellites described in the previous two sections on a panel of 18 common bean genotypes representing wild and cultivated germplasm of common beans, both Mesoamerican and Andean, which have been used as parents in the bean breeding program.

Methodology

The genotypes are described in the CIAT annual report (2000) and were the parents of 11 mapping populations being studied at CIAT for nutritional quality, phosphorus stress tolerance and other traits (Table 1). The genotypes were evaluated with a total of 178 new microsatellite markers (of which 119 were derived from the cDNA library and 59 were derived from the small-insert genomic libraries). The markers were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The amplification conditions are given in other parts of this annual report. The PCR products were resolved by electrophoresis for approximately one hour at 130 constant volts on silver-stained 4% polyacrylamide gels. Microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

Results and Discussion

The average rate of polymorphism was higher in the four inter-genepool (Andean x Mesoamerican) crosses than in the seven intra-genepool (Mesoamerican x Mesoamerican) crosses (Table 1). Among the Andean x Andean crosses, G21078 x G21242 was the most polymorphic and among the Mesoamerican x Mesoamerican crosses, G11360 x G11350 was the most polymorphic. This may reflect inter gene-pool introgression in one of the parents of each population. Among the inter-genepool crosses all were relatively similar in the level of polymorphism between the parents (from 33 to 36% on average). Both of these trends for polymorphism rates in intra and inter-genepool crosses were equally evident when using genomic and cDNA derived microsatellites.

Genomic and cDNA microsatellites detected about equal polymorphism (22.6%) overall. Similar number of average alleles per locus were found for microsatellites from the cDNA and genomic libraries. The discriminating power (D) of the gene-derived microsatellites (0.490 among polymorphic, 0.267 among all markers, including monomorphic) was slightly higher than for the genomic microsatellites (0.471 and 0.189, respectively). The discrimination power was positively correlated with the number of alleles produced at the locus. Null alleles were uncommon in both microsatellite classes.

Future plans

- Mapping of the new microsatellites will be pursued
- Assembling of microsatellite fingerprint data into the AceDB database, BeanGenes that were described in last year's annual report

Table 1. Polymorphism rate among 11 parent combinations for 178 microsatellite loci (119 cDNA and 59 genomic).

Population		GENIC CDNA		GENOMIC Small Insert		TOTAL
		Pol	% Pol	Pol	% Pol	% Pol
1	G 11360 X G 11350	24	20.2	11	1.86	19.5
2	G 21657 X G 21078	16	13.4	12	20.3	13.6
3	G 21078 X G 21242	23	19.3	13	22.0	18.8
4	G 14519 X G 4825	13	10.9	8	13.6	11.0
5	DOR 364 X G 19833	46	38.7	11	18.6	33.1
6	DOR 364 X G 3513	15	12.6	11	18.6	12.3
7	BAT 477 X DOR 364	10	8.4	16	27.1	9.1
8	BAT 881 X G 21212	16	13.4	4	6.8	13.0
9	G 24404 X RAD-CER	43	36.1	24	40.7	36.4
10	RAD-CER X G 24390	45	37.8	20	33.9	35.7
11	DOR 390 X G 19892	45	37.8	17	28.8	35.1
Total no. of microsatellites tested		119		59		17.8

1.3.5 Parental survey of phaseolin patterns in parents of RIL populations

MW Blair, MC Giraldo
SB-2

Introduction

The objective of this work was to characterize the phaseolin alleles found in two sets of common bean genotypes that are parents of recombinant inbred line populations at CIAT. Phaseolin is the major seed storage protein in bean seed and has been used as an evolutionary marker given that the genetic diversity at the phaseolin locus reflects the structure of the species: wild accessions generally have more diversity than cultivated genotypes and Mesoamerican and Andean beans have different alleles. Phaseolin analysis has been used to follow introgression and as a genetic marker in mapping studies. The genetic resource unit has experience determining the phaseolin pattern on denaturing protein gels.

Methodology

In the first part of this study, a total of 30 common bean genotypes were evaluated for phaseolin pattern. All the genotypes were parents of genetic mapping populations used in the Bean Germplasm Laboratory and Bean Breeding Program. In the second part of this study, several mapping populations were also analyzed for phaseolin pattern. Total seed proteins were extracted

from 0.01 g of peeled, finely-ground, oven-dried seed by a standard extraction technique used at the Genetic Resource Unit at CIAT. One microliter each of the protein extracts were separated with 6% separation / 12% stacking SDS-PAGE (polyacrylamide) mini-gels run at 180 V for 50 minutes and stained with Coomasie Blue dye. The phaseolin pattern was compared to known standards provided by C. Ocampo of the Genetic Resource Unit of CIAT.

Results and Discussion

A total of 4 phaseolin patterns were detected among the 30 genotypes (Table 1). These included the alleles for phaseolin "S", "T", "C", and "I". The alleles could be distinguished by the number and molecular weights of the protein bands on the SDS-PAGE gels (Figure 1). As expected, the "S" allele was common in the Mesoamerican genotypes while the "T" allele was common in the Andean genotypes. A single wild accession (G24390) had the "I" allele, while both a wild (G24404) and a cultivated (G21242) bean had the "C" allele.

The phaseolin protein pattern was polymorphic for nine populations of interest in the CIAT breeding program and so far the locus has been mapped in three of these populations. The phaseolin locus was already mapped in the BAT93 x Jalo EEP558 population (S x T) (Freyre et al., 1998). From that map as well as the one from Vallejos et al (1992) the phaseolin gene family is known to be located on chromosome b07.

Our results showed that in the core CIAT mapping population (DOR364 x G19833 – S x T alleles) the locus mapped to the expected location on chromosome b07 and fit the expected segregation ration of 1:1. Heterozygosity at this locus was very low (1.2%). This was not surprising given that the population of 87 RILs is in the F11 generation. In the advanced backcross population (Cerinza x (Cerinza (Cerinza x G24404))) – C/L x T), segregation was skewed towards the cultivated allele "T" allele and surprisingly there was introgression of both the "C" and "L" alleles from the wild parent. In the Andean micronutrient population (G21242 x G21078 – C x T), the locus also mapped to the correct location on chromosome b07.

Table 1. Phaseolin patterns identified for genotypes in parental surveys I and II.

Parental Survey I				Parental Survey II			
No.	Genotype		Phaseolin	No.	Genotype		Phaseolin
1	*G11360*	cult	S	19	DOR476	cult	S
2	G11350	cult	S	20	SEL1309	cult	S
3	G21657	cult	C	21	BAT93	cult	S
4	G21078	cult	T	22	JALO	cult	T
5	G21242	cult	C	23	ICA PIJAO	cult	S
6	G14519	cult	S	24	G40001	*wild*	*Globulina*
7	G4825	cult	S	25	VAX6	cult	S
8	G19833	cult	T	26	MAR1	cult	S
9	DOR364	cult	S	27	J117	cult	S
10	BAT477	cult	S	28	JAMAPA	cult	S
11	G3513	cult	S	29	G2333	cult	S
12	BAT881	cult	S	30	G19839	cult	T
13	G21212	cult	S				
14	G24404	wild	C				
15	RADCER	cult	T				
16	G24390	wild	I				
17	DOR390	cult	S				
18	G19892	wild	T				

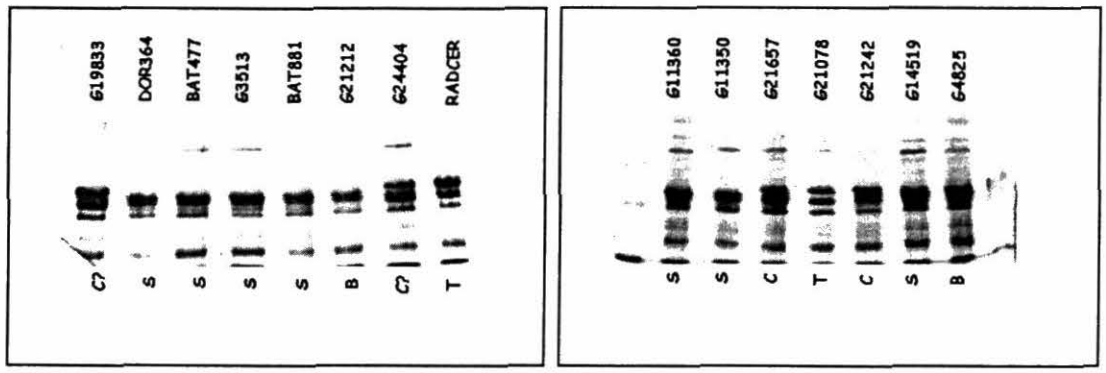

Figure 1. **Phaseolin pattern in 16 genotypes of common bean that are parents of mapping populations at CIAT.Future studies**

Mapping of the phaseolin locus in the Cerinza x (Cerinza (Cerinza x G24390))) (T x I), DOR390 x (DOR390 x (DOR390 x G19892)) (S x T) and G2333 x G19839 (S x T) populations.

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1.3.6 Sequence analysis of a cluster of resistance gene analogs associated with resistance to angular leaf spot (ALS) in the common bean (II)

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Introduction

A cluster of Resistance Gene Analogs (RGAs) that explain 47 and 64% of the resistance to two isolates of angular leaf spot (ALS) in the common bean is currently being analyzed at the sequence level. In doing so, we pursue three goals:

To facilitate the genetic dissection of the cluster in order to establish which member(s) of the cluster is(are) involved in the resistance to ALS

To generate new molecular markers that benefit breeding programs
To understand the difference in sequence that determines resistance in genotype G19833 in contrast with other genotypes

Once a full-length genomic copy of the member associated with resistance is obtained, it could be introduced in susceptible genotypes in order to demonstrate that they are effective resistance genes.

Last year we initiated the sequencing, at the genomic level, of a relatively large BAC clone (57M14) of the common bean that contains 4 members of the RGA7 family out of 7 found in a contig of 11 BAC clones. On the other hand, at the expression level, it is necessary to determine whether some members of the RGA7 family are being expressed in the plant as an indication of its functionality. Here we show the advances made at both levels of sequence analysis and the work in progress that is required to accomplish the proposed goals.

Materials and Methods

Sequencing of the BAC clone 57M14 was initially attempted by a transposon-insertion strategy using a kit from Epicentre, which randomly inserts an EZ::Tn <KAN-2> transposon, containing a selectable marker (kanamycin resistance) and sequencing primer-binding sites into BAC DNA (CIAT, 2001).

A second strategy was designed to complement the results obtained with the first one. It involved constructing a "subcloning library of amplified fragments from the BAC 57M14". The BAC clone was partially digested using a low concentration of *Mse*I, and the fragments were ligated to a common *Mse*I-adapter used for AFLP. The ligation was diluted and an aliquot used to perform a standard PCR reaction using the *Mse*I-adapter as a primer. Amplified products were separated by electrophoresis in an agarose gel; and those in the range of 500-0.2 bp were excised, purified and cloned in the pGEM-T easy vector system (Promega). The library was transformed in *Escherichia coli* DH5α cells by electroporation, and 384 clones were randomly selected. Plasmids were isolated by "the microwave protocol," a high-throughput method that allows microwave-mediated bacterial cell lysis in a 96-well format (Marra et. al., 1999) for use directly in sequencing reactions with T7 and/or SP6 primers.

Two different software programs are being used to edit and assemble sequences in contigs. Sequencher, which is the current software for editing sequences in the Project, includes several useful features such as fast contig editing and restriction-site analysis. For large-scale sequencing projects, however, the choice is Phred/Phrap/Consed (Gordon et al., 1998), which are used together for editing, assembling and viewing/editing, respectively. The results obtained from both packages were merged to arrive at the final inference.

To isolate the complete sequence of cDNAs corresponding to expressed members of the RGA7 family, we decided to perform a Rapid Amplification of cDNA ends (RACE) (Frohman, 1993), using the SMART RACE cDNA Amplification kit (Clontech) and following the User Manual instructions. RNA was extracted using Trizol® reagent from the first leaves of one-month-old G19833 plants that had not been challenged with any pathogen. Gene-Specific Primers (GSPs) were designed from the sequence of RGA7 so that they can produce overlapping 5'- and 3'-RACE products; thus they can also be used to amplify the original RGA7 sequence. An additional Nested Gene Specific Primer (NGSP) was designed for 3'-RACE (Figure 1).

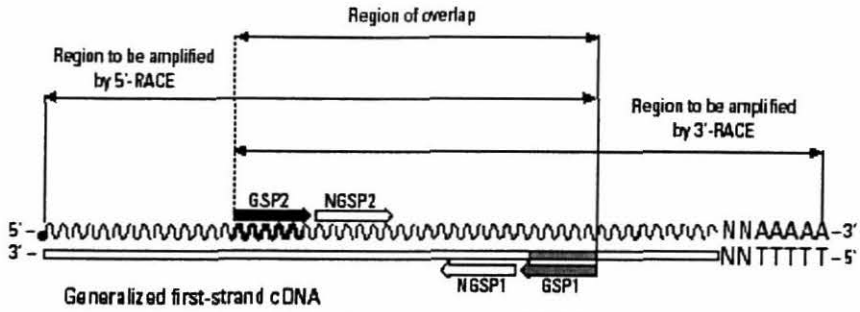

Figure 1. The relationship of Gene-Specific Primers to a cDNA template (adapted from SMART RACE cDNA Amplification kit User Manual PT3269-1 PR14596, Clontech).

Results and Discussion

Sequencing of the BAC 57M14. The complete sequence of the BAC clone 57-M14 is important to unveil the structural organization of cluster RGA7. We sequenced 501 clones —either at one or both ends—from the transposon insertion library that rendered about 55 kb of the total sequence. However, 50% of the clones were assembled on one of three contigs containing retroelement-type sequences. There was clearly a bias in the transposon-insertion reaction for this kind of sequence, which explains why we were not able to cover a greater portion of the total sequence. Therefore, a new library was constructed, this time containing subcloning-amplified fragments from the original BAC 57M14 (see above). After sequencing the 384 subclones, we reached 70 kb of the genomic sequence. Currently, we are working on the remaining sequence, which is estimated to be 10-15 kb long.

Twenty contigs were assembled, their sizes ranging from 690 bp—a contig containing a single clone—to 26.2 kb. We then performed GenBank searches with the BLASTX algorithm as a first step to identify contigs containing RGA-type sequences and additional putative coding sequences. Only 9 out of the 20 contigs contained sequences that, when translated, have significant homologies in the protein database (Table 1).

Six of the contigs correspond to RGA-type sequences. Considering that contig RGA7F may be the 3'end of one of the other RGA7 contigs, we can reason that BAC 57M14 contains 5 members of the RGA7 family. When 989 bp of the 5'coding sequence of these members was compared, their similarities ranged from 68-91%. The NBS sequence of the original RGA7 predicted that this RGA was of the TIR-NBS-LRR family (CIAT, 2000). Now, we have confirmed this because the 5' region of the RGA7 members contained in BAC57M14 is homologous to the TIR domain of R-genes. Only RGA7B contains all the domains of a TIR-NBS-LRR, and we are currently working on the annotation process involving intron-exon prediction, open reading frame determination, etc. We still have to complete the sequence of the other members in the contig, and this will be done when the remaining sequence of the BAC clone is determined.

Table 1. Features of the contigs with significant protein homologies in the GenBank.

Contig	Size (kb)	Features
RGA7A	5.5	5' region of a NBS-LRR R-gene; ORF interrupted by a retroelement-type sequence
RGA7B	26.2	Nearly complete NBS-LRR gene preceded by several retroelement-type sequences
RGA7C	2.3	5' region of a NBS-LRR R-gene
RGA7D	2.2	5' region of a NBS-LRR R-gene
RGA7E	1.4	5' region of a NBS-LRR R-gene
RGA7F	0.8	Contains leucine-rich repeats (LRR, 3' region of a NBS –LRR region)
10	1.2	Probable long-chain fatty-acid -CoA ligase (lipid metabolism)
13	2.4	Mutator-like transposase
Retro3/6/8	8.1	Three regions containing retroelement-type sequences

It is surprising to note that apart from RGAs, the BAC 57M14 does not seem to contain a large number of sequences coding for genes directly involved in cell function. Only one sequence in contig 10, probably coding part of an enzyme participating in lipid metabolism, is the exception. A great portion of the BAC sequence may be not coding, and another is related to retroelement-type sequences. This last finding is striking, although not uncommon. RGA7 seems to be located in a region enriched in transposable elements, which has also been the case for some R-gene clusters: *Xa21* (rice) and *Mla* (barley). In the former case, evidence for the involvement of transposition in the evolution of the cluster has been provided (Song et. al., 1997).

The origin of BAC 57M14 is the common bean cv. Sprite. However, the source of the resistance that we have found associated with RGA7 is var. G19833, one of the parents in the mapping population used at CIAT. Now, our aim is to isolate the corresponding copies of RGA7 members from G19833 and determine if one or several of them are conferring resistance to ALS. A genomic approach is currently being designed to reach this goal.

Isolation of RGA7 cDNAs. Trizol® reagent guarantees DNA-free preparations of RNA. The SMART RACE cDNA Amplification kit from Clontech allows the amplification of 5' and 3' end sequences of a known target as RGA7 from cDNA. After the cDNA synthesis reaction, we performed a PCR using both GSPs to amplify the overlapping fragment corresponding to the original RGA7 NBS sequence. Successful amplification of the expected band of ≈500 bp confirmed that the GSPs we designed worked well, that our method of cDNA synthesis was satisfactory, and that RGA7 is constitutively expressed in common bean leaves. We also tested the RACE technique on the expression of a housekeeping gene, a subunit of the Rubisco Activase. Primers designed from an EST for this gene, reported in the GenBank, permitted the amplification of the expected fragment in both 5'- and 3'-RACE (data not shown).

3'RACE amplification attempts for RGA7 produced multiple products, but at very low yields. Therefore we performed an additional (nested) PCR reaction on these primary products, using the Universal Nested Primer (UNP) provided with the kit and our NGSP. Multiple products were then observed at 0.5, 0.8, 1.2, 1.5 and 3.0 kb. By far, the band at 0.5 kb was most efficiently amplified. Cloning and sequencing analyses of these multiple products were achieved for the bands at 0.5, 1.2 and 1.5 kb, which correspond to the RGA7. However, they do not seem to correspond to full-length cDNA sequences even though the largest product contains the LRR motif. The band at 3 kb has the expected size of a full cDNA product, but we were not able to clone it. After gel excision, the 0.5 kb band was present as a background that competed in the cloning reaction with the desired largest band. Now, we are considering some of the multiple products an artifact in the cDNA synthesis, particularly the one of 0.5 kb, because we have found some regions in RGA7 with high TA content, which could lead to mispriming of the oligo (dT)

primer. Other explanations may be alternative splicing or the use of multiple polyadenylation sites although they seem less feasible.

On the other hand, the 5'RACE amplification failed to produce the expected band of 1.5kb even after several reamplification procedures were tested. In conclusion RACE is not a simple matter, and a number of factors influence the success of the procedure:

The R-genes are expressed at low rates in the cell although the members of some large RGA families are more easily detected (Acosta & Tohme, 2001). The efficiency of both 5'- and 3'-RACE amplifications depends on the abundance of the target transcript.
According to Clontech, "no method of cDNA synthesis can guarantee a full-length cDNA, particularly at the 5' end." The severer secondary structure may block the action of the reverse transciptase and/or the Taq DNA polymerase in some instances. Consequently, our next step is to improve the performance of RACE procedures to determine whether RGA7 is being expressed as a complete sequence, which is to be expected for an NBS-LRR type R-gene.

Future Activities

Finishing and annotating the complete sequence of BAC clone 57-M14
Isolation of members of the RGA7 family from parental line 619833, which contains resistance specificities in this family
Improvement of the RACE technique for weakly expressed genes. Critical points are the quality of RNA, cDNA synthesis and PCR amplification.

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1.3.7 Characterization and genomic implications of Ty1-copia group retrotransposon RNAse-LTR sequences in common bean (*Phaseolus vulgaris*).

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SB-02 Project

Introduction

Retrotransposons are mobile genetic elements that transpose through an RNA intermediate and are ubiquitous in plants. Retrotransposon have become a powerful tool for many tasks, including: diversity and linkage analysis, mapping, phylogeny, expression, retrotransposon based molecular marker studies, and an increasing interest in the field of retrotransposon mediated genomic evolution has become evident considering the insertional patterns of retroelements in promoter regions, resistance gene clusters, and the synergic activation of retrotransposons and stress genes.

We continued in sequencing and analysis of fragments from a previously generated library (Galindo, Gaitan and Tohme, 2001) created by using the technique implemented by Pearce et al. (1999) to isolate numerous and diverse RNAse-LTR fragments. Analysis of the sequences uncovered evolutionary relationships concerning bean retroelements.

Materials and Methods

Besides the clones selected for sequencing and analysis in the previous study (Galindo, Gaitan and Tohme, 2001), new clones from the library were selected to be sequenced and analyzed giving an overall number of 123 clones with the expected pair of primers (RNAse-MseI). Analysis was performed using Sequencher 3.0, Blast x 2.0, Clustal 1.8, Clustal x 1.62, MatInspector professional (Quandt et al., 1995) and PAQ (Baccam et al., 2001).

Results and discussion

From the 123 clones having the expected pair of primers in the flanking regions, 91 showed similarity to RNAse like sequences from the Genbank. Figure 1 shows alignment of some of the conceptual translations of the isolated RNAse sequences.

Small continous variations between sequences indicated that retrotransposons behaved as a quasispecies-like population. An example of this behavior is shown by sequences CIAT-Tpv403, CIAT-Tpv1151 and CIAT-Tpv459 (Fig. 2). The sequence CIAT-Tpv403 has a 98 similarity score (assesed by Clustal W) to CIAT-Tpv1151 (one nucleotide difference), and the latter bears a 98 score to CIAT-Tpv459; however, CIAT-Tpv403 and CIAT-Tpv459 have a similarity score of 97 (two nucleotide difference) meaning that these two sequences (when comparing this RNAse section) are two units away from each other, and at the same time, one unit away from CIAT-Tpv1151, as defined by a hamming sequence space (Eigen 1993).

```
CIAT-Tpv454              ADILTKHLPKDRFFLLRNELGIINSHTLS-- 29
CIAT-Tpv992              ADMLTKPLPKDMFFLLRNELGIIDSQTPS-- 29
CIAT-Tpv357              ADMLTKPLSKDRFYCLRNELGIIDMHDLS-- 29
CIAT-Tpv003              ADMFTKPLAEDRFNFLINELGIINVNCTLQ- 30
CIAT-Tpv930              ADIFTKPLAKDRFNFLLNELGIINVNCAS-- 29
CIAT-Tpv076              ADMFTKVVTRAKFEHCLDLVNILHI------ 25
CIAT-Tpv814              ADMLTKVVIRAKFEHCLDLVNILHI------ 25
CIAT-Tpv223              ADMLTKVVTRTKFEHCLDLVNILHI------ 25
CIAT-Tpv829              ADILTKVVTRTKFEHCLDLVNILHI------ 25
CIAT-Tpv1150             ADMLTKVLSGNKFFNCLDFIQL--------- 22
Most conserved           ** *** *    *       *
 Consensus               ADMLTKPLPKERFFFLRNKLGI
                                          E
P. consensus             ADIFTK-LP---F--LR--LG-
                    ML
```

Fig. 1. Alignment of RNAse 3' sections. Consensus aminoacids (most frequent aminoacid in a position) are indicated for the first 22 aminoacids. An asterisk indicates over 80% conservation in that position when comparing all sequences. Comparison to the consensus reported by Pearce et al. (1999) (P.consensus) is also shown.

```
CIAT-Tpv403              GCCGATATGCTGACGAAGGCATTGGGCAAGGAACGATTTTTGACGCTACGACACAAGTTG 60
CIAT-Tpv1151             GCCGATATGTTGACGAAGGCATTGGGCAAGGAACGATTTTTGACGCTACGACACAAGTTG 60
********* ************************************************
CIAT-Tpv403              GGGTTCTTGATCTTCACCTACCAACTT 87
CIAT-Tpv1151             GGGTTCTTGATCTTCACCTACCAACTT 87
**************************

CIAT-Tpv459              GCCGATATGTTGACGAAGGCATTGGGCAAAGAACGATTTTTGACGCTACGACACAAGTTG 60
CIAT-Tpv1151             GCCGATATGTTGACGAAGGCATTGGGCAAGGAACGATTTTTGACGCTACGACACAAGTTG 60
***************************** ******************************
CIAT-Tpv459              GGGTTCTTGATCTTCACCTACCAACTT 87
CIAT-Tpv1151             GGGTTCTTGATCTTCACCTACCAACTT 87
**************************

CIAT-Tpv459              GCCGATATGTTGACGAAGGCATTGGGCAAAGAACGATTTTTGACGCTACGACACAAGTTG 60
CIAT-Tpv403              GCCGATATGCTGACGAAGGCATTGGGCAAGGAACGATTTTTGACGCTACGACACAAGTTG 60
********* ******************* ******************************
CIAT-Tpv459              GGGTTCTTGATCTTCACCTACCAACTT 87
CIAT-Tpv403              GGGTTCTTGATCTTCACCTACCAACTT 87
                         **************************
```

Fig. 2. Alignment of RNAse nucleotide sequences from three clones filling three continuous points in a Hamming sequence space.

The inference of a quasispecies-like behavior led us to analyze possible clustering between the isolated RNAse sections. When the 92 sequences were submitted to Clustal X (Isolated sequences plus element Tpv2-6 isolated by Garber et al., 1999) it was difficult to limit clustering for some sequences. Therefore, we analyzed the same sequences using a whole new package "PAQ" (Partitional Analysis in Quasispecies) which has already been implemented for retroviral sequences (Baccam et al., 2001). Eight non-overlapping clusters were defined and eight sequences were left out of any cluster using an optimal radius value of 6 differences (Table 1). PAQ uses hamming distance to create groups where the degree of separation between each sequence reflects similarity, and the separation depends on nucleotide or aminoacid changes from one sequence to another. While Clustal relies on a progressive alignment where all the sequences become related, PAQ excludes under-represented sequences or sequences that are far from most common variants in sequences space, thereby not forcing relationships. PAQ only assumes that closely related sequences should differ by a small number of changes while additional pre-established evolutionary processes are the basis of programs like Clustal. These more complicated

assumptions make not work so well with scarce data, or when data comes from retroelements, which usually violate the assumptions because of their distinct evolutionary patterns.

Table 1. Clusters attained with PAQ.

Cluster	SEQUENCES	CENTER[a]	Av. Dist.[b]	Maximum number of aminoacid changes	Minimum number of aminoacid changes
1	CIAT-Tpv59, CIAT-Tpv242, CIAT-Tpv261, CIAT-Tpv375, CIAT-Tpv412, CIAT-Tpv437, CIAT-Tpv454, CIAT-Tpv490, CIAT-Tpv527, CIAT-Tpv534, CIAT-Tpv553, CIAT-Tpv558, CIAT-Tpv602, CIAT-Tpv731, CIAT-Tpv737, CIAT-Tpv812, CIAT-Tpv868, CIAT-Tpv870, CIAT-Tpv890, CIAT-Tpv923, CIAT-Tpv934, CIAT-Tpv966, CIAT-Tpv971, CIAT-Tpv974, CIAT-Tpv992, CIAT-Tpv1031, CIAT-Tpv1126, CIAT-Tpv1136	CIAT-Tpv527	d=14.55	12	0
2	CIAT-Tpv36, CIAT-Tpv134, CIAT-Tpv235, CIAT-Tpv293, CIAT-Tpv438, CIAT-Tpv443, CIAT-Tpv500, CIAT-Tpv571, CIAT-Tpv649, CIAT-Tpv707, CIAT-Tpv781, CIAT-Tpv817, CIAT-Tpv871, CIAT-Tpv877, CIAT-Tpv950, CIAT-Tpv1050, CIAT-Tpv1052, CIAT-Tpv1112	CIAT-Tpv438	d=2.41	5	0
3	CIAT-Tpv-11, CIAT-Tpv403, CIAT-Tpv436, CIAT-Tpv459, CIAT-Tpv818, CIAT-Tpv873, CIAT-Tpv906, CIAT-Tpv959, CIAT-Tpv996, CIAT-Tpv1065, CIAT-Tpv1151	CIAT-Tpv996	d=7.50	8	0
4	CIAT-Tpv-6, CIAT-Tpv264, CIAT-Tpv479, CIAT-Tpv496, CIAT-Tpv622, CIAT-Tpv625, CIAT-Tpv764, CIAT-Tpv975, CIAT-Tpv1004	CIAT-Tpv-6	d=5.25	7	1
5	CIAT-Tpv-17, CIAT-Tpv732, CIAT-Tpv766, CIAT-Tpv865, CIAT-Tpv1021, CIAT-Tpv1094, CIAT-Tpv1097	CIAT-Tpv-17	d=1.33	3	0
6	CIAT-Tpv76, CIAT-Tpv103, CIAT-Tpv223, CIAT-Tpv586, CIAT-Tpv814, CIAT-Tpv829	CIAT-Tpv223	d=7.00	7	1
7	CIAT-Tpv3, CIAT-Tpv407, CIAT-Tpv930	CIAT-Tpv407	d=9.00	5	3
8	CIAT-Tpv621, CIAT-Tpv955	CIAT-Tpv621	d=1.00	1	1
Excluded	CIAT-Tpv191, CIAT-Tpv258, CIAT-Tpv306, CIAT-Tpv357, CIAT-Tpv442, CIAT-Tpv669, CIAT-Tpv1150, Tpv2-6				

[a]Most representative sequence from each cluster.
[b]Average distance of the sequences from each cluster.

We also searched for the different regions corresponding to RNAse-ppt-LTR (an example is given in Figure 3). The regions presented different levels of variability but in general higher variability is usually present at the LTR sections since these regions are subjected to higher mutations rates than coding regions (Blush et al., 1997).

Analysis of LTR regions of several sequences obtained using primers from polypurine tracts showed that some regions presented homology to promoter regions located upstream and downstream of genes like PVNR2 corresponding to nitrate reductase and CH5B corresponding to chitinase. Therefore these promoter regions are assumed to contain degenerate retrotransposon insertions, most likely from solo LTRs. Residual regions of retrotransposons have also been found in barley with retrotransposon BARE-1 (Manninen and Schulman, 1993), and the integration pattern is a common feature on many elements (White et al., 1994; San Miguel et al., 1996). In this way LTRs have played and important role in the evolution of gene expression, actually becoming regulatory for basal genes (White et al. 1994).

Element	aa	stop	in	PPT	LTR	Ln
CIAT-Tpv454	29	TAA	42	AGGGAGAGAA	TTGTTCTGGTTTG	85
CIAT-Tpv992	29	TAA	45	GGGGAGAA	TTATTTTGGTTTG	36
CIAT-Tpv357	29	TAA	44	AGGGGGAGAAATA	TGTTGGGATTTG	54
CIAT-Tpv3	30	TGA	29	GAAGTGAAGAGA	TGCATCGAAGGT	108
CIAT-Tpv930	29	*CAG	68	AGGAAAGAGACAG	TGAACTTTGACT	34
CIAT-Tpv76	25	TGA	58	GAGAAGA	TTTGTATTAGGTGG	24
CIAT-Tpv814	25	TGA	58	GAGAAGA	TTTGTATTAGGTAG	231
CIAT-Tpv223	25	TGA	58	GAGAAGA	TTTGTTTTAGGTGG	56
CIAT-Tpv829	25	TGA	58	GAGAAGA	TTTGTTTTAGGTGA	117
CIAT-Tpv1150	22	TAA	65	GGAGGAGA	TTTATGAAGTTTGG	201

Fig. 3. Regions of isolated retrotransposon fragments. The number of aminoacids before stop codon (aa), the number of intervening nucleotides between RNAse and polypurine tract (in), and the number of isolated nucleotides from LTR section (Ln), are indicated. The Polypurine Tract (PPT) and the beginning of the Long Terminal Repeat (LTR) are also shown. An asterisk indicates that a base of the stop codon is mutated. The inverted repeat of LTR is in bold characters.

Additionally some of the presumed LTR regions (L814-90, L438-314) were analyzed with the program MatInspector to search for putative transcription factor binding sites. Besides finding typical transcription factor binding sites, high scoring homology was found to SBF-1 which is also present in the promoter region of bean defense gene CHS15 coding for chalcone synthase (Lawton et al. 1991), and to DOF recognition motifs which are also related to pathogen responsive gene expression (Yanagisawa and Schmidt 1999). These elements may provide evidence of recombination mechanisms leading to activation of retroelements by the same factors activating defense genes. Although the mechanism leading to acquisition of these regulatory sequences between both types of sequences is obscure, retrotransposons related to RGA clusters have been found in rice (Song et al., 1997), lettuce (Meyers et al., 1998) and bean (Acosta, unpublished results). The acquisition of these sequences by retrotransposons could also explain retrotransposon activation by stress factors (Hirochika et al. 1996), which is also a common feature of defense gene promoters.

Future plans

- Mapping of a population derived from a G19884*DOR362 cross using primers derived from several LTR regions from different retroelements using a modification of the SSAP technique (Waught et al, 1997).

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1.3.8 Diversity array technology (DArT) for cassava mapping

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Introduction

DArT is one of the latest techniques employing microarrays. It is based on the generation of an array or panel of DNA segments with unknown sequences of two or more genomic DNA individuals. These segments are hybridized with fluorescent-labeled DNA probes of two individuals used for creating the panel (Jacoud et al., 2001). In our case the panel was created with DNA sequences of only two mapping population parents. The technique has a wide range of applications, among which are tracking genome methylation changes, germplasm

characterization, genetic marker-assisted breeding, dilucidation of complex genomic mixtures and genetic fingerprinting. The fact that there is no need for previous sequencing and that this technique is characterized by rapid, high throughput makes it an inexpensive and suitable option for generating genetic map saturation in Neotropical plants such as cassava.

Materials and Methods

Generation of panels. Whole genomic DNA was extracted from four parents of two mapping populations: NGA-2 and CM 2177-2 for the first one; M Ecu 72 and M Col 2246 for the whitefly-resistance gene mapping population. Total DNA (100 µl) of each parent of the first population was bulked and digested with 2 units of the restriction enzyme PstI. Then the digestion product was bonded to its corresponding adapter and PCR amplified. The product of this amplification was further column- purified using QIAGEN PCR cleaning kit, then bonded to the pGEM T-easy vector, and used to transform competent *Eschlerichia coli* strain DH5α by electroporation. The positively transformed cells (white colonies) were transferred onto a freezing medium, where they grew at 37°C overnight. The culture was PCR amplified using T7 and Sp6 universal primers. Afterwards PCR products were alcohol precipitated and dried at room temperature. The same protocol was carried out with both parents, but this time using restriction enzyme MspI. With the other two parents, the procedure was repeated using the same enzymes. Once the PCR products were dried, they were re-suspended in spotting buffer. These were spotted onto microscope glass slides covered with polysine, using SPBIO MirraiBio's spotter. Slides were processed as per Jacoud et al. (2001).

Generation of the representation. DNA (100 ng) of each parent was digested with the two enzymes in separate reactions, bonded to their own adapter and PCR amplified. A fraction of that product was also digested with EcoT14I or BanII and PCR amplified. The product was then column purified (QIAGEN PCR purification kit) and labeled with Cy3 or Cy5 dyes, using an Amersham Megaprime Labeling kit, except that the enzyme was Kleenow fragments, exonuclease free, polymerase I. Five units were employed for each reaction.

Hybridization. Cy3 and Cy5 solutions were concentrated at 5µl and mixed with hybridization solution from Clontech, denatured at 94°C for about 3 min, and pipetted directly onto the microarray surface. They were immediately covered with glass cover slips and incubated at 60°C in a water bath hybridization chamber from Clontech overnight. After hybridization the slides were process washed in SSCXSDS solution. After washing, the slides were dried at room temperature by centrifugation.

Scanning and image analysis. Once dried, slides were scanned with Virtek Chipreader, and images were analyzed using Array Pro version 4.0.

Results

Four genomic libraries were generated; two libraries for each pair of two mapping population parents:

Library 1: CM2177-2 and NGA-2 (PstI) contains 1536 clones.
Library 2: CM2177-2 and NGA-2 (MspI) contains 768 clones.
Library 3: MEcu72 and Mcol2246 (PstI) contains 1536 clones.
Library 4: MEcu72 and Mcol2246 (MspI) contains 1536 clones.

Only the first library was taken to make 7 panels and hybridize them: 3 using PstI DNA probe, 2 using PstI and EcoT14I DNA probes, and 2 using PstI and BanII DNA probes. Eight clones of this library are possibly polymorphic. Table 1 summarizes the place in the library where clones are and to which parent they are related.

Table 1. Type of probe hybridized to panel that contains clones of CM2177-2 and NGA-2 PstI library, No. of possible polymorphic clones found in the library with the respective probe used in the hybridization of the array, place in the library or name of the polymorphic clone and its corresponding parent.

Type of Probe Hybridized	No. Possible Polymorphic Clones in Library	Place in Library	Parent
Digested with PstI	5	384/1*B18	NGA-2
		384/1*F2	NGA-2
		384/1*H10	NGA-2
		384/2*F20	NGA-2
		384/3*A11	NGA-2
Digested with PstI & EcoT14I	4	384/1*F2	NGA-2
		384/2*O5	CM2177-2
		384/3*A11	NGA-2
		384/3*I23	NGA-2
Digested with PstI & BanII	3	384/1*F2	NGA-2
		384/2*F22	NGA-2
		384/2*O5	CM2177-2

In this table it can be seen that the level of polymorphic clones found by this method is low. Only 8 clones seem to have different sequences between parents that correspond to 0.52% of the library. The DArT technique is not yet completely standardized so not all of the genomic libray clones were represented in the hybridization.

When using other probes, other polymorphic clones were found in the same library. For example, clone 384/2*F22 was found only when using probes PstI and BanII. On the other hand, some polymorphic clones were found when using different probes; e.g., clone 384/1*F2 was found using all three types of probes.

Seven polymorphic clones were sequenced and compared with the sequences database, but no matches with reported sequences were found.

Future Activities

- Continue to adjust application of DArT in cassava
- Analyze microarray images to obtain more precise data about the ratio of color signals in each spot to reach conclusions about polymorphisms of specific clones
- Identify CM2177-2 and NGA-2 genomic library polymorphic clones generated with the MspI restriction enzyme
- Identify M Ecu 72 and M Col2246 genomic library polymorphic clones
- Sequence polymorphic clones and analyze sequences by contrasting them to sequence databases
- Make panels with individuals derived from aforementioned cross and hybridize polymorphic sequences found with these panels to determine genetic markers in order to saturate genetic maps.

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1.3.9 Discovery of SNPs in *Phaseolus vulgaris*

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Introduction

The most abundant genetic polymorphisms between individuals are Single Nucleotide Polymorphisms (SNPs) and small insertions/deletions, both of which are emerging as a new generation of markers due to their abundance and amenability to fully automated genotyping. SNPs are single base-pair positions at which different sequence alternatives exist between two individuals. There are six possible SNP types, either transitions (A<>T or G<>C) or transversions (A<>G, A<>C, G<>T or C<>T). (http://www.cerealsdb.uk.net/snp.htm.). These polymorphisms can be used as simple genetic markers, which may be identified in the vicinity of virtually every gene. There is also great potential for the use of SNPs in detecting associations between allelic forms of a gene and phenotypes, especially for common diseases that have multifactorial genetics. Several different routes to the discovery of SNPs may be taken: The re-sequencing of PCR amplicons with or without prescreening, electronic SNP (eSNP) discovery in shotgun genomic libraries, eSNP discovery in expressed sequence tag (EST) libraries, and direct sequencing of DNA segments (amplified by PCR) from several individuals (Rafalski, 2002). The Project (SB-02: Assessing and utilizing agrobiodiversity through biotechnology) started discovering SNPs using direct sequencing from 10 different *Phaseolus vulgaris* genotypes.

Materials and Methods

Ten genotypes belonging to Mesoamerican and Andean pools (wild and cultivated) are being used for sequencing reactions to find polymorphisms. Several primer pairs were designed from sequences obtained from the GenBank, which amplified from 300-1000 bp for noncoding regions such as introns, CDs and some RFLP probes. Libraries were generated using degenerated primers, clones were sequenced and specific primers were designed to amplify all 10 genotypes. Primer pairs producing strong and unique bands for each genotype were used in sequencing reactions. The PCR products for each region/genotype were sequenced directly in both directions. The resulting sequences were edited, and aligmens using Sequencher Software version 4.1 and Clustal X were done to find the polymorphism between genotypes. Primers that produced more than one band were eliminated in this study.

Results and Discussion

A total of 46 primer pairs were standardized for magnesium concentration and annealing temperature. Almost all of them can be amplified using the same PCR program. Of the 46 primer pairs, no polymorphism was found in only 4 (9%); in 28% there were regions with insertion/deletion polymorphisms of at least 1 bp in size. We covered 20,964 bp of the *P. vulgaris* genome in which we observed 223 SNPs in total, and 111 (50%) of them were

polymorphic between DOR364 and G19833. An example of this can be observed in Figure 1, in which two SNPs are polymorphic between parentals and gene pools.

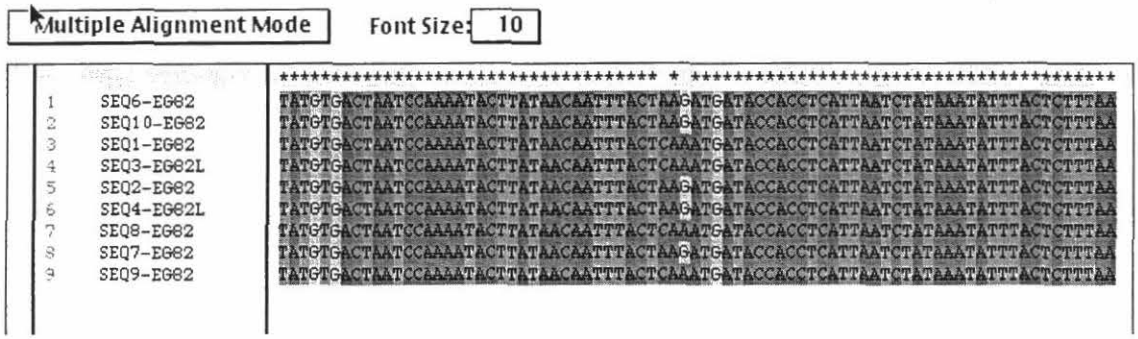

Figure 1. Clustal X alignment among ten genotypes using one intronic region.

Future Activities

Design primers from the polymorphic site to be used for *single-nucleotide primer extension*
Standardize high-throughput detection of SNPs, using the bead system on Luminex-100
Use polymorphic primer extension for SNP detection in mapping and diversity

References

Rafalski, A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Curr Opin Plant Biol 5:94-100.

1.3.10 Saturation of the cassava molecular genetic map using PCR-based markers: Progress on the mapping of SSR markers

Chikelu Mba, Tanya Garcia, Diego F. Cortes, Martin Fregene and Joe Tohme

Introduction

The utility of the molecular genetic framework map of cassava published five years ago (Fregene *et al.*, 1997) has been hampered by the preponderance of RFLP markers that were used in anchoring the map. On realizing that the map could be made a lot more useful for cassava researchers worldwide especially in the NARS of Africa, Asia, Latin America and the Caribbean, through the inclusion of PCR-based markers, CIAT's BRU has over the last few years been developing and mapping simple sequence repeat markers (Mba *et al.*, 2001 a & b; Fregene Pers. Comm.; Mba, Pers. Comm.). There have been two batches of SSR markers developed, one set from a cassava whole genomic library while the second batch were sourced from a cassava roots and leaves cDNA library.

This report summarizes the mapping of the series of 157 SSR markers developed from the cassava roots and leaves cDNA library.

Methodology

Mba *et al.* (2000 and 2001) had described the construction of the cDNA library, picking of clones, isolation and sequencing of putative SSR-containing clones, primer design and synthesis for clones containing SSR loci in suitable positions. The use of these SSR markers to screen the parents of the mapping population, TMS 30572 and CM 2177-2, and the initial screening of the 150-member F_1 mapping population progeny were also described in these 2 reports.

The SSR markers that had a unique allele in either or both parents of the mapping population, i.e. polymorphic, were used to screen the 150 progenies making up the F_1 mapping population. The segregation data of the markers that fitted the expected ratio of 1:1, presence: absence of the unique parental allele were used to place the markers on the framework map using the linkage analysis computer package MAPMAKER 2.0. (Lander *et al.*, 1987).

A LOD score of 2.0 or more was used for placing the markers within the existing linkage groups. All the data analyses procedures were the same as has been already described for the mapping of some SSR markers from a whole genomic library (Mba *et al.*, 2001). Specifically, the "compare", "try"," three point", "ripple" and "map" commands of MapMaker were used to determine the genome locations of the markers. The Kosambi function of this software was used all through.

Results and Discussion

Amplification of the SSR loci. Out of the total of 157 SSR markers, the genome loci corresponding to144 of them or 92% were successfully amplified at either of the annealing temperatures of 50°C, 55°C or 57°C. In all, 59 out of these 144 markers, or 38%, had at least an unique allele in one or both parents of the mapping population in which case they were classified as polymorphic while 85 of them or 54% had no unique alleles in either of the parents, making them monomorphic and hence of no use for mapping in this population (Figure 1).

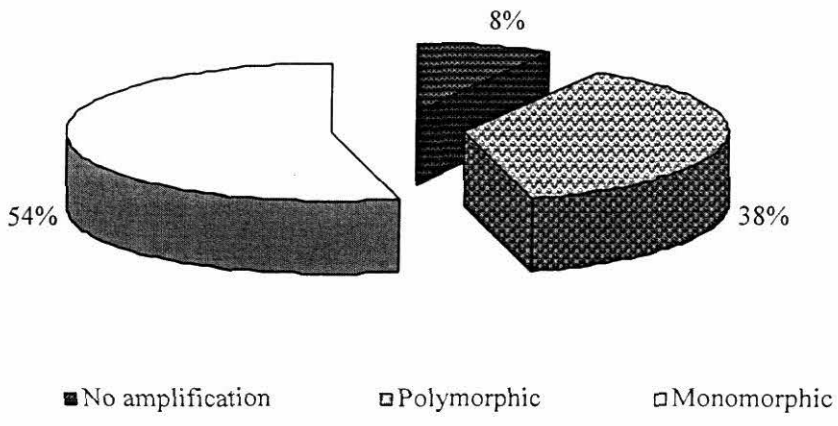

Figure 1. Distribution of 157 primer pairs used to survey the 2 parents of the mapping population

Amongst the polymorphic SRR markers, i.e. those with at least one unique segregating allele in at least one of the 2 parents of the mapping population, 3 types of polymorphism were observed, namely, one unique allele in TMS 30572 (54%), a unique allele in CM 2177-2 (46%) or at least a unique allele in both parents (20%), this last type being referred to as "allelic bridges". The results obtained are summarized in Figure 2 below.

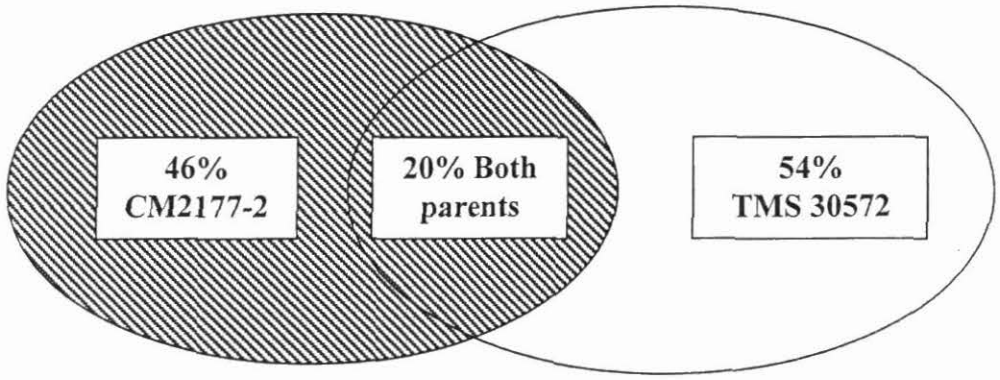

Figure 2: Types of polymorphism observed in the parents of the mapping population in percentages of total polymorphism observed

Data Analyses

Statistical tests. Chi-square tests of one degree of freedom at 0.01% level of significance were carried out to determine if the markers segregated according to the expected Mendelian ratios. It was found out that all the 41 SSR markers that were polymorphic for the female parent, TMS 30572 segregated according to the expected 1:1 ratio. All but 4 of the 35 markers that were polymorphic for the male parent, CM 2177-2, segregated according to the expected ratios of 1:1 and 3:1.

Genome location of the markers. The linkage groups initially established by Fregene *et al.* (1997) and followed by Mba *et al.* (2001) were maintained and the new SSR markers whose mapping is being described in this report fit into these groups.

From the present work, 52 new SSR markers derived from a cassava roots and leaves cDNA library were placed on the existing molecular genetic framework map of cassava (Figure 3). Forty-seven of these markers were uniformly distributed over 16 of the existing 18 linkage groups. Also, 5 of these were linked to a group of markers that are yet to be assigned to any of the existing linkage groups (shown on the bottom right hand corner of the linkage map, Figure 3). The distribution of these markers within the linkage groups is summarized in Table 1.

Table 2. Number of the new SSR markers according to linkage groups for both the female- and male-derived molecular genetic maps

Linkage group	Female-derived linkage map (TMS 30 572)	Male-derived linkage map (CM 2177-2)
A	1	2
B	1	1
C	1	1
D	5	5
E	2	0
F	3	0
G	3	3
H	2	5
I	1	0
J	1	0
K	3	3
L	2	2
M	1	2
N	1	0
O	0	0
P	1	1
Q	1	2
R	1	0
Not assigned	3	2

Out of the 52 SSR markers evaluated, 14 of them had polymorphic bands that segregated for both parents (Table 2), thereby forming 14 allelic bridges in 9 linkage groups, an example of which is shown using linkage group L in Figure 4. A criterion for assigning linkage groups is based on this concept of allelic bridges as this helps corroborate the veracity of each linkage group as being the same for segregations from both parents. In the linkage group L, 2 allelic bridges corresponding the map positions of two markers, SSRY236 and SSRY 250, are shown (Figure 4).

Table 3: List of the new SSR markers that segregated for both parents according to the linkage groups where they were located.

SSR Marker	Linkage Group
SSRY 191	H
SSRY 217	M
SSRY 222	K
SSRY 226	G
SSRY 236	L
SSRY 240	D
SSRY 242	A
SSRY 248	P
SSRY 251	D
SSRY 250	L
SSRY 295	D
SSRY 297	K
SSRY 299	Not assigned
SSRY 328	H

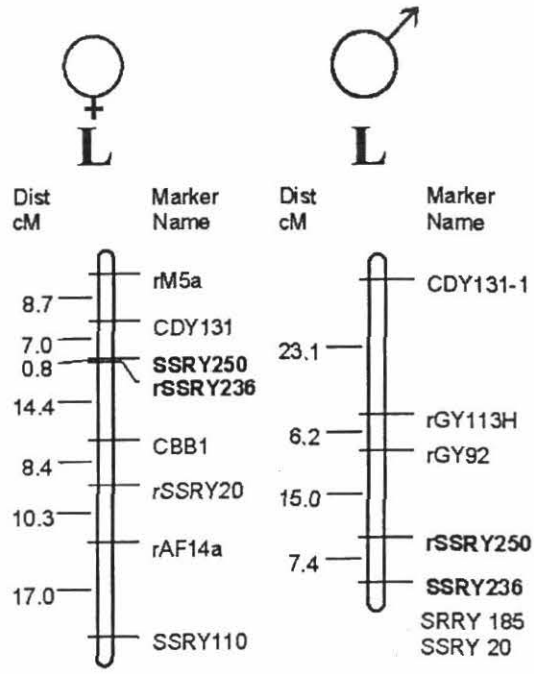

Figure 4: Linkage Group L showing 2 allelic bridges formed between the male- and female-derived linkage maps by 2 newly located SSR markers, SSRY236 and SSRY250

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1.3.11 Construction of an SSR Map of Cassava Based upon Linkage Analysis in a F_2 Cross Derived from Non-Inbred Parents and QTL Mapping of Early Bulking.

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CIAT
Funding: The Rockefeller Foundation

Introduction

The QTL mapping early bulking at CIAT has identified a number of major QTLs for this important trait (Okogbenin and Fregene 2002). Last year, a new F_2 mapping population was developed from an F_1 individual to validate the authenticity, magnitude, and action of these QTLs. Quantitative trait loci (QTL) mapping in single generation, full-sib pedigrees of allogamous crops is complicated by the inability to access all information on the genetic architecture of the quantitative trait in question. Furthermore, marker genotype in these mapping populations result from the independent meioses and crossovers in the maternal and paternal parents leading to separate maps for each parent which alters QTL mapping by redefining mating type at a locus level rather than all loci in both parents. (Groover et al. 1994; Van Eck et al. 1994; Grattapaglia et al. 1994).

The new genetic map of cassava was constructed using SSR markers, a relatively easier to use marker system. The use of SSR markers have considerably cut down the time required for the development of a cassava map compared to earlier efforts, from 3 years to a little over a year. Although, 3 F_2 crosses were developed, only one, the cross with the highest number of heterozygous markers in the F_1 parents, was chosen for map development.

Methodology

The F_2 population obtained from the K150 progeny of the cassava map population was selected as our F_2 mapping population because of the large number of markers that were heterozygous in K150, more than 60%, and the relatively large number of progeny, 372. Due to poor seedling development of certain genotypes (resulting in senescence in some few cases), only 268 plants of initial 372 in the F2 population were used for genotyping. The F_2 seedlings were initially germinated in the screen house at CIAT headquarters in Palmira under intensive management and care in February 2000. Seedlings were later transplanted to the field in July 2000 and harvested for planting materials at 11 months after planting (MAP) the following year (2001). Of the 268 genotypes used for mapping analysis, only 207 genotypes with relatively sufficient stem cuttings (12 stakes) of about 25 cm long each could be planted for QTL mapping experiment at Santa Elena, in a location, 25 Km from CIAT headquarters. Individual genotypes were sown on May 18, 2001 in single row plots of 6 plants each in randomized complete block design of two replications. Plants were planted at 0.8 cm between plants and 1m between rows. All plants were harvested 7 months after planting (MAP). Traits measured include: dry root yield, fresh foliage and harvest index.

DNA was extraction from individual genotypes of the F_2 population has been described earlier (CIAT 2001). For the parental survey, 186 SSR markers developed by Mba et al. (2001), 132 SSR markers from a cassava root and leaf cDNA library (Mba et al., unpublished data), and 154 SSR markers generated by Fregene et al. (unpublished data), a total of about 500 markers, were used. PCR amplification and page gel electrophoresis were as described by Mba et al (2001). For

linkage analysis, individuals of the F_2 population were by the three different genotypic classes expected for a F_2 population and Chi square values were computed to test for significant deviations from expected ratios (segregation distortion). Linkage analysis was using MAPMAKER/EXP 3.0 (Lander et al., 1987). Recombination fractions were converted to map distances, centiMorgan (cM), using Kosambi mapping function..

The mean of each genotype over two replications was used for the correlation analysis and QTL mapping Phenotypic correlations coefficients between yield and components were estimated and tested for significance ($P< 0.05$). Type III mean squares from ANOVA based on General Linear Model procedure (Proc GLM; SAS 1996) was used to calculate broad sense heritability estimates for each trait.

The locations of putative QTLs were determined by interval mapping analysis using MAPMAKER/QTL 1.1b (Patterson et al. 1988). Maximum likelihood estimates of both additive (a) and dominance (d) effects were calculated simultaneously during the genome scan for QTLs as performed by MAPMAKER/QTL. The gene action of a QTL, largely additive, dominant or recessive, can be determined by evaluating the relative likelihood of gene models. To test for additional QTLs, we fixed the position and effect of one QTL, the single QTL model (SQM), then re-scanned the genome searching for other QTLs, using a two-QTL model (TQM). Two-QTL map allows each locus to control its fraction of the variance while at the same time estimating the effect of the other. The multi-locus model was used explain how much of the phenotypic variance among the F_2 population for each trait was explained by fitting in the model, QTLs identified in SQM and other additional QTLs detected in TQM.

Results

A total of 122 SSR markers, or 25%, were polymorphic in the F_1 parent and could be scored in the F_2 mapping population (Fig 1). This number is close to what is expected, that is 50% x 50% (average percent polymorphisms of SSRs in cassava x percent polymorphisms expected in a F_1 genotype). Most of the markers surveyed had the expected segregation, a ratio of 1:2:1, for homozygous for parent A, heterozygous and homozygous for parent B respectively. Deviation from the expected 1:2:1 genotype frequency was significant ($P \leq 0.05$) for 33 (27%) of the 122 markers scored. The 122 markers were employed in constructing a linkage map (Fig2). The linkage map consists of 100 markers, 22 markers remained unlinked. The presence of so many unlinked markers suggests that the available SSRs still do not cover the entire cassava genome. The number of linkage groups in this map (22) exceeds the haploid number of chromosomes for cassava, indicating that the map is also unsaturated.

Phenotypic data for DR, FF and HI showing means, standard deviation, kurtosis, skewness, and W-test are summarized in Table 1. Data range for each trait measured revealed wide variation in the F_2 population as equally observed in the F_1. All of the traits studied showed continuous distribution as expected for quantitative traits. The heritability (H^2) estimates in the F_2 were 66% for dry root yield (DR), 68% for Fresh foliage weight (FF) and 78% harvest index (HI). The relatively high heritability of the three traits is in agreement with high estimates obtained in the F_1 (Okogbenin and Fregene 2002). A total of nine QTLs (LOD> 2.0) (three QTLs each) influencing FF. DR and HI were identified by interval mapping analysis on seven linkage groups (Table 2). Results revealed that two QTLs (*Dr3* and *Ff3*) fall within a single interval (NS 928 – SSRY 153) separated by only 4 cM (Table 2). The direction of the genetic effects of these two QTLs are similar, suggesting that they are probably not different QTLs, providing evidence for gene pleiotropy for DR and FF at this locus.

Seven highly significant two-QTL interactions were identified for FF, and 4 each for DR and HI (Table 3). Phenotypic variance (PV) explained for these interactions varied from 11 to 36% with LOD scores ranging between 2.74 and 8.97. Some of the QTLs identified in the single QTL model (SQM) significantly interacted with each other. In some instances, interactions led to highly significant increase in LOD and PV explained. For example, *Dr1* significantly interacted with *Dr13* resulting in LOD of 5.14 and explained PV of 17.2%, which were higher than the sum of LOD scores and PVE for both QTLs under the SQM. All additional QTLs identified in the two-QTL model (TQM) were fitted along with those identified in the SQM in a multi-locus model to determine total phenotypic variance explained among the F_2 progeny for each trait. The total PV explained based on multiple QTL model are 33% for foliage, 44% for DR and 37% for HI.

The gene action of individual QTLs was evaluated by comparing the fits of individual QTL models (Lander and Botstein, 1989). The three QTLs detected for FF revealed different gene actions: *Ff3* had a recessive gene model, *Ff5* dominance, while *Ff9* exhibited additive gene action. Two of the QTLs identified for DR (*Dr3* and *Dr13*) were consistent with a recessive gene model. Two other QTLs, *Hi2* and *Hi9* exactly fit a pure additive model. The additive effect, which is the measurement of the change in a population mean when an allele of a QTL is substituted, showed that *Hi9* increased harvest index while *Hi2* decreased HI. The third QTL for HI (*Hi12*) was recessive .

A comparison was made of QTLs identified in the experiments using F_1 and F_2 crosses. Results reveal that 1, 7, and 3 common QTLs were detected for fresh foliage, dry matter yield and harvest index respectively (Table 4).

Future Perspectives

- Test QTLs that are stable across generations and thatexplain substantial phenoytpic variance (>20%) in a different genetic background

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Table 1 Performances of three traits evaluated in the F_2 population

Trait	Range	Mean	Standard deviation	Skewness	Kurtosis	W-statistic
Fresh foliage (g)	112.50-3900.00	1152.15	583.26	1.08	2.34	0.94*
Fresh root (g)	213.24	213.24	120.62	0.27	-0.43	0.96*
Harvest Index	0.38	0.38	0.14	-0.4	-0.28	0.96*

Table 2. QTLs associated with dry root yield (DR),fresh foliage(FF) and harvest index (HI) in the F_2 population

Trait	QTL	Flanking markers	Length (cM)	Linkage group	LOD	QTL position (cM)	PVE	*a*	*d*	*d/a*	Mod
FF	Ff3	NS 928 – SSRy 153	16.3	3	2.95	0.0	7.6	-87.84	274.90	-3.13	R
	Ff5	SSRy 35 – SSRy 284	28.1	5	2.26	10.0	31.1	-414.21	-556.36	1.34	D
	Ff9	SSRy 12 – SSRy 91	31.0	9	2.16	38.0	5.5	-211.98	-21.83	0.10	A
DR	*Dr1*	NS 911-NS 847	17.7	1	2.18	12.0	9.2	0.3	73.70	245.67	DR
	Dr3	SSRy 928 - SSRy153	16.3	3	2.14	4.0	7.3	-21.53	52.40	-2.43	R
	Dr16	NS 33 - SSRy 100	16.3	13	2.25	18.0	6.0	47.35	-64.61	-1.36	R
HI	*Hi2*	NS 149 - SSRy83	7.3	2	6.67	0.0	15.0	-0.08	0.00	0.05	A
	Hi9	SSRy52 - NS 340	3.1	9	2.25	0.0	54.3	0.05	0.00	0.00	A
	Hi12	NS 74 - NS 389	44.4	12	2.10	0.0	4.9	0.01	-0.06	-6.00	A

Individual QTL loci are named by trait (abbreviation indicated in titles) and linkage groups. The LOD score (LOD) and percent phenotypic variance explained (PVE) by the QTLs are presented from the single-QTL model with unconstrained gene action. The additive effect (*a*) dominance deviation (*d*), and ratio of dominance to additivity (*d/a*) for each QTL are presented in their original units. The possible pure modes of gene action (Mode) for each QTL are indicated based on testing of additive (A) and dominant (D, R) models as described in Materials and methods (if $d = 0$, then A, if $d = a$ then D, if $d = -a$ then R). If a model reduced likelihood by 10-fold or more, it was deemed unlikely. When two pure modes of gene action could not be deemed unlikely, the more likely mode was listed first (e.g for *Dr1*, dominance (D) was most likely but recessivity (R) could not be deemed unlikely, thus the mode for this locus is denoted DR. QTL position is position of LOD peak given as distance from the first marker listed in the interval.

Table 3 Two-QTL interactions affecting Dry root yield, fresh foliage and harvest index

Trait	LG	Interval 1	QTL	QTL position (cM)	Interval 2	QTL	QTL position (cM)	LG	PVE	LOD
FF	9	SSRY 12 – SSRy 91	*Ff9*	38.0	NS 717 – SSRy 3	*Ff4a*	14.0	4	12.0	3.07
	9	SSRY 12 – SSRy 91	*Ff9*	38.0	NS 217 – NS74	*Ff12*	0.0	12	11.1	4.02
	9	SSRY 12 – SSRy 91	*Ff9*	38.0	SSRy50 – SSRy 281	*Ff15a*	40.0	15	11.9	2.74
	3	NS 928 – SSRy 153	*Ff3*	0.0	NS 717 –SSRy 3	*Ff4a*	14.0	4	16.0	3.97
	3	NS 928 – SSRy 153	*Ff3*	0.0	SSRy12 – SSRy91	*Ff9*	38.0	9	13.2	4.98
	3	NS 928 – SSRy 153	*Ff3*	0.0	SSRy 50 – SSRy281	*Ff15b*	28.0	15	15.4	3.56
	5	SSRy 35 – SSRy 284	*Ff5*	10.0	NS 717 – SSRy 3	*Ff4b*	8.0	4	36.0	3.48
DR	3	NS 928 – SSRy 153	*Dr3*	4.0	NS 717 – SSRy 3	Dr4	16.0	4	13.2	3.47
	16	NS 33 – SSRy 100	*Dr16a*	18.0	NS 74 – NS 319	*Dr12*	20.0	12	14.6	3.30
	16	NS 33 – SSRy 100	*Dr16a*	18.0	NS 33 – SSRy 100	*Dr16a*	12.0	16	15.4	3.23
	1	NS 911 – NS 847	*Dr1*	12.0	NS 33 – SSRy 100	*Dr16b*	18.0	16	17.2	5.14
HI	2	NS 149 – SSRy 83	*Hi2*	0.0	SSRy 182 – SSRy 148	*Hi8*	8.0	17	22.1	8.97
	9	SSRy 52 – NS 340	*Hi9*	0.0	NS 149 – SSRy 83	*Hi2*	0.0	2	17.0	7.64
	12	NS 74 – NS 389	*Hi12*	0.0	NS 267 – SSRy 1	*Hi18*	26.0	18	13.1	2.76
	12	NS 74 – NS 389	*Hi12*	0.0	NS 149 – SSRy 83	*Hi2*	0.0	2	18.3	8.25

In cases where multiple QTLs affecting a trait were found along the same linkage group, the QTLs are distinguished by letters indicating the temporal order in which they were discovered (e.g. *Ff15a* and *Ff15b*). The LOD score (LOD) and percent phenotypic variance explained (PVE) by the QTLs are presented from the two-QTL model with unconstrained gene

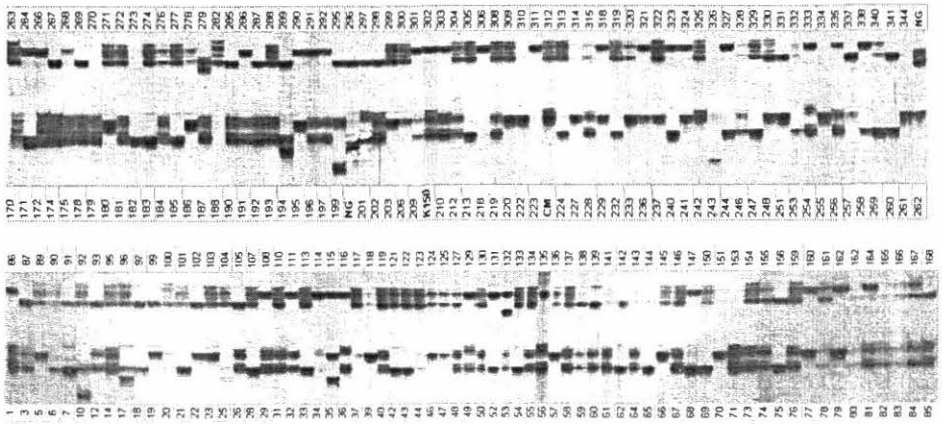

Figure 1. Silver stained polyacrylamide gel showing segregation of SSRY marker SSRY105 in individuals of the F_2 mapping population

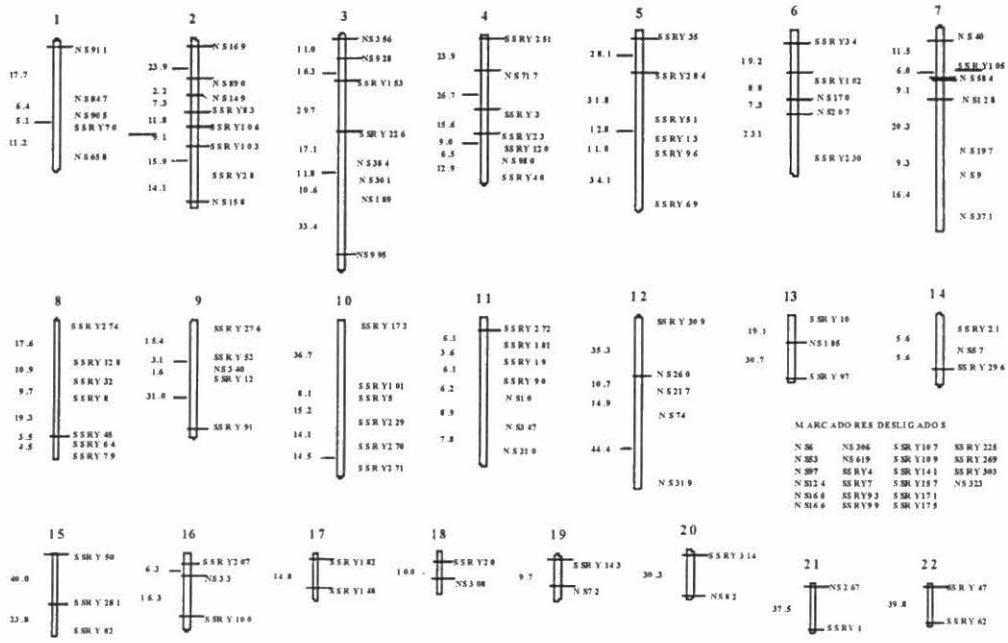

Figure 2. A linkage map of cassava (Manihot esculenta Crantz) based upon a F2 cross and SSR markers

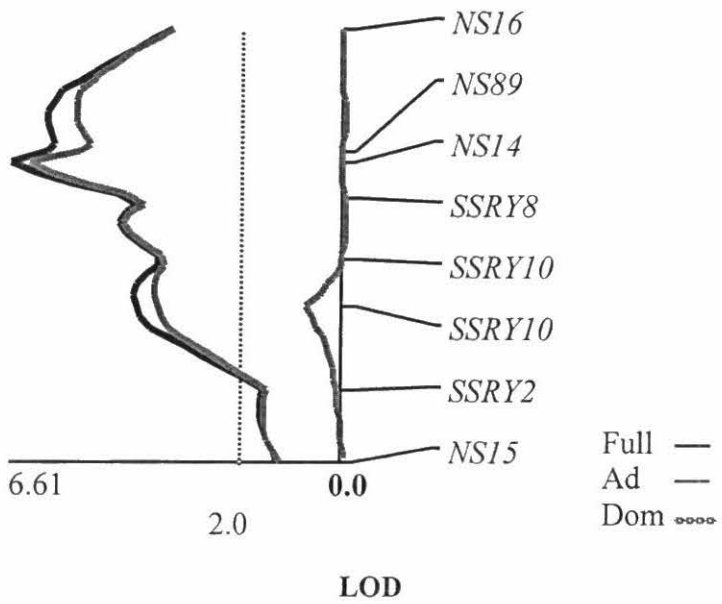

Figure 3. A first order QTL for harvest index detected by interval mapping.

Table 4. Putative QTLs that were common in the F_1 y F_2 mapping populations for fresh foliage, dry matter yield and harvest index

Trait	QTL F_1	QTL F_2
F.F	GP R (GY48)	GP 22 (SSRY47-SSRY62)*
DR	GP S (GY153-GY212)	GP 1 (NS911-NS847)
	GP G (GY6)	GP 3 (NS928-SSRY53)
	GP D (GY181-GY42)	GP 4 (NS 717-SSRY3)*
		(SSRY3-SSRY23)*
	GP J (K10)	GP 10 (SSRY5-SSRY229)*
	GP-L (CBB1; CDY131)	GP 18 (SSRY20-NS308)*
	GP UD (GY24)	GP 2 (NS149-SSRY83)
HI	GP E (NGY162)	GP 12 (NS74-NS319)*
	GP A (rBEST)	GP 20 (SSRY 314-NS82)*
	GP J (GY34)	GP 17 (SSRY182-SSRY148)

FF = fresh foilage yield; DR = Dry matter yield; HI = Harvest index. Gp = Linkage group. The marker interval where the QTL was found is indicated in parenthesis.

1.3.12 Progress Towards a PCR-Marker Based Map of Cassava

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SB-2
Funding: CIAT

Introduction

Progress towards a PCR-based map of cassava for gene tagging and marker-assisted selection has crossed a significant mile stone with the report of a SSR only map of cassava (CIAT 2002, this report). However, 20% of SSR markers evaluated remained unlinked and denotes that the map is not complete and more markers need to be developed. At the same time, saturation of the RFLP genetic map of cassava has continued with 57 SSR markers from genomic sequences (Zarate et al 2002, unpublished data) and another 45 SSR markers from cDNA sequences (Garcia et al 2002, unpublished data) added to the map.

To ensure that a PCR-based map of cassava can be achieved within the near future efforts have been geared this year to converting RFLP markers on the genetic map of cassava to single sequence conformation polymorphism (SSCPs). Furthermore, additional SSR markers have been designed from cassava genomic sequences reported in GeneBank, with particular reference to BAC end sequences developed at the Clemson University Genome Institute (CUGI).

Methodology

More than 100 RFLPs from the molecular genetic map of cassava have been sequenced. Primers were designed from 2 RFLP clones, CYP79D1 and CYP79D2 and used to amplify total genomic

DNA from the two parents of the mapping population and a sub-set of 20 progenies. PCR amplifications were carried out in 12.5-µl reactions containing 100 ng of DNA, 0.5µM of each primer, 10 X of *Taq* polymerase buffer (500mM KCl, 100mM Tris-HCI (pH 8.5), and 1 mg/ml gelatin), 2mM of $MgCl_2$, 0.5mM of dNTPs and 0.25 U of *Taq* polymerase. The PCR profile was: 94°C for 2 min, followed by 30cycles of 95°C for 1 min, 55 °C for 2 min and 72 °C for 2min. A final extension step of 72 °C for 10 min was added at the end. The PCR amplification was cleaned with the QIAGEN PCR clean-up kit (QIAGEN Inc. Los Angeles, CA) and digested to completion with 10U of Hinf I restriction enzyme for 3h at 37 °C according to the manufacturer's instruction (New England Biolabs, Cambridge, MA). The digestion product was electrophoresed on 6% polyacrylamide-MDE gels (Cambrex Bio Science Inc, Baltimore, MD) made up of 10% glycerol, 6ml 10XTBE, 25ml MDE gel solution, 65ul TEMED, 500ul APS, and 60ml deionized water, at 40Watts for 48h. The gel was stained as described by Slaubaugh et al (1997). Briefly, the gel was fixed for 3 min in 10% ethanol, 0.5% acetic acid, stained for 5 min in fixing solution plus 0.2% silver nitrate , washed in water for 1 min and developed for approximately 10-20 min in 3% NaOH and 0.27 % formaldehyde in water. Following staining, the gel was fixed for a further 5 min and washed in water.

The construction of a bacterial artificial chromosome (BAC) library from the white fly resistance variety MOLC72 has been described earlier (Tomkins et al 2001). A total of 2301 BAC ends were sequenced and 1755 good sequences were deposited in gene bank (Tomkins et al 2002. Unpublished data). The BAC end sequences were downloaded from GeneBank and following SSR motifs: (AT), (GA), (CA), (GC), (GT), (TCT), (GCT), (AGC), (GCC), (GGT), (ATT), (GGA), (TATG), and (GTGA) were searched for in the sequences using the DNAMAN software. The local BLAST facility at CIAT (http://gene2/BLAST/inicio.htm) was used to compare the sequences with each other to eliminate duplicated sequences. Primer design was using Primer 3.0, the primer picking software found at http://waldo.wi.mit.edu/cgi-bin/primer/primer3.

Results

SSCP analysis of the parents of the cassava mapping population and a sub-set of 20 genotypes of the same population revealed polymorphisms that segregated as single dose restriction fragment (SDRF) polymorphism. The entire mapping population is being analyzed at the moment. Primers will be designed from other sequenced RFLP markers that are currently on the map to also convert them to PCR-based markers. A draw back of the SSCP methodology is the need to always purchase costly MDE gel solutions (Cambrex Bio Science Inc, Baltimore, MD). Efforts are also being made to try normal polyacrylamide gel solutions in an effort to reduce costs. The sequenced RFLP markers being converted to SSCP markers can also be described as sequence tagged sites (STSs). These have the advantage that once they have been analyzed in any population the map location as well as the sequence is known.

A total of 141 BAC end sequences were found to contain SSR motifs. The distribution of SSR sequences was as follows: 67 dinucleotide, 46 trinucleotides, 4 tetranucleotide, and 24 other (mixture of different repeats) motifs, a mixture of two or three motifs (Table 1). Average length of the dinucleotide repeats was 8 repeats for the dinuleotide, 6 for the tri and 4 for the tetras. Primers designed will shortly be sent for synthesis and once available they will be placed on the existing map of cassava beginning with the parental survey.

Table 1. Di, tri, tetra nucleotide, and other SSR repeats found in the BAC end sequences

Type of SSR	Number	Percentage
AT	38	27
GA	24	17
CA	4	3
GC	1	0.7
TCT	11	8
GCT	3	2
AGC	3	2
GGT	4	2
ATT	12	8
GGA	13	9
TATG	4	2
Others	24	17
Total	141	

Future perspectives

Continue the SSCP analysis for the genetic mapping of STSs in cassava
Parental survey of the additional 141 primers and mapping of polymorphic ones.

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1.3.13 Developing and exploiting expressed sequence tags for cassava starch

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Preamble

This is a collaborative project between CIAT and the French Universities, Universite de Peripgan and Universite de Montpellier, both in France. This is a progress report on the development of expressed sequence tags (ESTs) for cassava starch biosynthesis.

Introduction

Cassava Starch. The annual global starch market is valued at about US$20 billion. Starch demand however depends on its form, there existing distinct market niches for native starch, and such value added products as modified starch and sweeteners. The different forms of starch find application in a myriad of uses in the food industry (as thickener, filler, binder, stabilizer and to improve food texture); and other industries such as paper and cardboard, adhesives, alcohol,

textiles, medicines, oil well drilling, chemicals and in manufacturing (Kay, 1987). The challenge to cassava researchers lies in the development of the cassava varieties that have starch species targeted to these different needs.

That cassava starch is of high quality is evidenced in the fact that it (cassava starch), and its industrially produced derivatives are increasingly replacing the other traditional sources of starch in food, paper, textile and adhesive industries. However, a major bottleneck to this trend of the industrialization of starch production from cassava has been the widely varying characteristics observed for major starch quality indices. At times such variations are observed not only between cassava cultivars but also in the same cultivar under different conditions such as environmental factors and time of harvest (Sriroth *et al.*, 1998).

The Cassava EST Project. The ever-increasing rapid development of genomics and bioinformatic tools is increasing our knowledge of plant genome structure, organization and gene function and one tool which holds a lot of promise in unraveling the complexities in gene expression is Expressed Sequence Tags (ESTs). These are usually short (300 to 500bp) single read from random cDNA. They form the basis for getting a global "still image" snapshot of gene expression (level and complexity) in a given tissue at a given time under given conditions and therefore represents the status of the activities of enzymes encoding for specific plant metabolic pathways.

This strategy of partially sequencing randomly selected cDNA clones has since evolved into an inexpensive and efficient gene discovery methodology (Ohlrogge and Benning, 2000). As far back as 1992, Uchimiya *et al.* had successfully matched the sequences obtained from a rice cDNA library to reported genes in the Genbank. It is little wonder then that the ESTs database is the fastest growing and constitutes the largest portion of the public DNA sequence database with approximately which as at December 2001 contained over 1.3 million ESTs spread over most of the plant genera (Walbot and Delseny, 2002). The large scale exploitation of ESTs as reservoirs for gene cloning, evaluation of tissue-specific gene expression, as markers for map based cloning and for the annotation genomic sequences has not only become routine in Arabidopsis (Györgyey *et al.,* 2000; White *et al.,* 2000) but has been extended to such other plant species as poplar (Sterky *et al.*, 1998); grapes (Ablett *et al.,* 2000); and in *Pinus* (Temesgen, *et al.,* 1998; Cato *et al.,* 2001). Linked with such a novel high throughput platform as the DNA microarray technology, ESTs become even more potent as a rapid route to the identification of genes and to linking sequence information to biological function (Richmond and Somerville, 2000).

The cassava starch ESTs project, a joint project between CIAT and the French Universities of Perpignan and Montpellier, aims at the development of at least 5,000 ESTs each from 2 root cDNA libraries sourced respectively from cassava varieties CM 523-7 and MPer 183. It is hoped that the annotation of these ESTs and in concert with the novel microarray technology, some functions will be assigned to the sequences. This will ultimately therefore lead to the use of these as tools for exploiting the genetic diversity that is available in cassava and by so doing lead to the expansion of the range of the potential uses of cassava starch. Also, it is envisaged that the ESTs to be generated shall be used to develop DNA chips and also converted to PCR primers to be used in saturating the existing molecular genetic framework map as has been demonstrated for *Pinus* (Cato *et al.,* 2001) and also as markers in germplasm fingerprinting.

Materials and Methods

Two cDNA libraries were constructed (Cortes *et al.*, 2001), one each from root tissues of CM523-7 (high starch content) and MPer 183 (low starch content). The authors had reported the detailed procedures for the cDNA synthesis and cloning using the Stratagene's cDNA Synthesis Kit, ZAP-

cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit in CIAT's SB-2 Annual Report for 2001, pages 281-282.

The *in vivo* mass excision of the pBluescript phagemid from the Unizap XR vector using ExAssist helper phage with SOLR strain was according to the Manufacturer's protocols contained in the manual.

Minipreps of the plasmid colonies were done according to the REAL Prep 96 protocols from QIAGEN® . These were then sequenced on the ABI PRISM® 3100 Genetic Analyzer from Applied Biosystems Inc. Sequencing was from the T3 end.

Results

This is a summary of results obtained and available to CIAT as of 26 June 2002. The table below summarizes the progress report on the isolation and sequencing of cDNA clones from the 2 libraries. Presently, the sequences for 3770 unique clones have been obtained. The sequencing of the rest of the clones is on going.

Status of sequencing of 2 cassava roots cDNA libraries

	CM523-7	Mper183
No. of clones picked	5376	4992
No. of clones sequenced	2304	2688
Average %age good sequences obtained	Approx. 85%	
No. of unique clones	1733	2037
Average %age redundancy	Approx. 21%	

Putatively interesting hits in genbank
The following shows the entries in the genbank that have given significant hits with some of the ESTs
xyloglucan endo-1,4-beta-D-glucanase (EC 3.2.1.-) XTR-6 - A. th.
nucleotide sugar epimerase-like protein - A. th.
sucrose synthase (EC 2.4.1.13) - fava bean
probable sugar transport protein F23E12.140 - A. th.
fructose-bisphosphate aldolase-like protein - A. th.
resistance protein RGC2J - garden lettuce
sucrose synthase-like protein - A. th.
GDP-D-mannose-4,6-dehydratase (MUR1) - A. th.
disease resistance protein RPS2 homolog T12H20.8 - A. th.
starch phosphorylase (EC 2.4.1.1) H, cytosolic F13I12.20 [similarity] - A. th.
pathogenesis-related protein 3 - kidney bean
UDPglucose 4-epimerase (EC 5.1.3.2) (clone GEPI48) – guar
fructose-bisphosphate aldolase (EC 4.1.2.13) isoenzyme C-1, cytosolic – rice
malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) (EC 1.1.1.40) (clone 064) - western balsam poplar x cottonwood
starch phosphorylase (EC 2.4.1.1) H, cytosolic isoform - fava bean
starch phosphorylase (EC 2.4.1.1) precursor - sweet potato

On-going Activities

Sequencing and analyses of the remaining clones (more than 5,000).

- Using the published sequences of genes involved in the starch biosynthesis pathway - ADP glucose pyrophosphorylase; Branching Enzyme and Granule Bound Starch Synthase (GBSS) – (Munyikwa *et al.*, 1997)as queries against all cassava starch ESTs sequences
- Broadening the genbank searches for homology
- Application of the ESTs and future perspectives
- Project aim of the development of at least 5,000 ESTs each from 2 root cDNA libraries is on target
- ESTs to be annotated and along with the microarray technology develop DNA chips
- Will become robust tools for exploiting cassava genetic diversity
- ESTs to be converted to PCR
- Project scope however is limited and to be fully meaningful, a much larger project based on cDNA libraries from a broader range of cassava varieties (starch quantity and quality yields) is an imperative.

Conclusion

Undoubtedly, some appreciable gains have been made in the development and deployment of molecular tools for cassava improvement. These include the RFLP-anchored framework map (Fregene *et al.*, 1997), the development and mapping of SSRs (Chavarriaga, *et al.*, 1998; Mba, *et al.*, 2001), and the BAC and EST libraries. Suarez *et al.* (2000) had also reported a small-scale development of ESTs from Polymorphic Transcription Derived Fragments (TDFs).

There is therefore still a compelling need to develop a lot more PCR-based markers (SSRs, ESTs, SNPs, etc.) and place them on the framework map. The on-going EST project also should form the impetus for developing DNA chips to complement the existing PCR-based markers. Saturation of the map to a density of at least 1 PCR-based marker per 10 cM of the cassava genome will vastly improve researchers' ability to identify key markers for specific traits through fine mapping.

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1.3.14 Identification of genomic regions responsible for conferring resistance to whitefly in cassava

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Introduction

Whiteflies are among the most serious pest and disease vectors that affect agricultural production on many crops around the world. In cassava (*Manihot esculenta* Crantz), the whitefly *Aleurotrachelus socialis* can cause from 70-80% yield loss due to direct feeding damage. The most important source of resistance genes is genotype M Ecu 72. Due to the importance of the whitefly as a pest and virus vector, it is necessary to understand the nature of the genes that confer resistance to the whitefly in genotypes like M Ecu 72. Therefore it is important to know the F1 segregation of crosses between M Ecu 72 (resistant genotype) x a very susceptible genotype (M Col 2246), using molecular markers. This would help accelerate selection of germplasm resistant to whiteflies and also to isolate resistant genes.

Materials and Methods

Plant material. From the cross M Ecu 72 (resistant parent) x M Col 2246 (susceptible parent), a total F1 offspring of 286 individuals (family CM 8996) was produced. These materials were sown and evaluated in the field at two different locations: Espinal-Tolima (CORPOICA-Nataima, 350 m elevation and sandy soils) and Santander de Quilichao-Cauca (990 m. elevation and acid soils) in May 2001 and March 2002. These evaluations will identify gene segregation in the offspring so that we will be able to select the resistant and susceptible materials.

The field evaluation was done using population and damage scales ranging from 1-6, where 1 is the absence of damage and population and 6 is high population and damage (curling, sooty mold, etc.). Three evaluations were performed in 2002 using the highest damage and population dates for information processing (Arias, 2002).

In vitro propagation. For the greenhouse evaluation, the seeds were multiplied using the in vitro propagation methodology developed by Escobar (1991) to obtain enough material in a short period of time (approx. 3 mo), half the time the plant requires to produce lignified stems. In addition optimal health conditions were achieved. The tips were cultured in 4E medium (Roca, 1984) in 16-ml assay tubes. The growing period was from 60-80 days. Following this period a second in vitro propagation in 4E medium in 100-ml flasks were performed to increase the number of plants per clone. After this the tips of each clone are cut for culturing in 17N rooting medium (Roca, 1984) for 30-40 days. Finally the plants are transferred to the greenhouse. This methodology not only allows the conservation of materials under optimal growing conditions, but it also supplies sufficient material in a reduced space.

Mapping. We used Simple Sequences Repeat (SSR) to find markers associated with resistance for mapping and ultimately clone the resistant genes. We used silver staining to visualize the allelic segregation of the markers.

Results

Field evaluation. Initial field evaluations showed that these materials (family CM 8996) had low levels of the pest because control plants (clones very susceptible to *A. socialis)* did not present high levels of damage or population (scale of 4-6). The harvest evaluation showed that root yield was between 4.5 and 86.5 t/ha, and many clones presented desirable characteristics (high percent dry matter, palatability, etc.). From this family, some materials having a high yield percentage were selected to increase the number of seeds and for evaluations with a higher number of repetitions. Due to the fact that pest population and damage produced by *A. socialis* in these clones were low, further studies are a needed in order to determine if there are indeed resistant materials to the pest. Currently, the family is under a second sowing cycle at the same locality in Tolima, and a high pressure exerted by the pest has been detected as control clones have high degrees of damage (4-6). Preliminary evaluations have demonstrated that some genotypes from the family present low levels of damage and population (up to 2). Therefore we might infer that these materials are resistant to the whitefly (*Aleurotrachelus socialis*) although further evaluations in the field and in the greenhouse are needed to study behavior for a longer period of time to corroborate these results. (Arias, 2002).

In vitro propagation. We grew the 286 genotypes obtained from the in vitro propagation in tubes with 4E medium (Roca, 1984). We obtained 5 clones per genotype, which are being grown on 17N medium and set out in the greenhouse. These clones are renovated every 4 mo to maintain fresh and healthy plants.

Mapping. Both parents (M Ecu 72 and M Col 2246) were evaluated with 343 cassava SSRs (Mba et al., 2001) including 156 cDNA SSRs (Mba et al., submitted). Approximately 155 of the SSRs were polymorphic in the parentals and were evaluated in the F1 (286 individuals) (Figures 1 and 2, Table 1).

Table 1. SSRs evaluated in offspring of M Ecu 72 x M Col 2246.

SSRs Evaluated	SSRs Polymorphics in Parentals	SSRs Polymorphics in M Ecu 72 (female)	SSRs Evaluated in Offspring to Date
343	155	104	90

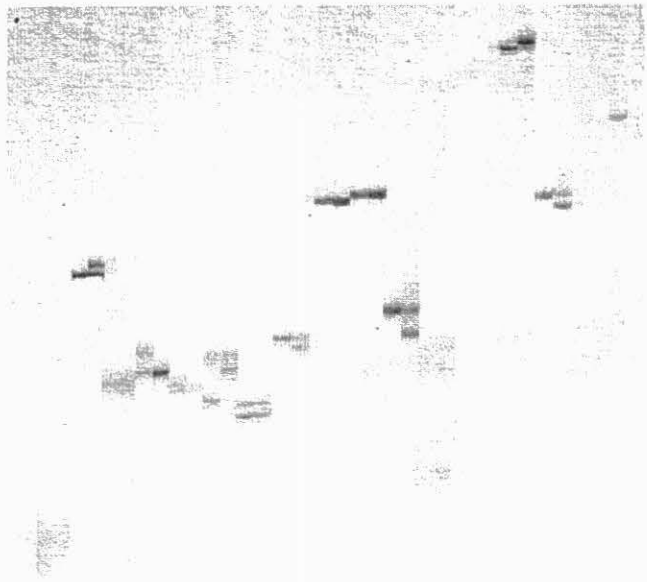

Figure 1. SSRs evaluated in parentals M Ecu-72 and M Col-2246.

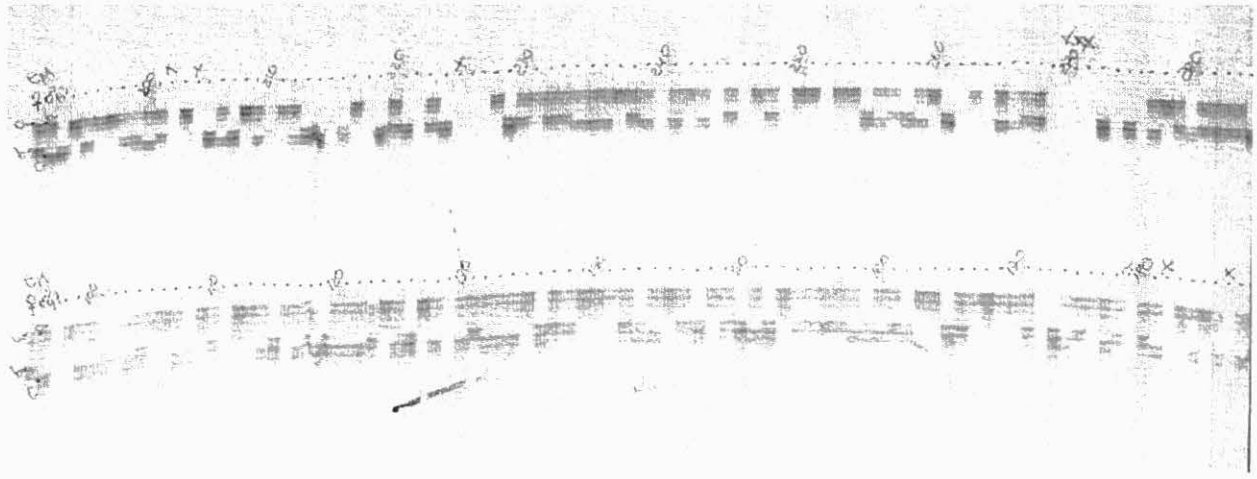

Figure 2. SSR cDNA 192 evaluated in F1 of M Ecu 72 x M Col 2246.

Future Activities

- Greenhouse evaluations of the family CM8996
- Field evaluation of the family CM8996
- Evaluation of the SSRs in the F1 (17 SSRs for the M Ecu-72 linkage map). Segregation data from these evaluations will allow the construction of a linkage map for resistance to whitefly.

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1.3.15 Development of Expressed Sequence Tags (ESTs) from TME3, the source of *CMD2*, the Dominant Cassava Mosaic Disease (CMD) resistance gene

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Introduction

Attempts to clone genes expressed down stream of the single dominant gene, designated *CMD2*, that confers high levels of resistance to the cassava mosaic disease (CMD) by the serial analysis of gene expression (SAGE) has led to identification of many differentially expressed tags – 11bp cDNA sequences. Two methods were employed to annotate the tags obatined, PCR amplification of a cDNA library, using the tag sequence as sense primer and a primer designed from the 3' end of the multiple cloning site of the vector (pYES, Invitrogen Inc.), and ESTs from CMD resistant genotypes. We describe here the generation of 4000 ESTs expressed in a CMD resistant genotype challenged with the virus.

Methodology

A cDNA library was constructed in pYES (Invitrogen Inc.) using mRNA from the CMD resistant bulk. Two microlitre of the cDNA library was electroporated into 40ul of *E.Coli* HB101 cells (Gibco BRL) and plated on LB agar plates + ampicillin (100ug/ml). A total of 5,000 colonies were picked into 70ul of LB media + ampicillin (100ug/ml) in 384 well plates. Plasmid isolation was by the MONTAGE 96-well plate system (Millipore Inc), 4 96-well plates or 384 clones were processed at a time. The 3' end sequencing of the cDNA clones was with a primer designed from the 3' end of the multiple cloning site of pYES (Invitrogen Inc.) and 5ul of plasmid miniprep. Sequencing PCR reaction was with the big dye terminator kit (Applied Biosystems) on a 9600 Perkin Elmer Machine or an MJ Research DNA engine (Tetrad). The sequence reaction was cleaned using the multi screen 96-well plate format (Millipore Inc.) and analyzed on a Shimadzu RISA 384-capillary sequencing machine. Sequences obtained were cleaned from vector

sequences by eye and combined into one single text file using a program written in perl, running on a SunSparc Station (Sun Microsystems Inc.). A program was written in perl to perform batch BLAST (Altschul et. al at 1990) similarity searches for sequence identification using the CIAT local BLAST site (http://gene2/BLAST/inicio.htm).

Results

The 3' end sequencing of about 5000 cDNA clones generated a total of 4000 ESTs. Homology with known genes and proteins deposited in public data bases were sought for using the local BLAST (Altschul et. al at 1990) at CIAT, the identity of about 2500 sequences could be ascertained with a good confidence level which corresponds to about 800 unique sequences. Redundancy found in sequences of known functions was about 30%. The ESTs were used for tag annotation and results are summarized Table 1. The most abundant tags were easily annotated, for example, identity of the ten genes that make up 5% of all expressed transcripts were found by ESTs, but annotation of less abundant tags is not as efficient. This suggests that the PCR method of tag annotation is a more powerful route to annotating SAGE tag compared to ESTs from regular cDNA libraries. On the other hand ESTs from a normalized cDNA library may be a more efficient means of tag annotation compared to non-normalized libraries. EST data will be be submitted to the Gene Bank.

Future Perspectives

1. Submission of the ESTs to GeneBank.

Table 1. Putative identity of SAGE tags annotated by cassava ESTs.

Tag Seq.	Suscep	Resist	Total	Putative identity
CCAGGTTGT	88	72	160	Chlorophyll A-B binding protein type II 1B, chloroplast precursor
CTGCAATGG	58	59	117	NONSPECIFIC LIPID-TRANSFER PROTEIN PRECURSOR (LTP) (ALLERGEN PYR
TTTGGATTC	58	37	95	Chlorophyll A-B binding protein type II 1B, chloroplast precursor
TTTGGGTGC	34	31	65	RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN PRECURSOR
GATTTCATT	29	26	55	Photosystem I reaction center subunit X, chloroplast precursor
ATGATATCA	18	23	41	THIAZOLE BIOSYNTHETIC ENZYME, CHLOROPLAST PRECURSOR.
GATTTGTGT	25	18	43	RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN PRECURSOR
AACTCCTTT	13	18	31	HISTONE H2B
TTCTTGTAT	33	16	49	CHLOROPHYLL A-B BINDING PROTEIN 7 PREC
TTCTGTTGA	24	16	40	Chlorophyll A-B binding protein 151, chloroplast precursor
TAGTCTTAT	18	14	32	PROBABLE NONSPECIFIC LIPID-TRANSFER PROTEIN AKCS9 PRECURSOR (LTP).
GCGTTGGTG	15	12	27	CYTOCHROME C OXIDASE POLYPEPTIDE III
AATGACCTT	1	12	13	TUBULIN BETA CHAIN
CGCCAGACA	3	11	14	ELONGATION FACTOR 1-ALPHA (EF-1-ALPHA).
CATTGTACA	8	11	19	Chlorophyll A-B binding protein 4, chloroplast precursor (LHCII
ATGTGGTCT	6	11	17	GERMIN-LIKE PROTEIN 1 PRECURSOR.
AAGAAGCTC	6	11	17	40S RIBOSOMAL PROTEIN S15A (PPCB8).
CGTAATCAG	30	10	40	PROBABLE NONSPECIFIC LIPID-TRANSFER PROTEIN AKCS9 PRECURSOR (LTP).
CCTGACCTC	23	9	32	Chlorophyll A-B binding protein 151, chloroplast precursor (LHCII
TTAATATGG	1	6	7	CYCLIN A/CDK2-ASSOCIATED PROTEIN P19
TACTTTGTA	13	5	18	Carbonic Anhydrase, Chloroplast Precursor (Carbonate Dehydratase).
GGTGTCTCT	13	5	18	40S RIBOSOMAL PROTEIN S4.
CGATTAAAA	1	5	6	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE (PPIASE) (ROTAMASE)
TTGGATCTT	0	4	4	HYPOTHETICAL 59.9 KD PROTEIN IN SGA1-KTR7 INTERGENIC REGION.
TAGAATCTT	1	4	5	superfamily: myrosinase-associated protein MyAP;
GCACAACAC	8	4	12	CHLOROPHYLL A-B BINDING PROTEIN 4 PREC
AGAACCACT	1	4	5	ELONGATION FACTOR TU, CHLOROPLAST PRECURSOR (EF-TU).
AATTTGATG	1	4	5	SUCCINATE DEHYDROGENASE [UBIQUINONE] I
AAGTGGTGC	0	4	4	60S RIBOSOMAL PROTEIN L17/protein tyrosine phosphatase e
GTGGTGGTA	0	3	3	60S RIBOSOMAL PROTEIN L2 (L8) (RIBOSOMA
GCTTCATTA	0	3	3	UBIQUITIN-CONJUGATING ENZYME VARIANT M...
CCTCAATCC	0	3	3	cholecystokinin B receptor - rat
ATTCCTGAT	0	3	3	putative protein [Arabidopsis thaliana]
AGGGAGGCA	0	3	3	PHOTOSYSTEM II CORE COMPLEX PROTEINS PSBY PRECURSOR (L-ARGININE
AAATTGAAA	0	3	3	unknown [Euphorbia esula]./recA protein A.thaliana

1.3.16 Functional genomics tools of post-harvest physiological deterioration in cassava

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Introduction

The development of genomics and bioinformatic tools is increasing our knowledge of plant genome structure, organization and gene function. Novel technologies such as Expressed Sequences Tags (ESTs) and cDNA microarrays are proving rapid ways to identify genes and to link sequence information to biological function.

Postharvest physiological deterioration (PPD) is a major constraint to the development of cassava for producers, processors and consumers alike. Extending the shelf life of cassava to 1-2 weeks is perceived as a goal that would have many benefits, particularly the sustainable livelihoods of small-scale rural farmers, food security and poverty alleviation.

The objective of this project is to identify the full set of major genes involved in the response by exploiting the powerful high-throughput analysis of cDNA libraries. This will enhance understanding of the problem and also provide the tools (clones) that could serve as components of gene constructs to modulate PPD. In this report we present an important step to make this goal achievable: the development of a cDNA library from different time-points during the PPD process in cassava roots.

Materials and Methods

Cassava roots from cv. CM 2177-2, the male parent of the F1 cross used to generate the molecular genetic map of cassava, was used as source of plant tissue for the RNA extraction in the construction of the cDNA library.

Roots from cv. CM 2177-2 were harvested from 10-month-old plants. The RNA was isolated form the following time-points: 0, 3, 6, 12, 24, 48 and 96 h after harvest. Poly (A)+ RNA was purified using magnetic poly A DYNAbeads according to the manufacturer.
The cDNA synthesis and cloning was done using the Stratagene cDNA Synthesis Kit, ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit according to the manufacturer.

Results

cDNA library evaluation. A total of 6 amplified cDNA libraries were evaluated by (a) determining the mean titer of the amplified library, (b) estimating the proportion of nonrecombinants by blue/white color selection, and (c) determining insert size. To determine insert size, 10 individual plaques were chosen at random from each library, and PCR was carried out using T3 and T7 primers. The number of PCR products larger than or equal to 0.9 kb were reported as percentages. Results are summarized below in Table 1. Libraries "Early 1" and "Late 1" are those constructed at CIAT in 2001; "P late lig 1," "P late lig 2," "P late lig 3" and "P early" are those constructed at Bath in 2000.

Table 1. cDNA library evaluation

Library	10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}		Mean Titer	% Non-recombinants	Insert Sizes
Plaques	Total	Blue	Total	Blue	Total	Blue	Total	Blue	Total	Blue			
Early 1	/	/	64	6	23	3	1	0	/	/	1.4×10^7	11.2%	80% ≥ 0.9kb
Late 1	77	2	6	0	/	/	/	/	/	/	6.9×10^5	2.6%	70% ≥ 0.9kb
P late lig 1	/	/	/	/	/	/	381	3	38	0	3.8×10^9	0.8%	70% ≥ 0.9kb
P late lig 2	/	/	/	/	/	/	457	< 1	90	0	6.8×10^9	< 0.2%	50% ≥ 0.9kb
P late lig 3	/	/	/	/	/	/	310	22	45	3	3.8×10^9	6.9%	10% ≥ 0.9kb
P early	93	10	10	1	/	/	/	/	/	/	9.7×10^6	10.5%	10% ≥ 0.9kb

The expected titer for an amplified library is in the region 1×10^9 to 1×10^{11}, with the number of recombinant plaques 10- to 100-fold higher than nonrecombinant plaques (i.e., 1-10% nonrecombinants). Thus libraries "Early 1" and "P late lig 1" would appear to be the most acceptable.

Genomic library construction. The following steps have been completed: (1) Extraction of DNA from leaves of cv. M Col 22; (2) optimization of Sau3A digestion conditions; (3) large-scale digestion of 50 µg DNA, where an aliquot of the digestion was run on a gel to ensure the results; (4) phenol extraction and ethanol precipitation of digested DNA to remove restriction enzyme; (5) digested DNA run on a preparative gel without ethidium bromide for size fractionation. The marker lane and a marker lane of an aliquot of digested DNA were then cut from the gel and stained with ethidium bromide, and then compared to the unstained portion of the gel to identify the region containing the fragments of interest. (6) The region of the gel containing fragments from 13-21 kb were cut out of the gel, and the DNA was recovered from the gel by electroelution into dialysis tubing and concentrated using Elutip columns. (7) In order to prevent ligation of any remaining smaller fragments that could subsequently ligate into the vector as a single large fragment, the DNA was treated with alkaline phosphatase (CIAP) (8) To remove the CIAP the DNA was purified using a Quiagen purification kit.

The Stratagene Lambda DASH II/BamHI Vector Kit has been ordered but has not yet arrived. On arrival the ligation and packaging reactions can be carried out.

Other

- Genomic DNA from 8 transgenic cassava lines prepared and transferred to Dr. H Li, Bath University.
- Total RNA extracted from leaves and roots of cv. NGA2 over a 5-day period. This RNA is intended for Northern analysis.
- Preparation of probes for Northern analysis – PAL, MecASP, MecCPI and la082 (cysteine protease)
- Sequencing of la082 (cysteine protease)

Future Activities

- Completion of genomic library construction and evaluation of genomic library
- Preparation of Northern blots using PAL, MecASP, MecCPI and la082 (cysteine protease) as probes
- Root treatments with salicylic acid, methyl jasmonate, ethephon and H_2O_2 and extraction of RNA from these roots for Northern blotting as above
- Completion of sequencing of la082 and submission of sequence data to Genbank.

1.3.17 cDNA-AFLP analysis of differential gene expression in the cassava- *Xanthomonas axonopodis* pv. *manihotis* interaction

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Introduction

Cassava bacterial blight (CBB) is a major disease, endemic to Latin America and Africa, which causes serious damage to the plant and results in severe yield losses. The causal agent is *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). The most appropriate and practical approach for controlling CBB is through host resistance. This resistance operates from the vascular system and seems to be polygenic and additively inherited; however, no resistance genes have been identified. Plants in general have a wide spectrum of cellular and molecular defenses including cell wall fortification, phytoalexin production and development of a hypersensitive response (HR) (Baker et al., 1997; Culver & Dawson, 1991; Hammond-Kosack & Jones, 1996). Resistance genes activate these defense reactions directly or indirectly. Understanding how these genes are involved in the recognition of and response against pathogen attack will allow us to understand how to manipulate resistance against a wide range of pathogens.

The objectives of this work are to:

- Identify differentially expressed bands between two different cassava cultivars, one resistant and one susceptible to CBB (M Bra 685 and M Col 1522, respectively), using the cDNA-AFLP technique
- Identify putative molecular disease resistance markers through analysis of cDNA-AFLP patterns at different postinoculation (pi) times with *Xam* strain CIO 151
- Isolate and sequence the fragments identified
- Compare the sequence data with the GenBank database to identify similarities
- Verify the differential expression of these fragments in time, through Northern blot and RT-PCR analyses?

Materials and Methods

Sample preparation and cDNA synthesis. Young plants were inoculated by stem puncture with *Xam* strain CIO 151. Stem tissues were collected at 24 and 72 h pi, and at 7, 15 and 30 days pi. Controls were healthy noninoculated plants and plants inoculated with sterile water. The tissue was ground in liquid nitrogen, and total RNA was isolated using the Proteinase K method (Hall et al., 1978). Poly (A) RNA was isolated using oligo (dT) coupled to DynaBeads (DYNAL). cDNA was synthesized using oligo (dT) primer and SuperScript II reverse transcriptase (GIBCO BRL) from 400-500 ng of mRNA as starting material.

cDNA-AFLP profiling analysis. The template for cDNA-AFLP was prepared according to Bachem et al. (1996) using *EcoRI* and *MseI* restriction enzymes and adapters. Preamplification was carried out with one *EcoRI* and one *MseI* single-chain adaptor with no (0) or one (1) selective base. The product was checked on agarose gel, and a 1/30 dilution was used for subsequent amplifications (with primers with 2 or 3 selective bases, GIBCO primers and Plant AFLP Kit, GIBCO, respectively). Selective amplification products were separated on a 6% polyacrylamide gel processed with the silver staining technique (Promega). Bands of interest were marked, cut and eluted in ddHO. AFLP fragments were reamplified by PCR, ligated to pGEM®-Teasy (Promega), transformed and sequenced using an automated sequencer (ABI Prism 377). The sequences were edited using Sequencher 3.0 (Gene Codes Corp.) and compared with GenBank databases through BLASTx and BLASTn.

Northern hybridization. The total RNA from each sample (7.5 to 25 µg of total RNA, containing ethidium bromide) was run on 1.2% agarose denaturing gels (1X MOPS, 3% formaldehyde). The RNA was transferred to nylon membranes (Hybond N+) in 10X SSC. Probe amplifications were cleaned and labeled with radioactive $dATP^{32}$ through the Multiprime DNA Labeling System (Amersham). Hybridizations were performed from 42-65°C, in formamide hybridization buffer (5X SSPE, 5x Denhardt's, 0.1X SDS, 50% formamide), rapid hybridization buffer (Amersham) or phosphate buffer (0.5 M NaHPO4 pH 7.2, 7% SDS, 1% BSA). Hybridized filters were incubated with autoradiographic film at –80°C up to 10 days. A hybridization test was performed with a nonradioactive Dig High Prime DNA Labeling & Detection Starter Kit II (Roche), following the manufacturer's instructions.

RT-PCR performance. RNA extraction for RT-PCR was performed as stated previously and cleaned with SV Total RNA Isolation (Promega) for DNA-free samples. cDNA synthesis was accomplished as described above, using 20 µg of total RNA as starting material. The complete cDNA reaction was diluted in order to decrease the amount of oligo dT primer in the reaction. Two µl of this dilution was used for PCR amplification.

Results and Discussion

The cDNA-AFLP technique was implemented with success using stem tissue from cassava plants. We evaluated 32 and 40 combinations of AFLP primers with 2 and 3 selective bases, respectively. These cDNA-AFLP profiles showed 40-70 bands per primer combination, ranging from 100-1500 bp, with ~3600 fragments screened (Figure 1). Differential expression was observed for 353 fragments putatively induced by the pathogen in the resistant variety, with an average of ~5 bands per combination. These fragments ranged from 130-650 bp.

We have sequenced and compared 231 bands with GenBank databases. Significant homologies with known or putative genes were found for 154 sequences, 105 of which are plant related. Only

34 of these showed homology with plant resistance or defense-related proteins (Table 1). We found fragments similar to resistance Cf-2 and I2 genes from tomatoes, and others with homology to putative resistance proteins. They appeared at 24 h pi in the resistant variety, indicating that their expression increased in the presence of bacteria. This differs from several authors who argue that resistance proteins are constitutively expressed in plant cells in order to recognize an invading pathogen (Staskawicz et al., 1995; Hammond-Kosack & Jones, 1996). Some of these bands showed expression in the susceptible variety also, but at late time points (15 d pi, bands E6, E18 and E45, Table 1), suggesting that these putative genes are present in both genotypes but are expressed earlier in the resistant variety.

Figure 1. AFLP PAGE stained with silver nitrate; primers E-ACA/M-CAG.

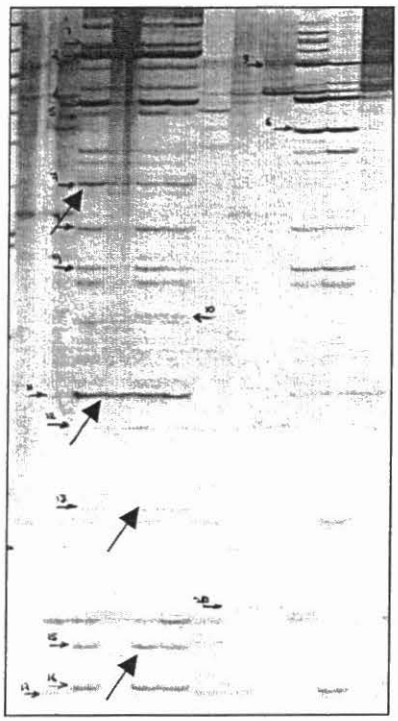

Table 1. Nucleotide homology and probabilities from BLASTx results for differentially expressed cDNA-AFLP bands.

Band	Bps	Best Match	E Value	Expression
Defense-Related Proteins				
E6	213	Disease resistance Cf-2-like protein (*L. esculentum*)	2e-05	24 h pi
E45	260	Resistance protein I2 (*L. esculentum*)	2e-08	24 h pi
M62	830	Putative resistance protein (*A.. thaliana*)	3e-24	24 h pi
E18	171	Resistance gene analog protein (*L. sativa*)	1e-05	24 h pi
M56	350	Putative receptor protein (*A.. thaliana*)	2e-14	24 h pi
M63	360	Putative translation initiation factor IF-2	7e-24	24 h pi
E1	218	Kinase protein SNF1(*Cucumis sativus*)	6e-25	24 h pi
E4	239	Protein AFR6 (*A.. thaliana*)	5e-12	24 h pi
E42	230	Similar to kinase protein (*A.. thaliana*)	7e-05	24 h pi
M37	210	Dormancy-associated protein (*P. sativum*)	4e-09	24 h pi
M1	250	Putative senescence-associated protein	1e-32	24 h pi
M22	314	Putative senescence-associated protein	3e-22	24 h pi
M82	280	Putative senescence-associated protein	7e-17	24 h pi
E13	261	ADP ribosylation factorlike protein (*A.. thaliana*)	2e-35	24 h pi
M5	376	Receptorlike serine/threonine kinase	5e-13	72 h pi
M80	260	Defense-related protein *(Brassica carinata)*	6e-11	15 d pi
E11.1	286	Pti-6 protein (*L. esculentum*)	3.1	15 d pi
M15	292	NPK1-related protein (*N. tabacum*)	4e-09	stress
Other plant proteins				
M32	421	Similar to kinase protein (*N. tabacum*)	6e-57	24 h pi
M48	250	mRNA for adenine nucleotide translocator (*L. albus*)	4e-28	24 h pi
Mb8	305	Chaperonin-like protein (*Z. mais*)	7e-29	stress
E19	318	Fumarate hydratase protein (*A.. thaliana*)	1e-29	24 h pi
E23	197	NAD-dependent sorbitol dehydrogenase protein	3e-26	24 h pi
Mb10	358	Soybean P450 cytochrome	2e-05	24 h pi

Fragments E6 and E45 showed significant homology with leucine-rich repeats (LRR) from Cf2 and I2 genes (LRR and NBS-LRR type, respectively). This motif is involved in protein-protein interactions and acts in specific recognition of avirulence proteins from pathogens (Staskawicz et al., 1995). Fragment E18 showed homology with a putative resistance protein in lettuce. This putative gene also has structural elements characteristic of NBS and LRR motifs (Shen et al., 1998). Other fragments were similar to serine/threonine or receptor protein kinases, which long to a different type of resistance gene that modulates the phosphorylation state of other proteins and is involved in signal transduction cascades that activate defense responses in plants. These results indicate that through the cDNA-AFLP technique we have isolated resistance- and defense-related fragments induced by *Xam*, corresponding to three different types of resistance genes in plants.

belong to a different type of resistance gene that modulates the phosphorylation state of other proteins and is involved in signal transduction cascades that activate defense responses in plants. These results indicate that through the cDNA-AFLP technique we have isolated resistance- and defense-related fragments induced by *Xam*, corresponding to three different types of resistance genes in plants.

Several fragments showed homology to senescence, apoptosis and dormancy-associated proteins, suggesting that they might be involved in the programmed cell-death mechanism included in hypersensitive responses. This defense reaction, very common in plants, creates a toxic media that prevents the establishment and expansion of the invading pathogen (Hammond-Kosack & Jones, 1996, Staskawicz et al., 1995). Another 59 fragments did not show significant homology to known sequences in the databases. These were differentially expressed starting at 24 h pi, indicating that were also induced by the pathogen and might represent novel sequences putatively associated with resistance to *Xam* in cassava.

To corroborate the difference in expression of the fragments, we prepared several Northern blots and hybridized them with labeled probes, but results were unsatisfactory. Although several methods for RNA extraction were used (special for recalcitrant tissues), there may have been some sort of contamination in the RNA restrict hybridization with probes and positive controls (cassava ubiquitin and ribosomal fragments). Given the foregoing, we decided to corroborate the fragments expression through RT-PCR and designed primers for 9 fragments and 2 positive controls (ubiquitin and ribosomal). RT amplification was standardized with primers for ubiquitin and ribosomal fragments, employing 4 RNA samples (4 different inoculation time points; measuring the number of PCR cycles that do not reach the curve plateau). However, amplification conditions with each pair of primers need careful standardization (temperature) in order to evidence real differences in the level of expression over time. Amplification with primers for fragment M15 was standardized at annealing 55°C and 30 PCR rounds. Comparison of M15 amplification products between varieties and inoculation time points is under way, as well as standardization of PCR conditions for the rest of the primer pairs.

Future Activities

- Amplify each fragmented pair of primers using RT-PCR and analyze differential expression over time
- Hybridize a cassava cDNA library with these fragments to isolate full-length clones
- Use this information to implement a detection method for resistance to *Xam* in other cassava varieties

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1.3.18 Using Microarray Profiling to Assess *Xanthomonas axonopodis pv. manihotis* (Xam) Genes Expressed during Plant Infection

Gloria Mosquera[1], Silvia Restrepo[1-2], Camilo López[2], Joe Tohme[1] and Valerie Verdier[2].
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Introduction

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is a major disease of cassava (*Manihot esculenta* Crantz) that was reported for the first time in South America but now has a worldwide distribution (Verdier, 1994). At present the bacterial factors controlling plant infection are not yet well understood. The DNA microarray method is a valuable tool and a very powerful technique that can provide information about DNA sequences that are simultaneously expressed in specific environments or under some stress or treatment. If the studied organism is not completely sequenced, entire sequenced genomes or anonymous sequences can be screened, using mRNA as the probe.

This study focuses on elucidating *Xam* genes that are regulated at different stages during cassava infection.

Materials and Methods

Plant material. Four-week-old cassava plants of cv. MCo1522 were inoculated with CIO 46 *Xam* strain; and punctured stems were collected at 24 h, 48 h, 7 days and 15 days postinoculation. All tissues were collected in liquid nitrogen and stored at -80°C until used.

DNA extraction and microarray construction. Strain CIO46 genomic DNA was partially digested and adapters were ligated to obtain fragments that could be amplified. Then one of the adapters was used as a primer to amplify DNA segments. The PCR product was cloned into the pGEMT-Easy (Promega) vector and transformed into *E. coli* DH5α. Clones were amplified using T7 and SP6 primers, and spotted onto a glass slide.

Probes and hybridization. Total RNA was extracted from inoculated stems (plant probe) and bacteria grown in culture media (culture probe), using a modified Kit SV (Promega). Then 30 µg of

total RNA were labeled using random primers, following a protocol proposed by the TIGR Institute (www.tigr.org). These cDNA-labeled primers were used as probes in slide hybridization. Slides were then scanned and analyzed using ArrayPro software.

Results

An efficient method for RNA extraction was developed, making it possible to obtain high-quality RNA samples in large quantities (Figure 1).

A genomic library was obtained with 1900 clones containing fragments from 500-1200 bp long (Figure 2).

An array containing anonymous sequences from genomic DNA of strain CIO 46 was constructed for use in different studies.

Labeling and hybridization protocols using total RNA from infected tissues and from bacteria grown in a medium (i.e., RNA concentration, labeling and hybridization temperatures) were standardized.

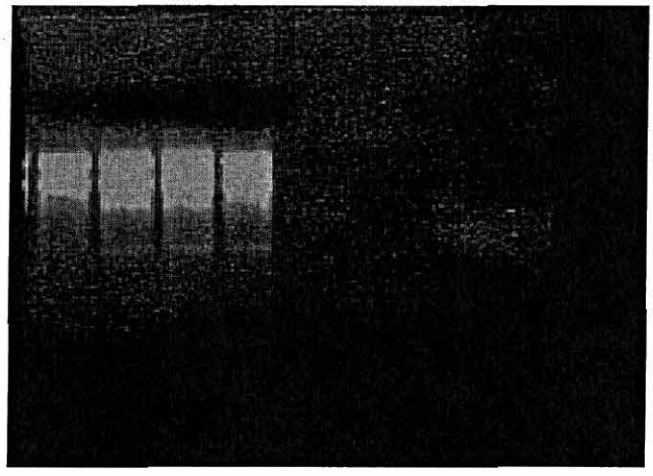

Figure 1. Bacterial RNA extracted from infected tissues.

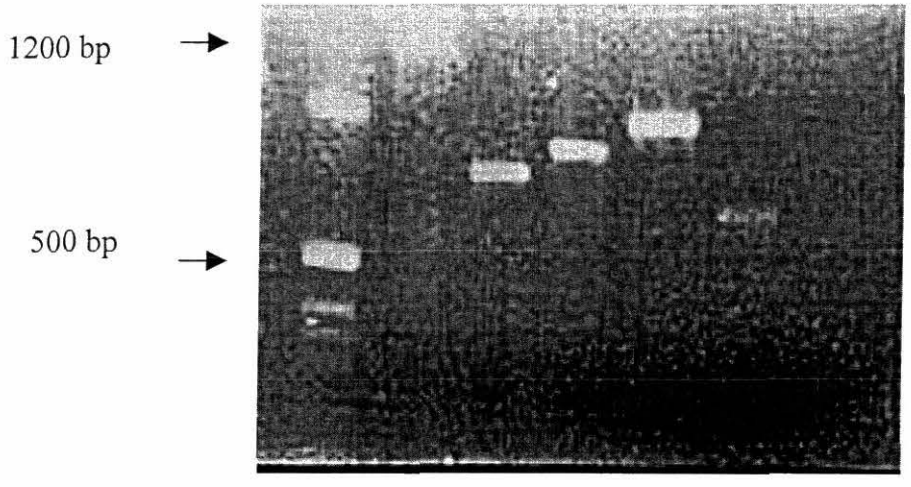

Figure 2. PCR from some clones of the CIO46 genomic library.

Conclusions

Many studies have been done to characterize microbial pathogenicity; however they were all developed in clinical microbiology. The first study performed using a plant pathogen, *Erwinia chrysanthemi*, was published recently (Okinaka et al., 2002). The methodologies used by these authors are very similar to those used in our study, showing that our approach is suitable for studying pathogenic pathways and strategies used by an organism whose genome has not been sequenced.

Ongoing Activities

Hybridize CIO-46 genomic library-microarrays with bacterial RNA extracted from different postinoculation times.
Sequence all clones that are expressed only in inoculated bacteria.and compare them with GenBank databases to assign putative functions.
Identify all bacterial genes that are up-regulated as a response to plant infection.

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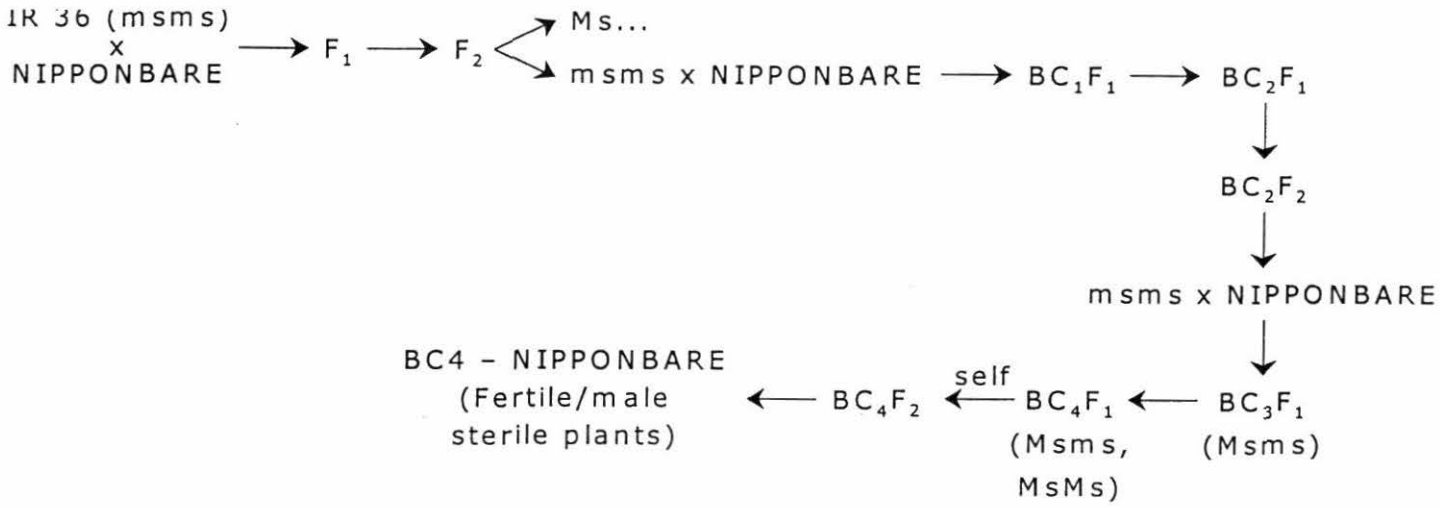
1.3.19 International rice functional genomics consortium

Cesar P.Martinez, James Carabali ,and J.Tohme
SB-2

Introduction

An international consortium of geneticists, molecular biologists and information scientists from Yale University, Cold Spring Harbor Laboratories, Brookhaven National Laboratory, and CIAT was assembled to address the following specific goals:

- To generate an extensive collection of rice lines, each containing an independent, dispersed insertion of a genetically-engineered Ds transposon;
- To determine the chromosomal position of each insertion by sequence of its flanking genomic DNA;
- To establish a database of that relates lines, sequences and phenotipic information ;
- To publicly distribute mutant lines and associated informatics.
- The overall idea is that by making use of this public information, research scientists worldwide can rapidly identify mutant alleles in genes of agronomic importance for functional genomic studies and crop improvement.



Breeding scheme to introgress the male sterility allele into NIPPONBARE

Materials and Methods

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

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

A simplified crossing method described by Sarkarung, 1991 was used. A seed sample from the IR36 source segregating for male sterility was planted under field conditions in CIAT; male-sterile plants were phenotipically identified at flowering time and used as the female parent. F2 seed was harvested from F1 plants and grown in the field for the identification of male-sterile plants, which were backcrossed to Nipponbare to produce BC1F1 seed. A second BC to Nipponbare was done and the BC2F2 population was grown in the field to allow the identification of male-sterile plants, which were used to produce the BC3F1 seed, and subsequently the BC4F1. This seed was planted again in July/02 to produce BC4F2 to observe for segregation. At this point the Nipponbare male-sterile plants look very similar to the fertile counterpart Nipponbare.

1.3.20 Isolation and characterization of sequences associated with resistance to spittlebug in *Brachiaria*

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

Introduction

The spittlebug is the most harmful pest of *Brachiaria* in America. One of the methods of controlling it, is the use of cultivars that are naturally resistant. We are interested in elucidating the molecular basis of resistance to spittlebug in *Brachiaria* using two strategies from different levels: At the genomic level, we isolate sequences bearing strong similarities with resistance genes (R-genes) named RGAs (Resistance Gene Analogs); (Acosta, I.; Tohme, J. 2001. CIAT SB-2 project annual report. P183-185. Cali, CO.). Given the need to establish the expression profile of the genes implicated in resistance when the resistant genotype is challenged with larvae of the insect, we followed two approaches: Look specifically for elements probably involved in the recognition of the insect and the activation of the resistance; and seek information about the defense response itself.

Mapping of RGAs from Brachiaria brizantha. A candidate gene approach was developed to characterize Quantitative Trait Loci (QTL) for resistance to spittlebug in *Brachiaria*. Based on the strong structural similarities between R-genes of the NBS-LRR (Nucleotide Binding Site-Leucine Rich Repeat) family, which includes the main part of cloned R-genes, we had isolated RGAs using degenerate primers targeting highly conserved regions (CIAT. 2001. SB-02 project annual report. Cali, CO). Now, these RGA sequences have been used as molecular markers to determine whether they are part of the spittlebug resistance QTL.

Isolation of sequences differentially expressed in resistant plants infested with spittlebug. To obtain sequences related with the defense process, we used a differential display technique that allows us to isolate fragments of cDNAs induced by the insect attack in a resistant variety. Sequence information of these fragments is used to hypothesize about its possible function in the defense response in order to get closer to the resistance mechanism.

Materials and Methods

Specific primers were designed for each of 8 RGA classes established previously (CIAT. 2001. SB-02 project annual report. Cali, CO) in order to use RGAs as PCR-based molecular markers. Five out of the 8 classes were informative polymorphic markers between the resistant and susceptible varieties (*B. brizantha* CIAT 6294 and *B. ruziziensis* BR4x44-03, respectively). One of these PCR-based markers is a Cleaved Amplified Polymorphic Sequence (CAPS), while the other ones represent dominant molecular markers (Presence/Absence). These polymorphic classes were evaluated in a population derived from an interspecific cross of *B. ruziziensis* BR4x44-03 X *B. brizantha* CIAT 6294. This population contains 215 individuals that have been scored phenotypically for resistance to one of the most widespread species of spittlebug (*Aenolamia varia*), and have been used to construct a genetic map (CIAT. 2001. SB-02 project annual report. Cali, CO). The RGAs were located on this map using Mapmaker (Lander, 1987). The correlation analysis between the segregation of RGAs and the resistance score was done with QGENE (Nelson, 1997).

To isolate sequences differentially expressed in a *Brachiaria*-resistant variety (CIAT 36062) when challenged with spittlebug, we employed a highly sensitive technique known as Differential Subtraction Chain (DSC) (Luo et al., 1999). Expressed sequences from treated and nontreated plants are put in hybridization, and sequences common to both states are suppressed. This is achieved by successive rounds of hybridization with a subsequent enriching of the sequences expressed only in the treated plants.

Two pools of plants from genotype CIAT 36062 grown under similar conditions were used for this assay. One pool was infested with *A. varia* larvae; the other was not treated. Infestation was done in superficial roots according to Cardona (1999) (Figure 1). These were collected at different stages of the infestation progress (1-30 days) from both pools, and total RNA was extracted from superficial roots with Trizol® reagent. We made two RNA pools that represent two populations of genes expressed in each condition during that time and are denominated "tester" (infested) and "driver" (not infested). Double-strand cDNA was synthesized using the SMART cDNA Synthesis Kit (Clontech) according to manufacturer's instructions. The cDNA was then digested with *Dpn*I and ligated with different adaptor sets for each cDNA pool to make its subsequent amplification by PCR possible.

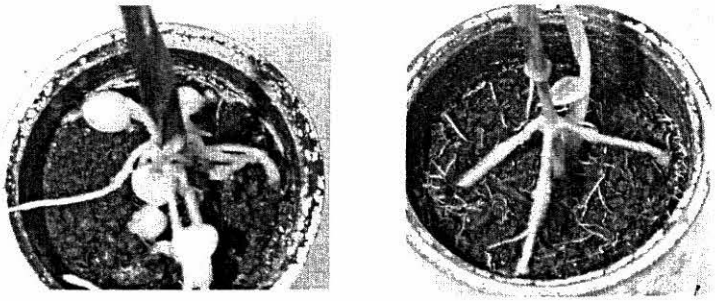


Figure 1: infestation system units consist in single steams propagules with profuse superficial roots. This substratum serves as feedings sites for nymphs.

We performed two separate hybridization experiments with different hybridization buffers: SSH (Diatchenko, 1996) and DSC (Luo et al., 1999), following the hybridization and PCR parameters suggested by Luo et al. (1999). After each round of hybridization, the level of subtraction was checked by PCR. Accordingly to these stages of subtraction we executed 3 and 4 rounds of hybridization for the SSH buffer and the DSC buffer respectively. PCR products from the last round of hybridization were considered to contain differentially expressed sequences from the tester; therefore, they were cloned in batch, and a 96-clone library was constructed for both types of hybridization. Sequences for the complete libraries were obtained, and homologies were searched for in the Gene Bank, using the BLASTN and BLASTX algorithms (Altschul et. al., 1997).

Results and Discussion

Mapping of RGAs from B. brizantha. Segregation of 5 RGA markers on the mapping population *B. ruziziensis* BR4x44-03 X *B. brizantha* CIAT 6294 did not show significant association with the trait of interest (resistance to *A. varia*). The highest explicative value for the resistance phenotype was 5%, corresponding to RGA 5B. Some AFLP markers used in the construction of this framework map show much better associations (25%) with resistance to spittlebug (CIAT. 2001. SB-02 project annual report. Cali, CO). Despite the small size of such markers (ranging from 100-300 bp), we attempted to sequence them in search for R-gene candidates; however, no apparent homologies were found (data not shown). Moreover, these QTL-containing regions are not saturated in molecular markers; thus they may still contain RGA-type sequences that were not isolated in the set we tried to map. Consequently our next step is to define new classes of RGAs isolated from less complex sources of DNA, such as cDNA or the arrayed genomic library that is currently being

constructed in the SB-02 Project. In this way we expect to obtain more diverse and representative RGA sequences that may be included in a genomic region implicated in the resistance of *Brachiaria* to spittlebug.

Isolation of sequences differentially expressed in resistant plants infested with spittlebug: Two "subtraction libraries" were constructed using DSC (see methods). They were supposed to contain sequences being expressed exclusively in resistant *Brachiaria* plants that had been challenged with spittlebug. It is highly probable that such cDNA fragments correspond to genes involved in the resistance response to the insect attack. Sequence analyses of 171 clones (Table 1) revealed 35 sequences with known homology, 4 hypothetical proteins (HP), 2 unknown proteins and 16 sequences with no homology. We isolated sequences that participate in the R-gene-mediated defense response studied in other plant systems (Figure 2). Indeed, we found Hypersensitive Response (HR)-related sequences (Table 1), suggesting that localized programmed cell death, a common strategy to arrest the propagation of pathogens, is also important in the resistance to spittlebug. This episode provokes a hormone response that alerts the entire plant; accordingly, we found sequences that are part of the ethylene, jasmonate and salicylic acid pathways triggered during resistance (Cheong, 2002). This evidence encourages us in the search for R-gene type sequences involved in the resistance mechanism of *Brachiaria*. Additionally, we found other general response elements to feeding, wounding (Reymond, 2002) and stress responses (Table 1), which are well correlated with the type of attack that the insect executes in the plant (mechanical wounding).

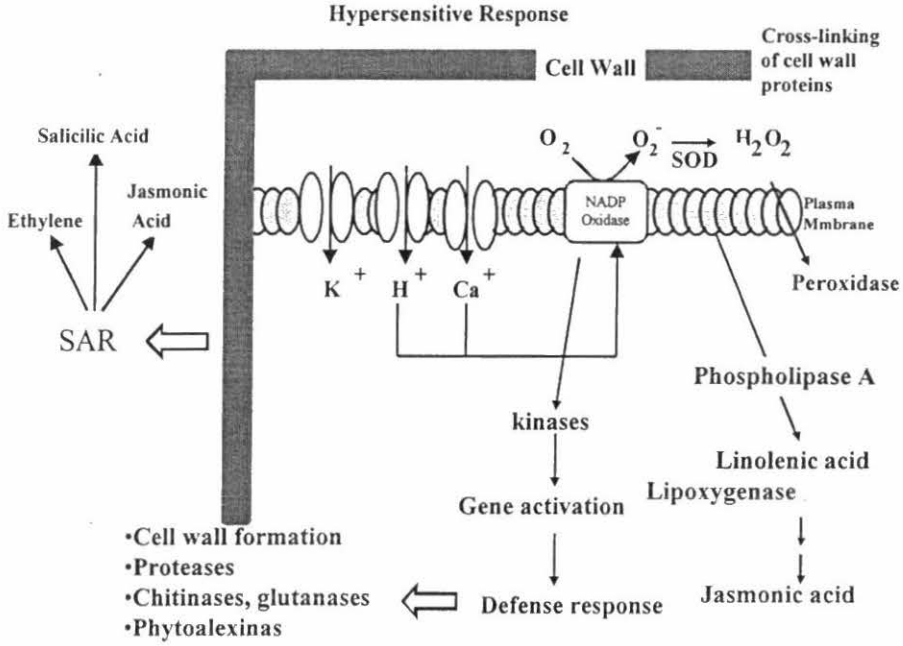


Figure 2: Outline of Hypersensitive response elements. Adapted from: B. F. Matthews, Sept 2002, Expression of Genes in Soybean Roots in Response to the Soybean Cyst Nematode, Soybean Genomics & Improvement, USDA -ARS. In http://www.ndsu.nodak.edu/virtual-genomics/conference_2002.htm

Unfortunately, a high number of redundant clones representing 4 types of rRNA artifact sequences were also isolated (not shown). These sequences are an artifact of the cDNA synthesis even though we used a poly-T primer for that step. One way to prevent this in future experiments is to use mRNA as a template for cDNA synthesis. When we separated the subtraction final products in polyacrylamide gels (data not shown), we were able to see from 50-65 different bands for each hybridization. Evidently, the 96-well libraries were too small to represent well the entire set of differential sequences present after subtraction, particularly in this case where the concentration of rRNA-enriched products can "mask" the cloning of less abundant fragments. However, we applied this strategy to make a rapid inspection of the contents and quality of the subtraction. Now our goal is to construct larger libraries that exclude rRNA fragments by cloning smear excisions from polyacrylamide gels. In that way we expect to broaden the light that initial analysis of subtraction products has shed on the mechanisms of resistance to spittlebug in *Brachiaria*.

Table 1 Functional groups of the differentially expressed sequences. Some sequences may be part of two functional groups because some of these pathways have a cross talk.

Transcription Factors
MADS Box
PUR alpha 1
SCARECROW
Signaling Proteins
Ca dependent carrier
Serine Threonine Kinase
Detoxification
Glutathion S conjugate ATPasa
LOX 1 (Lypoxigenase)
LOX 2 (Lypoxigenase)
Hydrolytic Enzymes
Beta glucanase
Cell Wall Modifying Enzymes
Alpha Tubulin
Beta Tubulin
Methyltransferase
O-Methyltransferase
Xyloglucan endotransglycolase
Stress Related Proteins
Cold acclimatation and water stress protein
Retrotranscriptase 1
Retrotranscriptase 2
SAMS (S-adenosyl L-methionine synthetase)

Hypersensitive Response Proteins
Carbonic Anhydrase
Cystein Proteinase
Jasmonate Pathway
LOX 1 (Lypoxigenase)
LOX 2 (Lypoxigenase)
Phospholipase
Wounding Induced Proteins
Fatty acid desaturase
Glutathion S conjugate ATPasa
Beta glucanase
Unclassified Proteins
CAB (Chlorophyll a/b binding protein)
Clone RG64 (sequence associated to Pi2)
Developmental Protein
dTDP Glucose dehydratase
Fructose bifosfate aldolase
GTP- Dehydratase
HP 1
HP 2
HP 3
HP 4
Safener Binding Protein
Unknown prot 1
Unknown protein 2

Future Activities

- Isolation and mapping of new RGA sequences from less complex DNA sources
- Final sequence screening of the subtraction libraries
- As found by Cheong et al. (2002), transient-induced expression of genes by feeding and wounding appears in 0.5-6 h after treatment, preceding the sustained expression of other effector genes. This prompted us to design a new assay that will include a subtler infestation method and a new subtraction experiment to cover the first 24 h postinfestation. In this way we will look for regulatory elements upstream of the expressed sequences that

we report here. Upstream elements have a particular interest in searching for polymorphism between susceptible and resistant varieties.

- The subtractive hybridization performed did not allow detection of constitutive mechanisms of resistance. Therefore we plan to perform a subtractive hybridization of cDNAs from a susceptible and resistant variety, challenging them with the insect to find out whether there is a constitutive mechanism of resistance.

Acknowledgments

We want to thank Guillermo Sotelo and César Cardona for supplying plant materials, their collaboration in the infection process and the useful discussion on the setting up of the experiments.

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1.3.21 Detection of differentially expressed genes related to apomixis using cDNA subtraction coupled to microarray hybridization

D.F. Cortés, [1] J. Miles[2] and J. Tohme[1]
SB-2 Project, [2]

Introduction

The identification of differential gene expression between two organisms or cells types is a frequent goal in modern biological research. The possibility of determining mRNA expression differences between apomictic and sexual genotypes using the novel tools of functional genomics is a powerful way to access the gene expression involved therein.

Several recent and rapid PCR-based methods, including Subtractive Suppression Hybridization (SSH) and Representational Differences Analysis (RDA), are being used to clone genes that are differentially expressed between genotypes. Recently, cDNA microarrays were developed and used to differentiate gene expression quantitatively by hybridization of a complex mRNA-derived probe onto an array of PCR products. Microarrays allow thousands of genes to be monitored simultaneously for expression level and compared between tissues.

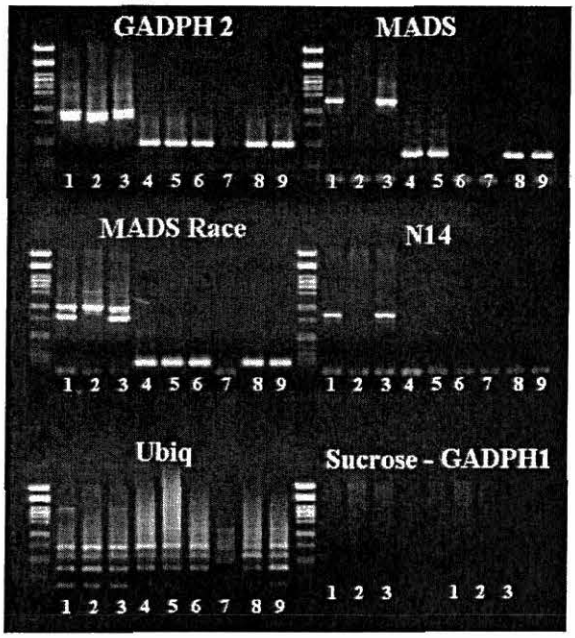
Here we show the advancement in the merging of cDNA subtraction technique with microarray analysis as a potential method for detecting unique differentially expressed genes related to apomixes in *Brachiaria*.

Materials and Methods

In order to verify the efficiency of the subtraction procedure carried out previously between *B. decumbens* (apomictic) and *B. ruziziensis* (sexual) (CIAT, 2001), dot blot analyses of subtractive cDNA clones were hybridized with radioactively labeled cDNA prepared from inflorescence tissues of apomictic and sexual genotypes. As has been reported (Wan et al., 2002) although the SSH is a helpful technique for isolating differentially expressed genes, a high percentage of clones in the subtractive cDNA library may not be differentially expressed.

From the subtractive library that contains the putative genes expressed only in the apomictic genotype, 140 cDNA clones were randomly selected after subtraction efficiency was determined. The selected clones were sequenced with an ABI prism sequencer using AmpliTaq DNA polymerase and a Big Dye Terminator Cycle Sequencing Kit (PE Biosystems)

Some of the sequenced clones with significant similarities found in the sequences databases were used to design primer and PCR amplification onto genomic DNA and cDNA prepared from *B. decumbens* (apomictic) and *B. ruziziensis* (sexual). PCR amplification was also carried out on previously characterized cDNA bulks of apomictic and sexual genotypes.



Results and Discussion

Of the sequenced cDNA clones, only 45 showed homology with known proteins of maize, wheat, rice, tomatoes, *Arabidopsis* and *Hordeum vulgare*. The rest of the sequences showed no similarity with the Genbank sequences. PCR amplification of the cDNA clones that had significant homology with the MADS-box transcription factor, glyceraldehyde 3-phosphate dehydrogenase, sucrose synthase, polyubiquitin and SCAR N14 linkage and the apomixis loci were successfully carried out using the corresponding PCR primer pairs for these genes. The results of the amplifications of these genes are shown in the figure below. These cDNA clones can now be mapped to see if there exists some linkages between them and the apomictic locus in *Brachiaria*. The clones can also be used as probes for screening a full-length cDNA library in order to obtain the full sequences for these clones.

It is noteworthy that the N14 SCAR that we have been using as a marker to discriminate between the apomictic and sexual genotypes of *Brachiaria* failed to amplify any corresponding locus in RNA extracted from the inflorescences of both genotypes. This would then lead us to suppose that the sequences for this SCAR are not part of a gene expressed in the inflorescence and forms the basis for future work to determine exactly where the gene is expressed in the plant.

Future Activities

- Sequencing clones of interest. Sequence the remaining set of clones identified as being differentially expressed and homologous.

- The identified and sequenced clones will be used as probes against Northern blots to confirm their differential expression.

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Wan et al. 2002.

1.3.22 Study of gene expression during embryo sac development in *Brachiaria* sp.

D.F. Cortés, [1] D. Bernal, [1] J. Miles[2] and J. Tohme[1]
SB-2 Project, CIAT; [2] IP-5

Introduction

Apomixis is found in over 300 species of at least 35 different plant families (Bashaw & Hanna,1990). Scientists and geneticists have studied the two broad categories of apomixis—gametophytic and sporophytic—because of their widespread occurrence and potential usefulness in plant breeding. The genetic analysis of apomixes provides researchers with various inordinate challenges because of ploidy levels, lack of sexual progeny, lethality and accurate identification and classification of progeny.

Methods for classifying progeny accurately in genetic studies are being developed, and currently several methods are available to researchers, none of which provides a complete picture. These include phenotypic analysis, cytoembryological study, microbiological methods and a variety of markers. A combination of these methods may be used to classify progeny accurately.When the genetic cause of apomixis is identified, apomixis could be applied to plant breeding programs as a means of permanently fixing hybrid vigor or capturing desirable genotypes immediately. Several theories of the genetic control of apomixis have been put forth. Studies suggest one major gene or linkage group with the possibility of modifying genes. None of these theories has proven to be completely satisfactory due to the lack of inheritance data, inaccurate progeny classification and recombination.

Recent advances in functional genomic technologies have provided a particular way to monitor gene expression on a genomic scale and under different conditions. Based on the recent gene expression technologies available, we are working on the identification of genes expressed during early embryo sac development, which could be related to the apomictic reproduction mode in *Brachiaria*.

Phenotypic identification and material selection. Selection of 7 apomictic and 6 sexual materials in the field was done by phenotypic detection of the reproduction mode. Phenotypic detection of apomixis involves the classification of a known heterozygous maternal parent and its progeny (grown from seed) with no observed segregation; or, in the case of the sexual reproduction mode, the presence of several progeny with complete heterozygosity. Phenotypic selection was also combined with the presence or absence of SCAR N14, which is linked to the apomixis locus.

RNA extraction. From each of the apomictic and sexual genotypes selected, 300-400 embryo sacs were obtained by cytological methods. Once the tissue was frozen in liquid nitrogen, the total RNA was extracted using Trizol® Reagent (Invitrogen). Equal amounts of high-quality RNA were pooled for apomictic and sexual material to obtain one bulk of total RNA from apomictic genotypes and another from sexual genotypes.

Full-length cDNA libraries. Two full-length cDNA libraries were constructed from the apomictic and sexual bulks. Double-strand cDNA synthesis was performed using the template switch technique (SMART™ PCR cDNA Synthesis Kit; Clonthech). The PCR-amplified cDNA was cloned using the Creator™ SMART™ cDNA Library Construction Kit.

Future Activities

- Construct forward and reverse cDNA subtraction libraries to identify genes expressed only in the apomictic or sexual bulks.
- Carry out cDNA microarray spotting. Clones from each library will PCR amplified and spotted onto replicate glass slides using a SPBIO spotting robot.
- Perform microarray hybridizations with Cy3- and Cy5-labeled cDNA derived from poly(A)+ of genotypes used in the cDNA subtraction library construction
- Include microarray hybridizations with labeled PCR product of the SCARs markers, linked to the apomixes loci in the *Brachiaria* genetic map.
- Generate cDNA chips with clones derived from the full-length cDNA libraries of both sexual and apomictic genotypes.
- Identify clones of interest. Computer analyses of the microarray hybridization output will be use to identify clones that change their pattern of expression between sexual and apomictic genotypes.
- Perform microarray hybridization and sequencing of the full-length cDNA chips, using as probes cDNA clones identified as interesting in the cDNA substraction cDNA chip.
- Use the identified and sequenced clones as probes against Northern blots to confirm their differential expression.

OUTPUT 2 Genes and genes combinations made available for broadening the base of mandated and non mandated crops

Activity 2.1 Transfer of gene and gene combinations using cellular and molecular techniques

Main Achievements

- The genetic transformation of tepary bean hybrids carried out in 2001 with the *Agrobacterium* strain LBA4404 pTOK233, was confirmed through southern blot. The transformation methodology used was developed at CIAT and permits the screening of large populations of hybrids. This is specially useful for breeding common bean genotypes amenable to Agrobacterium-mediated transformation.

- Interspecific hybridization between the common bean and the tepary bean genotype, NI576, (which is genetically transformable using *Agrobacterium tumefaciens*) was achieved and hybrid populations developed. Results from the evaluation of these populations for stable transformation suggest that some hybrid lines have received genes from tepary bean involved in conferring competence to *Agrobacterium*-mediated genetic transformation of mature seed meristems.

- The time consuming process of culturing and applying selective conditions to transformed tissues of common bean has been automated through the use of a modified and simplified temporary immersion methodology. This will lead to performing the transformation experiments more efficiently and uniformly.

- Field evaluation of F_6 interspecific populations between common and tepary beans developed through congruity and double congruity backcrosses clearly showed for the first time that drought tolerance can be effectively introgressed into common bean from this species and using these hybridization methodologies.

- Two new cassava cultivars (ICA-Negrita and SM1219-9) were transformed using *Agrobacterium* and Biolistics. Putative transgenic plants are positive for Southern of PCR-products probed with GUS gene.

- Southern blot analysis of transgenic rice using isoschizomer restriction enzyme pair indicated a differential methylation pattern between RHBV resistant and susceptible plants. Results may suggest a possible association of the RNA mediated RHBV resistance in these plants and a potential post-transcriptional gene silencing due to transgene methylation in these plants.

- Evaluation for RHBV resistance in the greenhouse suggest a possible protection encoded by the anti-sense NS4 transgene conferred by a different mechanism respect to the resistance encoded by the nucleoprotein transgene, since most resistant plants showed highest levels of NS4-RNA expression.

- Progeny plants containing stable inheritance of high level of expression of the anti-fungal PAPy123 transgene were advanced at the T2 generation, and are currently being evaluated for sheath blight resistance.

2.1.1 Towards the development of efficient genetic transformation protocols in common bean

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SB-02 Project

Introduction

Although transgenic common bean plants have been developed through particle bombardment (Russell *et al.*, 1993; Aragao *et al.* 1996, 1998 and 2002), a methodology for *Agrobacterium*-mediated transformation is still needed in order to efficiently apply recombinant DNA technologies in common bean breeding and research.

Compared to particle bombardment. *Agrobacterium*-mediated 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.

Agrobacterium-mediated transformation of common bean has not been possible up to date, despite the many experienced scientists and labs that have attempted it around the world. From the genus *Phaseolus* only the transformation the tepary bean (*P. acutifolius*) has been clearly demonstrated. Two methodologies have been used for it: the inoculation of green nodular callus tissues (Dillen *et al.*, 1997) or of meristems of mature seeds (Mejía Jiménez *et al.*, project SB2 Annual Reports 2000 and 2001).

The *Agrobacterium* mediated mature seed meristem transformation (AMMSM-Transformation) methodology developed at CIAT, did not require the previous induction of a callus tissue and thus allows the faster production of a transgenic plant, and the screening of large populations of genotypes with less effort than the previously developed methodology.
However, the application of the AMMSM-transformation methodology to more than 30 wild and domesticated genotypes of common bean during the year 2000 and 2001 yielded no transgenic callus tissues or plants. Transformation experiments done with tepary bean genotypes and hybrids (see annual report of 2001) suggested that nuclear genes of the genotype NI576 are involved in conferring competence to *Agrobacterium* mediated transformation of hybrids between responsive and non-responsive genotypes. For this reason we started interspecific crosses between common bean and the NI576 genotype of tepary bean, to transfer the supposed traits for *Agrobacterium* transformation competence to common bean.

The objective of this activity is to breed one genotype of common bean, which can be efficiently transformed with *Agrobacterium tumefaciens*, from which the transgenes can be transferred fast an easily to common bean cultivars.

During the year 2002 we continued to produce interspecific populations and test them for competence to *Agrobacterium* mediated transformation. We are currently also investigating the improvement of genetic transformation methodologies through particle bombardment.

Methodology

Hybrid progenies involving the NI576 tepary bean genotype, and common bean genotype Bayo Madero (among others used as facilitators of the interspecific cross) were generated through double congruity backcrossing (Mejía Jiménez *et al.*, Annual Reports 2000, 2001 and 2002).

Mature but not dry pods were used as source of sterile explants (whole mature seeds without one cotyledon) for 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).

Results

Confirmation of the genetic transformation of the tepary bean hybrids inoculated with *Agrobacterium* tumefaciens.

During 2000 and 2001, a novel methodology for the genetic transformation for *Phaseolus* beans was developed. Several hygromycin resistant meristematic tissues were obtained from F_2 seeds of two tepary bean hybrids (G40022 x NI576 and G40065 x NI576) inoculated with the *Agrobacterium* strain LBA 4404 pTOK233. However, plants could be regenerated from only three of them. These plants and their progeny expressed GUS in most of their tissues. The transformation of these plants was confirmed during this year through southern blot (Fig. 1).

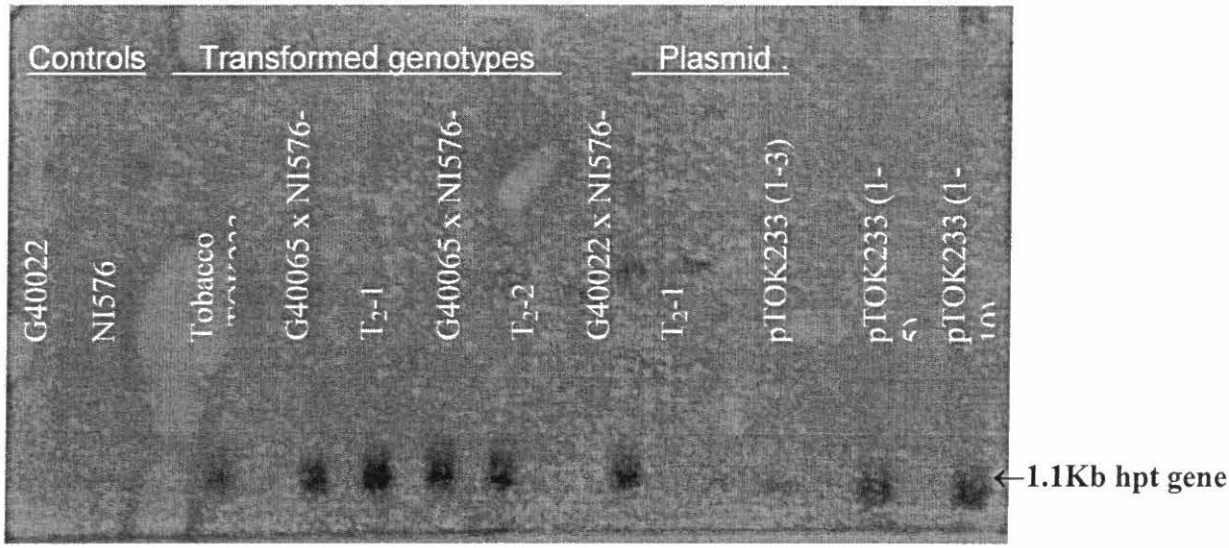

Fig. 1 Southern analysis of non transformed plants (G40022 and NI576) and T_2 progeny of two GUS-positive and hygromycin resistant tepary bean hybrids G40065 x NI576 and G40022 x NI576 obtained through the inoculation of mature seeds with the *Agrobacterium* strain LBA4404pTOK233. Total DNA of the plants was digested with BahmH1 and probed with a labeled fragment corresponding to the hygromycin phosphotransferase (hpt) gene.

This AMMSM-transformation methodology is different from the one developed previously (Dillen et al. 1997), and since the induction of a callus tissue is not necessary for applying it, it is specially useful for screening genotypes or populations of hybrids for competence to *Agrobacterium*-mediated transformation.

This is the first demonstration that meristems found in mature seeds of a *Phaseolus* bean species can be transformed stably with *Agrobacterium*.

Further development of fertile hybrid progenies between common and tepary bean using the tepary gentoype NI576

In order to breed common bean genotypes which are responsive to the AMMSM-transformation methodology, we have started interspecific crosses in which we included the tepary bean genotype, NI576, as a putative source of genes involved in conferring competence for transformation. We also included tepary bean genotypes, which were selected as the best in forming meristematic callus (a trait that can be useful during selection of transformed tissues), G40022 and G40065 and the best-identified genotype of common bean, which also forms meristematic callus, Bayo Madero. The development of fertile interspecific hybrids between the genotypes NI576 and Bayo Madero was possible only through a complex series of backcrosses (see annual reports 2000 and 2001) we performed starting from advanced congruity backcross hybrids developed years before (Mejía-Jiménez *et al,* 1994).

Since the stable introgression of tepary bean traits into common bean genome can be very difficult, and with the DCBC only a small proportion of the donor genotype NI576 is expected to be transferred to the receptor genotypes, several backcrosses may be necessary for obtaining a fertile genotype which combine the desired traits. A strategy for accumulating traits that may positively affect the in vitro culture and genetic transformation behavior of a genotype is being followed. This consists in screening hybrids obtained from both cytoplasms for competence for *Agrobacterium*-mediated transformation and in intercrossing them again. We are using transient or stable gus expression as well as callus induction and maintenance characteristics as screening tools.

Several hybrids populations with different complexity grades, produced by intercrossing hybrids with common or tepary bean cytoplasm which involve the genotypes NI576 and Bayo Madero, have been produced during 2002 and are being tested with binary *Agrobacterium* strains carrying marker genes for AMMSM-transformation. No transgenic plants have been obtained from these experiments, but several lines with tepary bean cytoplasm (A-DCBC8-1; see common x tepary bean interspecific crosses report) have been identified that after selection, produced meristematic (regenerable) calli that express GUS. Also promising lines with the cytoplasm of common bean have been identified (V-DCBC5), which produced calli expressing marker genes after transformation. This has been never achieved before with a common bean genotype (see annual reports of 2000, 2001 and 2002).

Improvement of the *in vitro* culture steps following the transformation of the explants through the use of temporary immersion systems (TIS)
The temporary immersion tissue culture methodology is a relatively young innovation for the way cells, calli or tissues are cultured *in vitro*. Instead of culturing them over a semisolid medium, or immersed in a liquid medium, which must be agitated or aerated in order to allow an appropriate gas exchange, in the TIS methodology the tissues are immersed only temporarily once, or several times a day, in a liquid culture medium, maintained wet but not in direct contact with the medium, most of the time. This is achieved using different types of vessels and by transporting the culture

media from one vessel to another using hydraulic or pneumatic methods (Teisson and Alvard, 1995; Escalona *et al.* 1999).

We have implemented the TIS methodology for culturing transformed bean tissues (through particle bombardment or *Agrobacterium*) in the phases following the transformation: meristematic-callus induction and selection. The advantages of applying the TIS methodology in transformation has been several: fewer media changes are needed, selective conditions are applied more uniformly to all explants thereby reducing the number of escapes (non transformed tissues that resist selective conditions) and reduction of tissue recontamination (when they have been inoculated with *Agrobacterium*). Also, a better aeration of the tissues is achieved producing healthier and bigger putatively transformed calli after finishing the selection. This last advantage of the TIS culture methodology seems to be very important for AMMSM-transformation, since the small size of the calli obtained after selection was the most important constraint impeding the fast and efficient regeneration of several of the meristematic calli that resisted selection after transformation (annual report 2001).

Towards the improvement of the genetic transformation methodologies of common bean through particle bombardement. Particle bombardment has been the only transformation methodology that has permitted the recovery of transgenic plants in common bean. This methodology however still has several impediments that prevent its routine application or would hinder the commercialization of the transgenic cultivars developed by this way. The most important impediments are: (i) the low rate of stable transformation, (ii) the need for the elimination of the antibiotic marker genes from the process or from the final product, and (iii) the occurrence of multiple insertions of the bombarded DNA molecules in the same DNA locus.

The two last constraints could be solved if the transformation efficiencies could be increased, and large populations of independently produced transgenic plants are developed. The lines that show the desired patterns of DNA insertion, and the desired levels of expression of the transgenes, could then be selected from these populations. In order to increase the transformation efficiency while using particle bombardment, we are testing the bombardment of plasmid DNA or DNA fragments in different configurations, the use of the TIS system for culturing bombarded tissues and the screening of several agronomically important lines or cultivars of common bean to identify the ones that respond best to the tissue culture and transformation treatments applied.

Conclusions

The transformation of the hygromycin resistant and GUS positive plants recovered after the inoculation of mature seeds of tepary bean hybrids with *Agrobacterium* during 2001 has been confirmed through southern blot.

Gus expressing hygromicin or geneticin resistant meristematic callus tissue has been obtained after selection of common x tepary bean hybrids inoculated with binary *Agrobacterium* strains

Future plans

- To further develop hybrid progenies using the genotypes NI576, and Bayo Madero as parents
- To screen the hybrid populations developed in order to identify genotypes that can be efficiently transformed through the AMMSM-transformation methodology
- To improve the efficiency of transformation of common bean cultivars through particle bombardment using plasmids or DNA fragments in different configurations, and temporary immersion tissue culture systems

- To initiate co-transformation experiments (with marker genes and agronomically important genes in different strains or plasmids) in order to produce antibiotic marker-gene free transgenic plants.

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2.1.2 Interspecific hybridization of common and tepary bean through double congruity backcrosses

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Introduction

Several traits found in tepary bean (*P. acutifolius* A. Gray) accesions are important for common bean breeding. For example tepary bean genotypes have been identified to be a source for resistance to drought (G40020 and G40023), the bruchids (*A. obtectus* and *Z. subfaciatus*;

G40199), leaf hopper (*Empoasca kraemeri*; G40019 and G40036) and bacterial blight (*Xanthomonas campestris pv phaseoli*; G40020). Also the accesion NI576 could be a source for genes for competence to *Agrobacterium* mediated transformation (see report on bean transformation).
Crossing of common with tepary bean has been traditionally difficult. Using facilitator genotypes of both species, and applying recurrent and congruity backcrossing (CBC; the alternate backcross of the hybrids with genotypes of each species for several cycles; Haghighi and Ascher, 1988), we could produce fertile progeny (Mejía-Jiménez *et al.* 1994), from which the VAX lines with high levels of resistance to bacterial blight were developed (Singh and Muñoz, 1999).

However the production of fertile hybrids involving most of the above mentioned tepary bean accessions, has been attempted by recurrent and congruity backcrossing but it has not been possible.
Using a modification of the CBC methodology, we called Double Congruity Backcrosses (DCBC), and advanced CBC hybrids developed previously as bridge, we could develop fertile interspecific hybrids involving the tepary bean accession NI576 (Annual report 2001).

In 2001 we started with the use of advanced DCBC hybrids, as bridge to facilitate the cross of other accession of tepary bean, G40019, G40036, G40199 and G40023. During 2002 and through the use of this backcrossing strategy, fertile progenies involving all these tepary bean accessions were produced. These are now be tested by the for introgression of the desired traits.

Methodology

Double congruity backcross hybrids with the cytoplasms of common or tepary bean (V-DCBC and A-DCBC hybrids) were developed starting from advanced congruity backcross hybrids with cytoplasm of common bean developed previously (Mejía-Jiménez *et al.*, 1994), as described in the annual report 2000 (Mejía Jiménez et al. 2000; Table 1 and 2).

Difficult to cross genotypes of tepary bean are crossed first to the most advanced fertile DCBC hybrids available of the same cytoplasm, to generate fertile hybrids, which are then crossed to the most advanced DCBC hybrids with common bean cytoplasm in order to produce fertile or self sterile hybrids, which have to backcrossed to obtain fertile progeny.

Embryo rescue was applied when necessary to recover viable hybrids from aborting embryos (Mejía-Jiménez *et al.*, 1994).

Results

Introgression of drought tolerance from tepary bean to common bean through CBC and DCBC
Drought tolerance has been always a breeding target in the common x tepary bean interspecific crosses made at CIAT. Due to this, accessions of tepary bean selected as resistant to drought by J. White (like G40023, G40020 and G40068), were included in the crossing program realized between 1988 and 1993 (Mejía-Jiménez *et al.*, 1994; Tables 1 and 2), in which we applied the CBC strategy. Starting from the most advanced hybrids obtained in that program we performed a series of new crosses which included intercrosses between CBC5 hybrids and between the obtained hybrids, followed by crosses to the cultivar Bayo Madero or to DCBCs hybrids involving the accession NI576 of tepary bean (Tables 1 and 2).

In 2001 F3 progeny of these hybrids was provided to the IP1 project for screening for drought tolerance. This year the IP1 project reports the results of the second year of screening of F6 populations derived from these hybrids. Several lines derived from CBC or DCBC showed

resistance to drought, and one of this yielded 40% more than the tolerant check, SEA 5 (see Beebe *et al.*, in this Annual Report). The complex pedigree of two of the most productive of these lines is shown in table 1.

This is the first clear demonstration that drought tolerance in common bean can be improved through interspecific crosses with tepary bean. It is also a demonstration, that complex multigenic traits such as drought, can been effectively introgressed from species of the tertiary gene pool to common bean through the congruity and double congruity backcross strategies.

Table 1: Pedigree of two of the common x tepary interspecific hybrid lines developed through CBC or DCBC identified by the IP1 project as drought tolerant (See Beebe *et al.* in this annual report)

BKI 11=

Bayo Madero

[CBC5 Pijao X G40001 X MAM38 X G40001 X Pijao X G40020 X Pijao	(CBC3 Pijao X G40001 X A775 X G40001 X Pijao	X CBC3)] Pijao X G40001 XA775 X G40001 X A800

MMNNI 14=

(CBC5 Pijao X G40001 X MAM38 X G40001 X Pijao X G40020 X Pijao	X CBC5) Pijao X G40001 X A775 X G40001 X MAR1 X G40001 X Pijao	X (CBC5 Pijao X G40001X A775X G40001 X MAR1 X Pijao X G40001 X A800	X CBC3) Pijao X G40001 X MAM38 X G40001X A800	X	[(G40065 X NI576)	X [(CBC5 Pijao X G40001 X MAM38 X G40001 X Pijao X G40020 X Pijao	X CBC5) X Pijao X G40001 X A775 X G40001 X MAR1 X G40001 X Pijao	X (CBC5 Pijao X G40001 X MAM38 X Pijao X X G40020 X Pijao	X (CBC3 Pijao X G40001X A775 X G40001 X MAR1	X CBC3)] Pijao X G40001 X A775 X G40001 X A800	X NI576

Accesions involved: Common Bean: Pijao (ICA Pijao), MAM38, A775, A800, Bayo Madero
Tepary bean: G40001, G40020, G40065 (cultivated). NI576 (wild)

This year new F3 progenies of more advanced DCBC lines and other involving other common bean cultivars or breeding lines selected for drought tolerance the previous year, were provided to the IP1 project for further screening.

Development of fertile progenies between common and tepary bean through DCBC involving accessions of tepary bean selected for agronomic traits

Table 2 shows a summary of the interspecific crossing program between common and tepary bean carried out since 1988. Until 1993 five generations of CBC were developed involving the common and tepary bean genotypes shown in the table, and others from which no fertile progeny was obtained (Mejía-Jiménez et al. 1994). From these hybrids the VAX lines were developed (Singh and Muñoz, 1999). The DCBC crossing program was started after many unsuccessful attempts to cross the tepary bean accession NI576 (as male) with common bean directly, or using the intercrossed CBC hybrids as bridge (hybrids with code M). Hybrids between common bean and a tepary bean hybrid involving NI576 could only be developed on the tepary bean cytoplasm (A-DCBC-1). These hybrids were self sterile and had to be backcrossed in order to obtain fertile progeny (A-DCBC-2). A-DCBC-2 hybrids were used for producing the first DCBC hybrids with common bean cytoplasm: V-DCBC1. These hybrids were partially fertile (such as the MMNNI14, table 1). At this time and despite the many backcrosses, the levels of introgression from the tepary genome are much less than expected. For example the seed and flower color of the A-DCBC-2 hybrids did not influenced the seed or flower color of the V-DCBC1 hybrids or that of its progeny.

For increasing the introgression of tepary bean alleles in the hybrids with common bean cytoplasm, several further cycles of DCBC on both cytoplasms were carried out (Table 2).

Fertile hybrids involving the also difficult to cross accessions of tepary bean G40019, G40036 and G40199, have been developed by crossing them with A-DCBC6-1 hybrids, and by crossing the resulting self fertile hybrid with V-DCBC5 hybrids. Self fertile and cross fertile hybrids were produced. More recently more hybrids involving G40199 have been developed (A-DCBC8-1). The most important results of the whole program are: The level of difficulty to perform the backcrosses is reduced with the advance of backcrosses mainly in the *P. acutifolius* cytoplasm The frequency of appearance of morphological markers from the male parent, such as leaf petiole size (in the case of hybrids made in the *P. acutifolius* cytoplasm), hypocotyl and flower color, and bracteoles, seeds or flower size, in F1 hybrids is increasing. The fertility and vigor of the F1 hybrids tend to increase

During 2002 several hybrid progenies with common bean and tepary bean cytoplasm which involve in their pedigrees the tepary bean genotypes G40019, G40036 and G40199 have been provided to the project IP1. These are being screened for resistance to drought, *Empoasca* and bruchids (see IP1 annual report).

Regarding the hybrid lines invoolving the accession NI576 tested for *Agrobacterium* mediated transformation through the innoculation of meristems of mature seeds, regenerable meristematic calli which express marker genes have been selected from several lines with the cytoplasm of tepary bean (A-DCBC8-1). Also promising lines with the cytoplasm of common bean have been identified (V-DCBC5), which produced calli expressing marker genes after transformation. This has been never achieved before with a common bean genotype (see annual reports of 2000, 2001 and 2002).

Conclusions

CBCs and DCBCs allowed the introgression of genes of tepary bean involved in drought tolerance to common bean lines.

DCBC allows the production of viable and fertile or cross-fertile common x tepary bean hybrids from genotypes that were before incompatible using other crossing techniques.

Promising results, regarding competence to genetic transformation through *Agrobacterium,* were obtained after screening of DCBC interspecific hybrid populations.

Table 2. **Interspecific crosses program between *P.vulgaris x P. acutifolius* in development since 1988. The column of the middle shows the code assigned to each hybrid generation. Accessions of common or tepary bean involved in the program are in bold; CBC= congruity backcross hybrids (Haghighi and Ascher 1988). DCBC= Double congruity backcross hybrids; V-DCBC= DCBC with the cytoplasm of common bean; A-DCBC= DCBC with the cytoplasm of tepary bean; the DCBC hybrids with odd numbers result from a cross between two DCBC with different cytoplasms. These are in general self-sterile (total self-sterile hybrid generations are shown in italics). The DCBC hybrids with the even numbers result from a cross between two DCBC with the same cytoplasms. These are in general self-fertile.**

Cytoplasm of *P. vulgaris*		
FEMALE PARENT	**HYBRID GENERATION**	**MALE PARENT**
ICA Pijao	x ↓ *F1*	**G40001**
(ICA Pijao x G40001) F1	x ↓ BC1	**A775, MAM38**
BC1	x ↓ *CBC2*	**G40001**
CBC2	x ↓ CBC3	ICA Pijao, MAR1, A800
CBC3	x ↓ CBC3	CBC3
CBC3	x ↓ *CBC4*	G40001, G40020
CBC4	x ↓ CBC5	ICA Pijao, A800
CBC5	x ↓ CBC5 x CBC3	CBC3
Bayo Madero	x ↓ BKI	CBC5xCBC3
CBC5xCBC3	x ↓ M	CBC5xCBC3
KI	x ↓ *CBC6*	G40022
CBC6	x ↓ CBC7	BBK
CBC5 x CBC3	X ↓ V-DCBC1	A-DCBC2-1
V-DCBC1	x ↓ V-DCBC2	BKIM=(Bayo Madero x KI)x M
V-DCBC2	x ↓ *V-DCBC3-1*	A-DCBC4
V-DCBC2	x ↓ V-DCBC3-2	GNV3*
V-DCBC3-1	x ↓ V-DCBC4	V-DCBC3-2

Cytoplasm of *P. acutifolius*		
FEMALE PARENT	**HYBRID GENERATION**	**MALE PARENT**
G40065	x ↓ F1	NI576
(G40065 X NI576) F1	x ↓ *A-DCBC1*	(CBC5 x CBC3)x (CBC5 x CBC3)
A-DCBC1	X ↓ A-DCBC2-1	G40065
A-DCBC1	X ↓ A-DCBC2-2	NI576
A-DCBC2-1	X ↓ *A-DCBC3*	CBC7
A-DCBC3	X ↓ A-DCBC4	A-DCBC2-2
A-DCBC4	X ↓ *A-DCBC5*	V-DCBC1
A-DCBC5	X ↓ A-DCBC6-1	A-DCBC4
G40199, G40019, G40036	X ↓ A-DCBC6-2	A-DCBC6-1
A-DCBC 6-3	X ↓ *A-DCBC7-1*	V-DCBC4
A-DCBC 6-3	x ↓ *A-DCBC7-2*	V-DCBC3-2

V-DCBC4	x ↓ *V-DCBC5*	A-DCBC6-1
V-DCBC5	x ↓ V-DCBC6	V-DCBC4
V-DCBC5	x ↓ *V-DCBC7-1*	A-DCBC6-2
V-DCBC6	x ↓ V-DCBC7-2	A-DCBC7-1
V-DCBC7-1	X ↓ V-DCBC8-1	V-DCBC6
V-DCBC7-2	X ↓ V-DCBC8-2	V-DCBC6

A-DCBC 7-1	X ↓ A-DCBC8-1	A-DCBC 6-3 G40199
A-DCBC 7-2	x ↓ A-DCBC8-2	A-DCBC6-3
A-DCBC 8-2	X ↓ A-DCBC8-3	A-DCBC6-3
A-DCBC8-2	x ↓ *A-DCBC9*	V-DCBC5 Y V-DCBC6
A-DCBC9	x ↓ A-DCBC10	A-DCBC8-2

Future plans

- To continue with the DCBC strategy to produce more advanced DCBC hybrid generations
- To perform additional DCBCs with hybrids that have already shown in the field resistance to drought, *Empoasca,* or *Acanthoselides* or that have shown competence for *Agrobacterium* mediated transformation.
- To measure the introgression of DNA fragments from the tepary bean genotypes into the fertile hybrid populations produced using AFLP techniques.

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2.1.3 Genetic transformation of cassava: Confirmation of transgenesis in clone 60444 and analysis of CRY1Ab protein in transgenic lines. Preliminary data on transformation of farmer-preferred cultivars SM1219-9 and CM3306-4

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Introduction

CIAT has developed transgenic cassava plants since the beginning of the 90s, using *Agrobacterium*-mediated transformation and the commonest cassava regeneration system—somatic embryogenesis. The Friable Embryogenic Callus (FEC) system has been implemented for routine transformation of new clones with either *Agrobacterium* or a particle gun to introduce insect-resistance genes. The real usefulness of transgenic cassava will, however, rely not only on our ability to deliver plants to the farmers, which implies dealing with intellectual property right issues, but also to modify genetically the genotypes they prefer. Given that cassava is a highly heterozygous crop, genetic transformation of a model clone like 60444 (formerly known as TMS 60444) and the transfer of transgenes to other clones through crossing are impractical due to time constraints. Obtaining a variety by classical breeding usually takes about ten years; therefore transgenic plants have to be obtained for varieties selected by farmers—an approach that requires adjusting the in vitro culture system for each clone. We report the molecular analysis of transgenic plants of the model clone 60444 and preliminary data on the transformation of farmer-selected cultivars using *Agrobacterium* and biolistics.

Materials and Methods

FEC lines from clones 60444, M Col 2215, CM 3306-4 and SM 1219-9 were submitted to transformation with *Agrobacterium* strains C58C1-pBIGCry and LBA4404-pBIGCry; and shooting with plasmids pBIGCry and pSGMC. Cotyledons of somatic embryos from clone M Col 2215 were also assayed for transformation with *Agrobacterium* strain LBA4404-pBIGCry. Plasmid pBIGCry contains three genes: *npt*II, *cry*1Ab and *gus*-intron; while plasmid pSGMC contains the last two plus *pmi* for selection on mannose. The selection regime of transgenic tissues and assays for transient and stable *gus* expression are summarized in Table 1. Modified ELISA tests (Envirologix, ME, USA) were run to quantify the amount of CRY1Ab protein expressed in young leaves of transgenic lines 55, 80 and 92.

Results and Discussion

Four lines of putatively transgenic plants were obtained with model cv. 60444: 27, 55, 80 and 92. All lines were established in the greenhouse. Two of them (55 and 92) still express the *gus*-intron gene very strongly. It should be noted that the FEC from line 80 tested positive for *gus* expression (stable expression; Table 1) before the third round of selection. Southern blots of PCR products showed positive signals for lines 27, 55 and 80 when probed with *cry*1Ab. Line 92 was not included in this test although it expresses *gus* strongly (Figure 1). Southern blots of genomic DNA detected the *gus*-intron and/or the *npt*II genes in lines 55, 80 or 92. Line 55 and 80 had at least two inserts of the T-DNA; line 92 had only one (brackets in Figure 1C). Transgenic tobacco showed at least one insert, while nontransgenic rice showed two nonspecific hybridizing bands. The *gus*-intron probe detected a rearranged T-DNA insert of higher molecular weight in lines 55, 92 and transgenic tobacco. No band was observed in line 80 (it does not express *gus*). Most probably this T-DNA was rearranged while stored in *Eschlerichia coli* or *Agrobacterium* as all

independent transgenic lines contain the same size insert. Plasmid pBIGCry must have undergone a rearrangement as Southerns of genomic DNA— hybridized with the probe *cry*1Ab, digested from plasmid pBT1291, from which *cry*1Ab was originally subcloned—do not show the expected T-DNA (data not shown). Similar results were obtained with transgenic tomato transformed with pBIGCry (H Ramirez and L Fory, personal communication).

Taken together, the molecular evidence indicates that there are at least three transgenic cassava lines derived from cv. 60444 (lines 55, 80 and 92). These lines may contain rearranged T-DNA carrying the *cry*1Ab gene. The rearrangement in pBIGCry must be confirmed by sequencing.

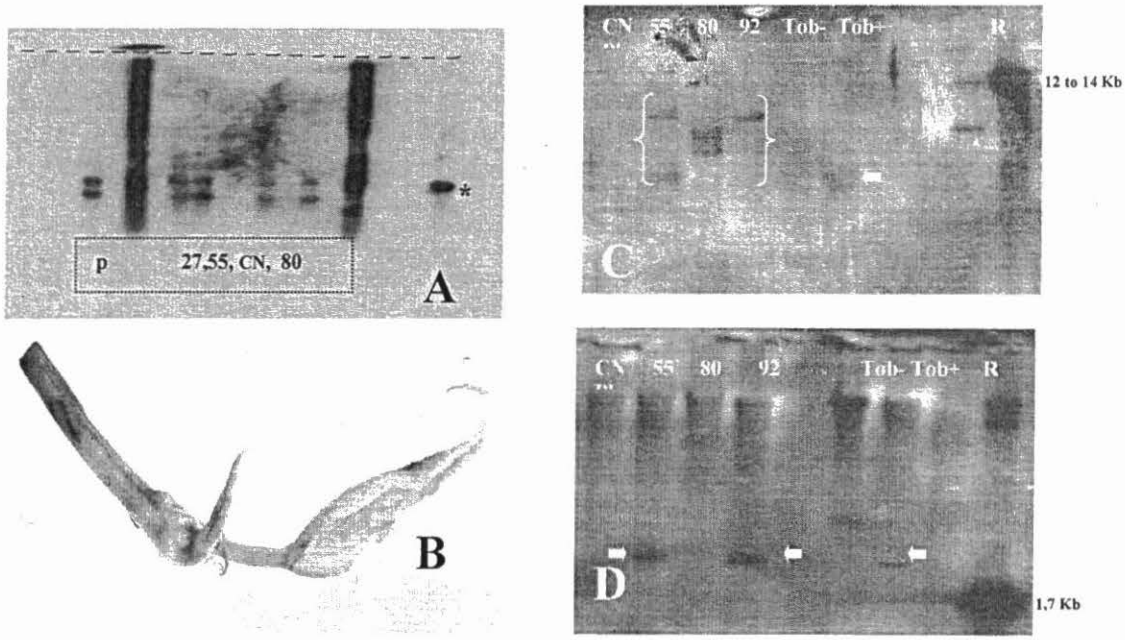

Figure 1. (A) Southern blots of PCR products probed with *cry*1Ab (* = 1.2 kb; p = plasmid pBIGCry; CN = negative control). (B) Line 92 expressing *gus*. (C) Southern blots of genomic DNA from lines 55, 80 and 92, restricted with BamHI or EcoRI, to determine the no. of insertions (brackets and arrowhead). Southern probed with a 0.7 kb PCR-derived *npt*II probe (CN= negative control; Tob- = tobacco genomic DNA; Tob+ = transgenic tobacco transformed with pBIGCry; R = rice genomic DNA; Plas = plasmid PBIGCry cut with same enzymes – 12 to 14 kb). (D) Southern blots of genomic DNA for same individuals as in C, restricted with *Pst*I to release part of the T-DNA. Southern blots probed with a 0.65 kb PCR-derived *gus* probe. A 1.7-kb band is expected as shown in the line of plasmids. Arrowheads point to a larger, rearranged T-DNA cassette, inserted in lines 55, 92 and transgenic tobacco, while no band appears in line 80.

Although we suspect that the T-DNA of pBIGCry may contain rearrangements, the CRY1Ab protein may still be expressed. The concentration of this protein in transgenic cassava lines 55, 80 and 92 was estimated as the average of two measurements (OD_{450}), separated in time, using the standard curve depicted in Figure 2. The results indicated that for line 55 only, the amount of CRY1Ab protein may be close to $0.18 \times (10)^{-6}$ ng of protein per gram of fresh tissue. However, these results need to be interpreted cautiously. As stated above, besides possible rearrangements in the T-DNA, the *cry*1Ab gene was not detected by Southerns of genomic DNA in these transgenic lines. Estimations of CRY1Ab concentration varied from test to test, although line 55 always showed OD readings above the background level. In one case the background was so high that the raw absorbance of the negative control exceeded those of lines 80 and 92. Therefore, transcription and translation of the *cry*1Ab gene, if present in lines 55 and 92, need to be confirmed by Northern Blot, RT-PCR and Western Blot.

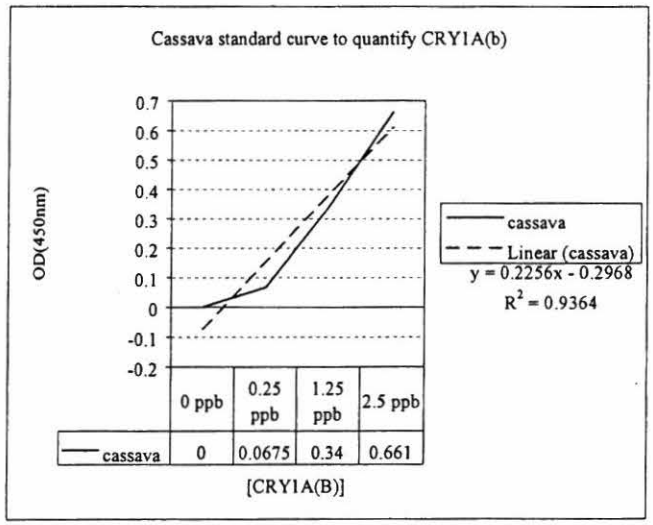

Figure 2. Standard curve of absorbance (450nm) of cassava leaf extracts and known CRY1A(b) concentrations. The dotted line represents the linear regression, adjusted data curve, from which estimations were made.

Bioassays are still pending to test the effectiveness of this *cry* gene to control Lepidoptera, specifically *Eryinnis ello* (cassava hornworm). The results of bioassays tests with transgenic tomato (H Ramirez, personal communication) indicated that this *cry*1Ab gene does not confer resistance against a tomato leaf miner.

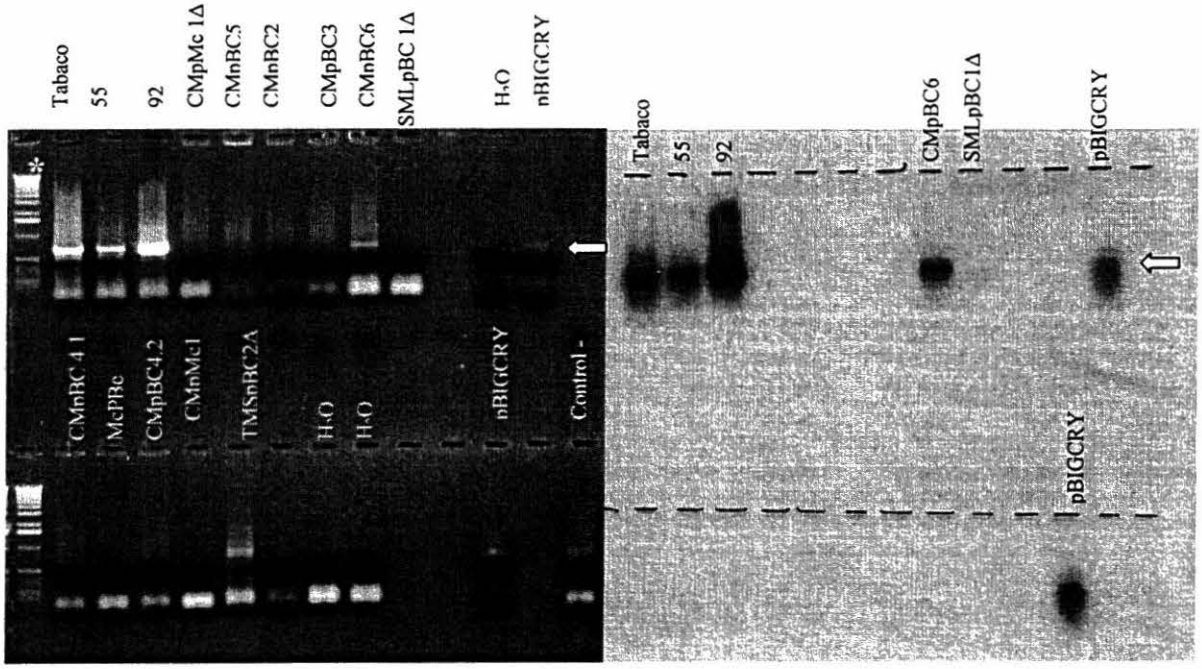

Figure 3. (Left) PCR products of putative transgenic lines of several cassava cultivars amplified with *gus*-specific primers. The expected 720-bp band (arrow) appears between the 506 and 1018 bp bands of the lambda marker lane(*). (Right) Southern of the gel on the left, probed with PCR-amplified *gus* probe from pBIGCry. Arrowhead points to the expected 720-bp band in positive controls and two new transgenic lines.

Transformation experiments of new farmer-preferred cassava cultivars resulted in the isolation of more than 20 putatively transgenic lines (transformation experiments ID 261001, 221101 and 190302, described in Table 1). Two lines from two clones—CMpBC6 from clone CM 3306-4 (from shooting experiment 261001) and line SMLpBC1Δ from clone SM 1219-9 (from experiment 221101)—were positive for Southern tests of PCR products of the *gus*-intron gene (Figure 3). The former was obtained after shooting; the latter from *Agrobacterium*-mediated transformation with strain LBA4404—both with plasmid pBIGCry. Positive controls from tobacco and cassava (lines 55 and 92 from clone 60444 mentioned before) indicate the position (720 bp) of the expected band in Southerns. We are still in the process of testing more putative transgenic lines through preliminary screenings via PCR. Once we have plants in the greenhouse, Southerns of genomic DNA for at least two of the genes present in pBIGCry or pSGMan (*npt*II, *cry*1Ab, *gus*-intron or *pmi*) will determine the true transgenic status of these new lines.

Future Activities

- Induction of more FEC lines, at least four times a year, to replenish old cell lines and to establish new ones with new farmer-selected cultivars.
- Implementation of cryopreservation for long-term storage of FEC lines
- Bioassays with the cassava hornworm.
- Sequence T-DNA of pBIGCry to confirm status of *cry*1Ab gene
- Find new sources of cry genes to replace the one in use

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Table 1. Selection regime of transgenic tissues and assays for transient and stable *gus* expression for selected transformation experiments performed during the last three years (antibiotic abbreviations: Cef = cefotaxime; Gna = geneticin; Par = paramomycin).

Lines/ Experiments	Inoculation or Shooting (*) Date	GusAssay (transient expression)	Antibiotic for Eliminat-ing Bacteria	Antibiotic – First Round of Selection	Antibiotic - 2nd Round of Selection	*gus* Assay (stable expression)	Antibiotic -3rd Round of Selection	Plant Regenera-tion: No. of PutativelyTrans-genic Plants in vitro/Greenhouse	*gus* Assay (stable expression)
27	27 April 2000	Negative	Cef (500 mg/l)	Par (25µM)	Gna (20 mg/l)	Negative	-	>20 plants in vitro; 10-20 plants greenhouse	Negative
55 and 80	09 May 2000	Positive	Cef (500 mg/l)	Par (25µM)	Gna (20 mg/l)	Positive	Gna 50 mg/l	>20 plants in vitro. 10-20 plants greenhouse	Positive for 55 only
92	20 Oct. 2000	Positive	Cef (500 mg/l)	Gna (7 mg/l)	Gna (20 mg/l)	Positive	-	>20 plants in vitro. 10-20 plants greenhouse	Positive
080801	*08 Aug. 2001	Positive	- ·	Mannose 1% or Gna (10 mg/l)	Mannose 1% or Gna (10 mg/l)	Positive	-	Approx. 25 in vitro	Positive
261001	*26 Oct. 2001	Positive	-	Mannose 1%	Mannose 1%	Negative	-	Approx. 6 in vitro	Negative
221101	22 Nov. 2001	Positive	Cef (500 mg/l)	Gna (10 mg/l)	Gna (10 mg/l)	Negative	-	Approx. 11 in vitro	Negative
190302	19 Mar. 2002	Negative	Cef (500 mg/l)	Gna (20 mg/l)	Gna (20 mg/l)	Dead	Dead	n.a.	n.a.
Time (days)	0	2-3	5-15	30-90	30-150	-	30	120-240	-
Time range from inoculation to transgenic plant = 217-528 days									

2.1.4 Preliminary evaluation of the expression of the transgen bar in cassava plants maintained under asexual propagation for 10 years

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Introduction

In 1992 the first transgenic cassava plants were obtained at CIAT through *Agrobacterium*-mediated transformation. Only one line (53-5.2) was shown to contain the T-DNA from pGV1040 carrying the genes *npt*II, *uid*A and *bar* (Sarria et al., 2000) although four more lines (108, 137-3.1 y 207) were also preserved as putative chimerical transgenics, based on their tolerance to PPT (Phosphinotricin; herbicide Basta = glufosinate-ammonium). Since then, these plants have been clonally propagated in vitro and in the greenhouse. It is important to monitor the activity of the transgene, especially in clonally propagated crops, where chimeras may disappear after several cycles of propagation. On the other hand, silencing may be occurring to shut down the expression of initially active transgenes. We evaluated the presence of the *bar* gene and its transcription, as well as the tolerance of these transgenic lines to aspersion with the herbicide Finale (glufosinate-ammonium) at different concentrations (Echeverry 2002).

Materials and Methods

The research was divided into three components: (1) Detect the presence of *bar* by PCR and Southern Blot analysis, (2) detect transcription of *bar* by RT-PCR and (3) evaluate *bar* activity phenotypically by aspersion and in vitro tolerance tests. We checked the aforementioned lines plus transgenic tobacco, carrying the *bar* gene from same plasmid, as well as nontransgenic controls (cassava M Peru 183 and tobacco).

The conditions for amplifying the *bar* gene by PCR were as follows: 1 µg of target DNA, 2 µM of each primer (*forward* 5'-CGCTATCCCTGGCTCGTC-3' and *reverse* 5'-CGACCACGCTCTTGAAGC-3'; expected product at 205 bp), 200 µM of each dNTP, 2 mM of $MgCl_2$, 1X buffer 10X, one unit *Taq* polymerase for a final volume of 25 µl. For tobacco, the conditions were the same, except that $MgCl_2$ was increased to 1.5 mM. The amplification profile was 3 min denaturation at 94°C, followed by 35 one-minute cycles of denaturation at 94°C, 2 min annealing at 55°C and 2 min extension at 72°C. The final extension cycle was at 72°C for 5 min. Agarose gels were blotted onto nylon membranes for Southern blot analysis using a radioactive bar probe, PCR-amplified from pGV1040.

In those individuals in which *bar* was detected, we ran RT-PCR to detect transcription using Superscript II Invitrogen® to produce cDNA, previous treatment of RNA with DNAse. 450 ng of transgenic tobacco cDNA and 1.5 µg of cassava cDNA were PCR amplified according to the following protocol: 2 µM of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$, 1X buffer 10X, 1 unit *taq* Gibco-BRL®, for a final volume of 25 µl. The cDNA amplification profile was 1 min denaturation at 94°C, 30 one-minute cycles of denaturation at 94°C, 2 min annealing at 62°C and 3 min extension at 72°C. A final extension was performed at 72°C for 10 min. Southern blots were performed for hybridization of PCR products.

For those individuals that were RT-PCR positive, leaf disks were cultured on a basal medium containing 0.5 ppm BAP and Finale at a final PPT concentration of 8 mg/l. Similarly, plants from

these individuals were sprayed with Finale at 8 mg/l, 200 mg/l and 1500 mg/l. Two sprayings were done for each concentration at 15-day intervals.

Results and Discussion

We detected a 205-bp, PCR-amplified band only in line 53-5.2, transgenic tobacco and plasmid pGV1040, which corresponded to the expected product of the *bar* gene. The Southerns confirmed this finding (Figure 1). No bands were detected in lines 108 1.1, 137-3.1 and 207, suggesting that they are not true transgenics, not even chimeras, given that PCR should be able to amplify the gene, even in chimeras. These lines are probably somaclonal variants that escaped selection with PPT, which, in part, explains their initial tolerance to herbicides (Sarria et al., 2000). RT-PCR also detected a 205-bp band in 53-5.2, tobacco and pGV1040 when hybridized to a radioactive, PCR-derived *bar* probe (Figure 2). The background observed in Figure 2 was unavoidable for all amplifications. These results confirmed that 53-5.2 is a true transgenic that still transcribes the *bar* gene although at lower levels than the comparable transgenic tobacco.

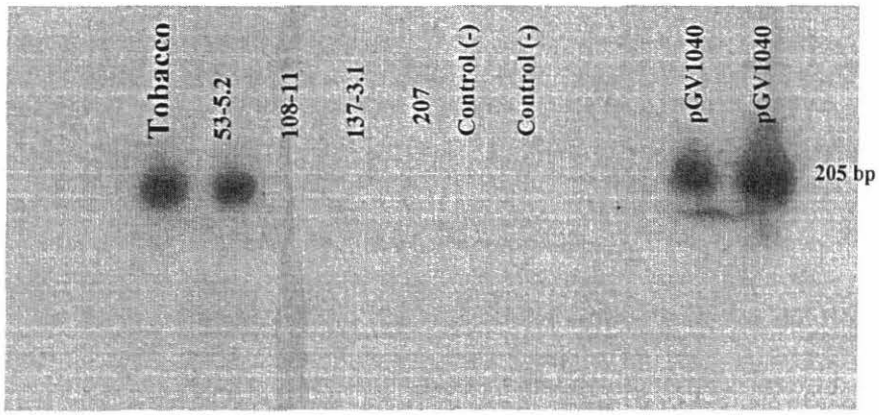

Figure 1. Southern blot of PCR products from transgenic cassava lines and tobacco-carrying pGV1040. The expected 205-bp band from the *bar* gene amplified from pGV1040 appeared only in tobacco and cassava line 53-5.2. One negative control corresponds to the nontransgenic cassava clone M Per 183.

In vitro leaf-disk cultures showed total necrosis in negative controls of cassava and tobacco, while transgenic tobacco and line 53-5.2 survived in medium with PPT (8 mg/l) (Figure 3). Leaf disks from 53-5.2 remained green but did not grow, while tobacco disks increased in size. This experiment agreed with the detection of RT-PCR products from the *bar* gene in both transgenic plants.

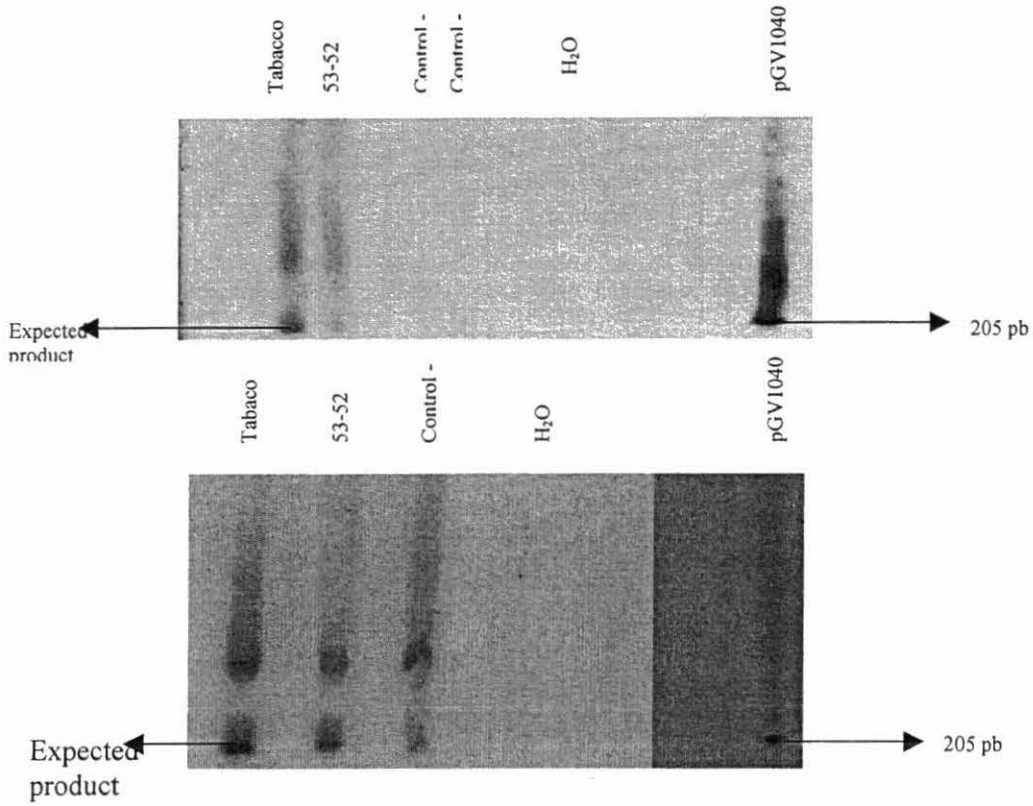

Figure 2. Two independent Southern blots of PCR products from cDNA (RT-PCR), probed with the *bar* gene, for transgenic cassava and tobacco. There are nonspecific hybridizations above the expected band.

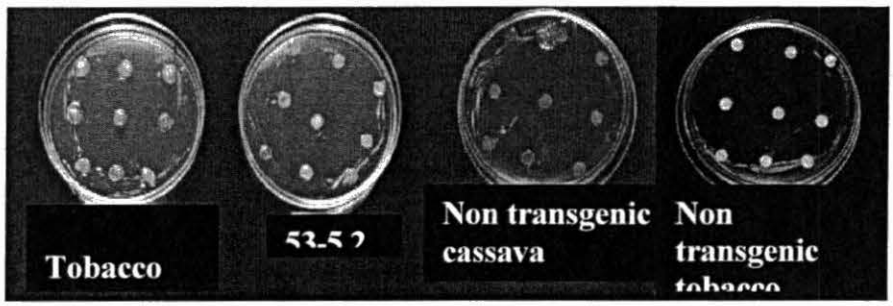

Figure 3. Leaf disks from transgenic and nontransgenic plants cultured onmedium containing 8 mg/l of PPT. Transgenic tobacco disks remained green and grew. Similarly, leaf disks from 53-5.2 also appeared greener than nontransgenic controls.

Spraying with 8 mg/l PPT had no effect on plants (not shown). Controls and transgenics did look similar. At 200 mg/l PPT, transgenic and nontransgenic cassava still looked similar. Both plants lost most of their leaves although they recovered with time (the shoot apex continued producing

new leaves). At the same concentration, transgenic tobacco remained green, retaining all its leaves, while nontransgenic tobacco dried out and died (Figure 4). The difference between transgenic and nontransgenic tobacco was striking, indicating that the level of *bar* transcription detected with RT-PCR was enough to confer tolerance to the herbicide applied at this concentration. The same cannot be said for the transgenic line 53-5.2; the observed transcription did not seem to be sufficient to protect this line from the herbicide sprayed at this concentration. Finally, aspersion of PPT at 1500 mg/l (for field application) killed both cassava transgenic and nontransgenic plants and nontransgenic tobacco. The only one to survive was the transgenic tobacco plant (Figure 4) although its leaves turned yellow and the lower ones dried out and died.

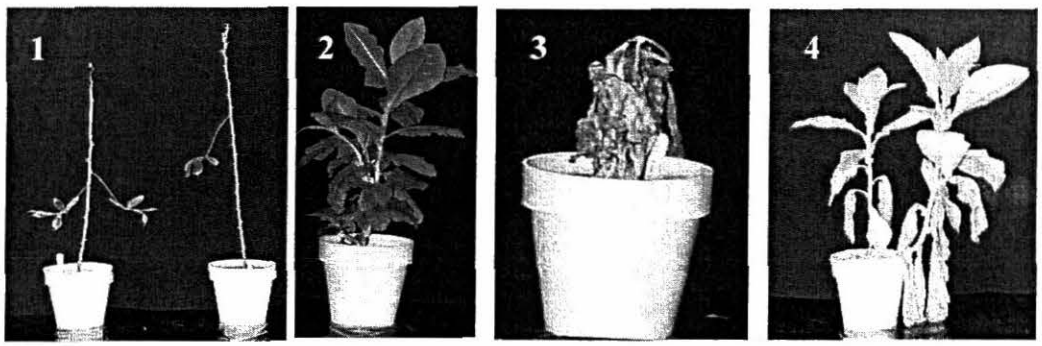

Figure 4. Spraying transgenic plants carrying the *bar* gene, and their respective controls with the herbicide Finale. (1) transgenic and nontransgenic (right) cassava plants sprayed with 200 mg/l PPT; (2) and (3) transgenic nontransgenic tobacco plants, respectively, (2 and 3 were sprayed with 200 mg/l PPT); (4) transgenic tobacco sprayed with 1500 mg/l PPT.

In conclusion, the transgenic cassava line 53-5.2, derived from clone M Per 183, contains and transcribes the *bar* gene although this expression is not enough to confer tolerance to the herbicide Finale (200 mg/l PPT). Transcription of the *bar* gene in this line seems to be enough for leaf disks to survive in vitro on medium containing 8 mg/l PPT.

Future Activities

- One last test will be performed with cassava line 53-5.2. It will consist of rooting cuttings from *in vitro* plants on media containing PPT, at concentrations from 4-16 mg/l.
- Similarly, one last Southern blot analysis of genomic DNA will be performed to detect the *bar* gene inserted in 53-5.2 and tobacco.

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2.1.5 Production of waxy cassava starch via the down regulation of GBSSI gene

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Introduction

Higher incomes from cassava in marginal areas of the developing world where the crop is generally found will require the industrialization of the crop and the development of novel industrial products from cassava with the aid of biotechnology. There are several novel products that can be produced from cassava, including modified starches such as 100% amylopectin or 100% amylose starches, from the down regulation of the granule-bound starch synthethase (GBSS) gene or the starch-branching enzyme (SBE) gene. Industrial applications of either pure amylopectin or pure amylose starches, such as the production of high-value biodegradable polymers from pure amylose starches or the use of 100% amylopectin in thickeners, pastes and glues, have a market with unlimited growth potential.

With funds from the Colombian Ministry of Agriculture and Rural Development, a project has been initiated to engineer industrial varieties genetically with an antisense construct of the GBSSI. GBSS, which is the predominant starch synthase gene, catalyses the conversion of ADP-glucose to amylose through the linkage of an ADP glucose to a preexisting glucan chain. Antisense disruption of the GBSSI gene has been employed to create potato transformants with 70-100% amylopectin via the down-regulation of the GBSSI gene (Salehuzzaman et al., 1993).

Materials and Methods

Isolation of a cassava GBSS cDNA clone. More than 87, 000 clones of a cassava root and leaf cDNA library cloned in the vector pCMV SPORT (GIBCO BRL Inc., USA) was gridded onto high-density filters (Mba et al., 2000, unpublished data). The library was screened using a potato GBSS cDNA clones, a gift from Dr. Christine Gebhardt (Max Planck Institute, Cologne, Germany). The potato GBSS gene was labeled with [^{32}P] dATP by random primer labeling and hybridized overnight to the cDNA filters according to standard protocols for Southern hybridization used in cassava (Fregene et al., 1997). The filters were washed twice with 2X SSC + 0.1% SDS at 60°C for 5 min, and autoradiography was at –80°C using 2 intensifying screens.

Construction of transformation cassettes. Primers were designed from published sequences of a full-length cassava cDNA of the GBSSI gene (Salehuzzaman et al., 1993) that incorporate *BamHI* and *XbaI* restriction enzyme recognition sites to enable subcloning of the cDNA clone in the antisense orientation into the multiple-cloning site (MCS) of the vector pRT101. The primers were used to amplify the cDNA clone obtained above, and the PCR product was cleaned using the QIAGEN PCR Clean Up Kit (QIAGEN Inc., Los Angeles, CA), digested with the appropriate enzymes. A 2.1kb *BamHI/XbaI* fragment was subcloned in the sense and antisense between the 35S promoter and the 35S polyadenylated terminator region of vector pRT101, a gift from Dr. Ryohei Terauchi, Iwate Biotechnology Research Center, Kitakami, Japan. The 35S promoter, GBSS gene in antisense orientation and the termintor region were excised using the restriction enzyme Hind *III*, separated on a special gel, Symergel (Diversified Biotech Inc., USA), eluted and cloned into the Hind *III* site of the binary vector pBIG101 having the GUS-intron and *nptII* reporter genes, a gift from Dr Richard Sayre, Ohio State University, Columbus.

Results and Discussion

Three GBSS cDNA clones obtained from screening the cassava library were sequenced, and one was found to be a complete cDNA clone. The cDNA clone has the ATG start codon 81 bp down stream from the beginning of the cDNA sequence and a stop codon about 100 bp from the poly-A tail. PCR amplification with the designed primers yielded a fragment about 2.1 kb in size that corresponds to the full-length GBSS cDNA clone (Figure 1).

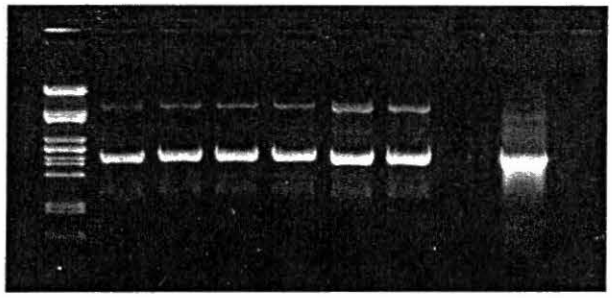

Figure 1. PCR amplification of the GBSS cDNA clone using primers designed to introduce restriction enzyme sites at the ends of the gene. The first lane on the right is molecular weight marker Lambda DNA, digested with *Hind III*, the next six lanes are PCR amplification of the GBSS gene, and the last lane is a control, PCR product of the GBSS potato gene.

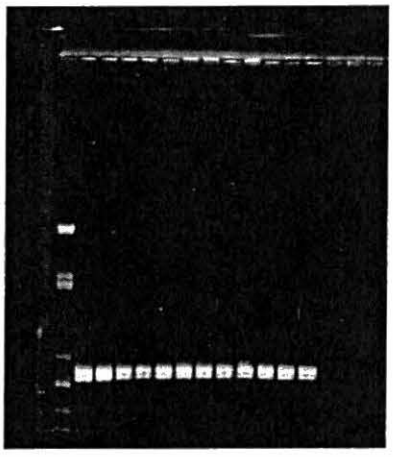

Figure 2. *Hind III* digested pRT101 plasmid containing the cassava GBSS gene in antisense orientation. The fragments of about 2.7 and 2.6 kb in size, respectively, represent the GBSS gene flanked by the 35S promoter and the polyadenylated terminator sequence and the rest of the pRT101 plasmid.

The resulting PCR fragment was digested with *BamHI* and *XbaI* restriction enzyme digestion and cloned into the MCS of pRT101. The GBSS gene, promoter and terminator sequences were excised with *Hind III*, and the two resulting fragments (sizes 2.7 and 2.6 kb) were separated by electrophoresis (Figure 2). The bigger fragment was eluted and cloned into the *Hind III site* of pBIG101. This is the construct that was used in the particle gun and *Agrobacterium*-mediated transformation.

Future Activities

- *Agrobacterium* transformation and regeneration of the industrial cassava cv. *Reina* with the antisense construct of GBSSI cloned in pBIG101.
- *Genetic transformation*. Genetic transformation will be done by particle bombardment and *Agrobacterium* transformation of Friable Embrygenic Callus (FEC) cultures of four cassava clones: MCol2215, CM3306-4, SM1219-9 and MNig11; and if FEC is available, the clone Reina as well.
- The first reporter gene assays (*gus* assays) with FEC are expected at the end of Dec. 2002. Once transformed calli have been revealed to have stable incorporation of the construct, regeneration of the transgenic calli will be initiated.

Training Component

The project is training a Colombian undergraduate project student (Gina Jazbleidi Puentes, Universidad Nacional-Palmira) in the tools and methodology of genetic transformation in cassava.

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2.1.6 RHBV (Rice Hoja Blanca Virus) nucleoprotein gene expression and its association with RNA mediated cross protection in transgenic rice

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Introduction

Rice hoja blanca virus (RHBV) is a major virus disease of economic importance affecting rice in northern South America, Central America and the Caribbean. Previous reports described the generation and selection under field conditions of transgenic RHBV resistant Cica 8 plants carrying the nucleoprotein (RHBV-N) viral gene. The transgenic RHBV resistance encoded by the RHBV-N gene appears to be RNA mediated, where plants do not expressed the transgenic protein but low levels of RNA (Lentini et al., 2002). One potential mechanism underlying the transgenic resistance is through gene silencing.There is increased evidence that transgene expression in plants is characteristically unpredictable, and depends on many factors, including the position of integration, the number of transgene copies and the structural integrity of the integrated DNA. Transgene silencing can occur at the transcriptional or post-transcriptional level. DNA methylation is often associated with transgene silencing, although it is presently unclear whether methylation causes

silencing or is a consequence (Ingelbrecht et al., 1994; Fu et al., 2000). Last year, we reported the evaluation in the field for two semesters of transgenic Cica 8 lines were forty five entries derived from line A3-49-60-12-3-3 were highly resistant showing scores 1 to 3. The transgenic resistance is stably inherited in crosses with other varieties (Annual Report 2001), and currently F_4 generation plants are being evaluated for yield potential and other agronomic traits to incorporate the best lines into breeding. This year we report the studies directed to understand the resistance mechanism underlying RHBV transgenic resistance via analyzing the RHBV-N gene expression in transgenic resistant plants.

Materials and Methods

Southern blot analysis. Two transgenic Cica 8 lines with known resistance (A3-49-60-12-3-57 resistant, and A3-49-60-12-3-31 susceptible) were selected. Genomic DNA was isolated from young leaf tissues (McCouch et al., 1988), and 20 µg DNA were digested with restriction endonucleases (Bam HI and KPN I), restriction fragments were resolved through 0.8% agarose gels, transferred onto Hybond-N$^+$ membranes (Amersham), and hybridized with Alpha-[^{32}P]-dATP-labeled hybridization probe specific to the RHBV-N gene prepared using the multiprime DNA-labeling system (Amersham) (Sambrook et al., 1989).

DNA Methylation Analyses – PCR Technique. Total genomic DNA was extracted at 20 days after germination from individual plant using a Dnasy plant mini kit (Qiagen), and 200 ng of DNA was digested for 5 hr at 37°c with 30 units of restriction endonucleases methylation sensitive or insensitive. Two sets of primers were used. The first set amplified the complete RHBV-N coding region. The second set amplified a 2.0 Kb fragment including the whole transgene, from the promoter to the terminal region (35S promoter-RHBV-N coding region-nos terminal) (Figure 1). All PCR products were separated by size on a 1.5% agarose gel stained with ethidium bromide. The fragments were transferred onto Hybond N$^+$ membranes (Amersham). The probe was synthesized by random-primer, and labelled by nick translation, in the presence of alfa^{32} dATP. Hybridization and high stringency washing were carried out according to standard procedures (Sambrook et al.,1989).

Results and Discussion

Southern blot analysis. The Southern analysis of genomic DNA showed multiple N gene fragments larger than the 1.4 kb expected size indicating that the transforming DNA did not integrate as a complete unit in the A3-49-60-12-3-3 line . The multiple banding patterns for the N gene suggest integrative fragmentation and rearrangements. The complex multiple banding pattern has been inherited through seven generations of selfing suggesting the integration at one locus. The detection of the corresponding 1.4 kb N sequence by PCR and RT-PCR, suggests the presence of at least one copy of the full length N gene sequence and the lack to resolve the corresponding 1.4 kb N fragment by Southern appears to indicate the lost of a restriction site(s) which could be due to methylation at one or both Bam HI/Kpn I flanking sites of the N gene sequence. Bam HI and Kpn I are inhibited by methylation of the same cytosine. Additionally, Kpn I is also inhibited when methylated at the adenine. Bam HI does not cut GGAT^{m4}CC; GGAT^{m5}CC; and GGAT^{hm5}C ^{hm5}C . Kpn I does not cut GGT^{m6}A^{m5}CC; and GGTAC ^{m4}C. If one assumes that the Kpn I and Bam HI sites are methylated, a tandem direct repeat is predicted at 5.4 and 5.9 kilobases, and tandem inverted repeat is predicted at 5.4 and 7.4 kilobases, respectively (Lentini et al., 2002).

Methylation Analyses – PCR Technique. Polymorphism was observed between the transgenic resistant and susceptible line sister lines when genomic DNA was digested with methylation

sensitive or insensitive restriction enzymes (Hae III, Bg II, PstI and Dpn I) and then amplified by PCR (Figure 2A). These results suggest possible differential DNA methylation between these two lines. Progeny plants derived from each of these transgenic lines need to be evaluated to elucidate if these polymorphic restriction pattern is reproducible and associated with the resistance/ susceptible trait. The original plasmid DNA was also analyzed following the same procedure. As expected, when restriction enzymes cutting inside the RHBV-N gene were used (Bam HI, Bg II, Pst I) and then the plasmid –DNA was PCR using a set of primers flanking the RHBV-N coding region (sk and RHBV 10, Figgure 1) no amplified RHBV-N fragment was resolved (Fugure 2B). In contrast, when plasmid –DNA was digested with enzymes no cutting the gene cassette (ClaI) or cutting outside the PCR amplified region (Eco RV and SphI), the complete RHBV-N was amplified using the same set of primers (Figure 2B). However, when restriction isoschizomer enzyme pairs (Mbo I and Sau3aI, Figure 1) were used a differential amplification pattern was noted (Figure 2B). Mbo I cut the methylated séquence GATm5C and not cut the Gm6AT. Sau3A1 no cut GATm5C but cut Gm6AT. No plasmid-DNA digestion was obtained when Mbo I while Sau3AI did cut the DNA. These results suggest a possible methylation at the adenine. It is important to determine the level of methylation of the plasmid-DNA sequence used for the transformation in order to understand better the potential methylation pattern in the RHBV-resistant/ susceptible transgenic rice plants. Fu et al. (2000) used a similar strategy in rice and identified at least three distinct types of silencing effects associated with different methylation patterns, including a novel form of transcriptional silencing involving methylation of cytosine residues only at non-conventional sites in the coding region.

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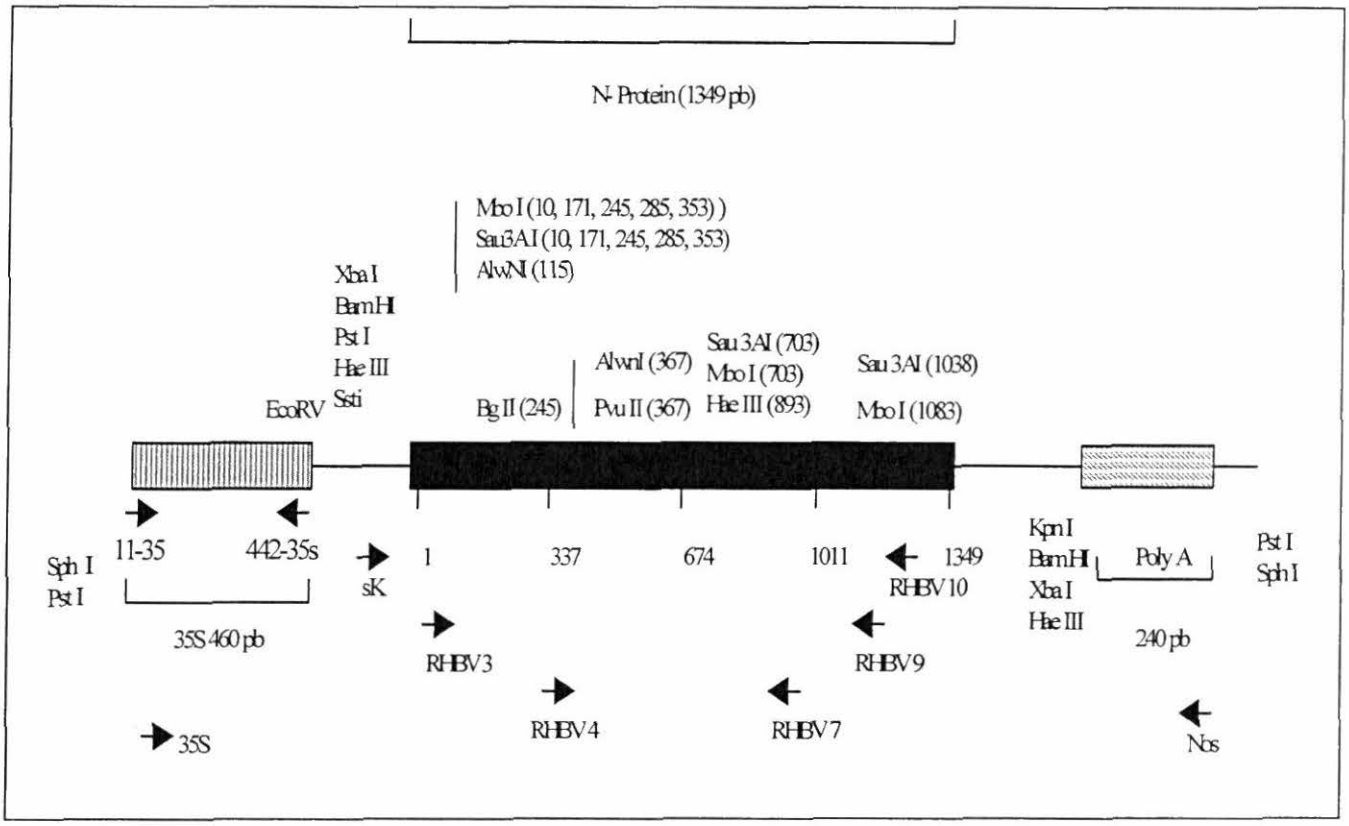
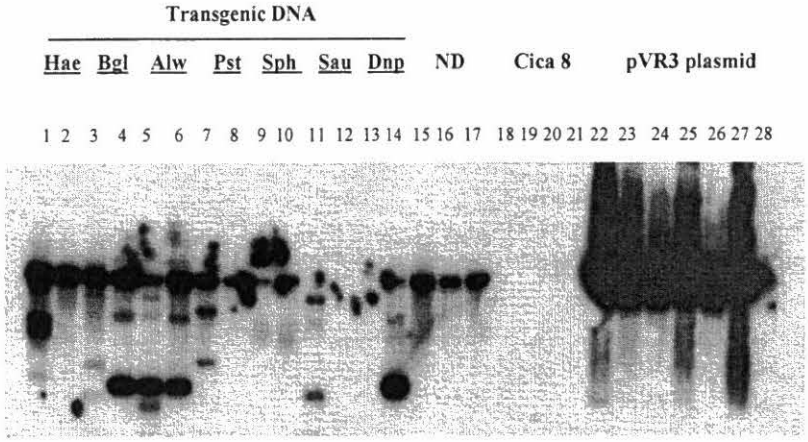
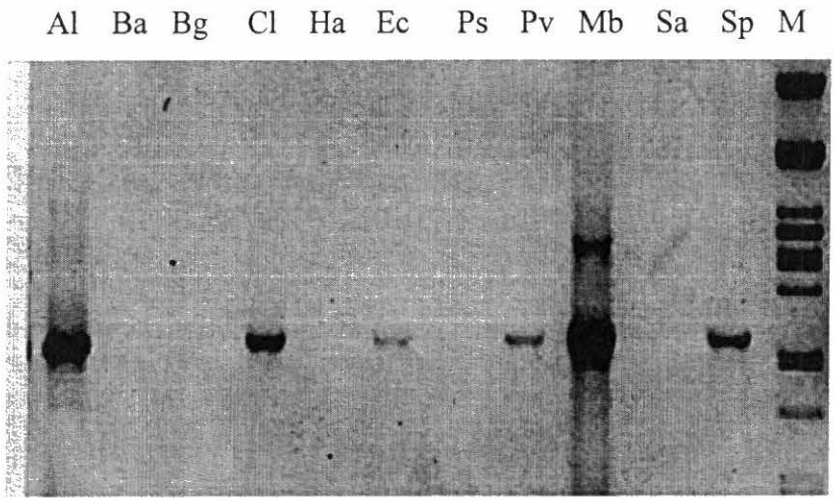


Figure 1. Map of restriction showing the RHBV N gene. Using the restriction sites Kpn I and Sst I, the entire 1.4 kb inserted in the sense direction between the cauliflower mosaic CaMV 35 S promoter and the NOS polyadenilation. In detailed show direction, sites restriction and primers used for amplification of the region codificant, promotor (11-35; 442-35S) and all cassette (35S, Nos).



(A)



(B)

Figure 2. Southern blot analysis of DNA first digested with methylation sensitive or insensitive restriction enzymes cutting inside or outside the RHBV-N gene sequence, and the amplified by PCR.

(A) Genomic DNA of lines A3-49-6012-3-57 (resistant) and A3-49-60-12-31 (susceptible) was first digested with either of one these enzymes (Hae II, Bgl II, AleNI, PstI, SphI Sau3A1, or DpnI) or not digested and then amplified with a set of primers to amplified the complete RHBV-N gene sequence including the promoter and terminal region of the gene. Lanes 1,3,5,7,9,11, and 13 correspond to A3-49-6012-3-57 DNA. Lanes 2,4,6,8,10, 12, and 14 correspond to A3-49-60-12-31 DNA. Lanes 15-17 correspond to non-digested transgenic DNA. Lanes 18-21 correspond to non-transgenic Cica 8 control. Lanes 22-28 correspond to the pVR3 plasmid.

(B) Plasmid *pVR3* DNA was first digested with restriction enzymes (Al) Alwn1; (Ba) Bam HI; (Bg) Bgl II; (Cl) Cla I; (Ha) Hae III; (Ec) EcoRV; (Ps) Pst1; (Pv) Pvu II, (Mb) Mbo I, (Sa) Sau3A1, (Sp) Sph I, and then amplified by PCR. M. Ladder Marker λ.

2.1.7 Characterization of transgenic rice containing the RHBV non-structural 4 (NS_4) gene from the RNA 4

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Introduction

The genome of the Rice Hoja Blanca Virus (RHBV) consists of four species of Single Strand RNA (ssRNA) designated RNA 1, 2, 3 and 4. The RNA 4 encodes a major non-structural protein (NS_4), which accumulates in the tissues of plants infected with the RHBV. The differential plant-insect NS_4 expression, and the similarity of NS_4 sequence with well characterized helper proteins described for other insect-transmitted viruses, suggest that NS_4 might be involved in the RHBV transmission from the plant to the plant-hopper, or in the virus movement from cell to cell. The main goal for the expression of the RNA 4 in transgenic rice is to determine the function of the major NS_4 protein and study the potential for a novel and different method of producing viral resistant plants. Last year, we reported the transformation efficiency and recovery of transgenic indica varieties and identified 55 transgenic plants carrying the NS_4 sense and anti-sense orientations by Southern analysis (Fory et al., 2001, CIAT SB-2 Annual Report). The analysis for the gus-intron, hpt, and NS_4 transgenes, RT-PCR and Northern analyses for the NS_4 of the first generation of transgenic (T_1) plants derived from selfing of the original transformed lines (T_0 generation) and identified as transgenics by Southern blot, indicated that not all T_1 plants inherited the three transgenes. These molecular analyses suggest that in some of the lines the NS_4 gene may had been integrated in a separate locus, allowing the elimination of the antibiotic resistance and/or marker genes through sexual crossing in advanced generations. The initial evaluations of RHBV resistance in the transgenic plants showed that most T_1 plants from transgenic Palmar line 4 transformed with plasmid pIC004 showed intermediate levels of resistance (Fory et al., 2001, CIAT SB-2 Annual report, 2001). In this report we present the results for the evaluation of RHBV resistance in the first generation of transgenic Cica 8, Palmar, Cimarron, Fedearroz 50, Nipponbare and the breeding line CT11275 plants transformed with five constructs (pIC002, pIC004, pIC007, pIC008 and pIC009), which differ in the NS4 sense and anti-sense orientation, and the promoter sequence (35S CaMV promoter Vs maize ubiquitin promoter).

Materials and Methods

Rice Genetic Transformation. The genetic transformation was conducted following the procedure described in Fory et al., (CIAT SB2 Annual Report 2001), using the plasmid constructs described in Tabares et al. (CIAT SB2 Annual Report 2000).

Molecular Analyses of the Transgenic Rice Plant. Southern blot, PCR, RT-PCR, and Northen analysis were conducted as described in Fory et al., (CIAT SB2 Annual Report 2001).

RHBV resistance assays. Fourteen independent events of transgenic lines represented by 16-18 plants each per line were evaluated for RHBV resistance as described in Fory et al. (CIAT SB2 Annual Report 2001). In all cases, only transgenic plants showing non-rearranged single integration NS4 gene copy by Southern blot were selected for RHBV resistance evaluations. Cica 8 plants were inoculated at 15 days after planting, and Plants at 13 days after planting since the non-transgenic

plants of this variety show intermediate RHBV resistance. Evaluations were conducted once a week starting 5 days after removal of viruliferous insect vectors, up to 54 days after inoculation.

Results and Discussion

Cica 8 transgenic plants generated using the constructs pIC002, pIC007 and pIC009 were susceptible to the RHBV. Between 69 to 100% of the plants showed symptoms on more than 50% of the leaf area. The reaction to the virus was similar to that observed for pIC002-12 line, used as the susceptible control. In contrast, Cica 8 line 9 containing the anti-sense NS4 gene from plasmid pIC008 showed about 30% of the plants with less than 10% of leaf area with disease symptoms (Table 1). In some of these plants, the integration of the NS4 gene has been demonstrated using Southern blot and PCR. Similarly, Palmar line 4 transformed with anti-sense NS4 gene derived from plasmid pIC004, showed a significant 63% reduction in the number of plants diseased respect to the non-transgenic control. In this case, 53 % of the transgenic Palmar line 4 showed little or no disease symptoms (<10% leaf area affected (Table 1). These results corroborate last year results where the same line showed 54% reduction in disease (Fory et al., CIAT SB-2 Annual report, 2001). While susceptible plants expressed low levels of RNA from either the sense or antisense-NS4 gene as indicated by RT-PCR analysis, resistant plants showed high expression of the NS4 gene as revealed by regular Northern analysis (Fory et al., CIAT SB-2 Annual report, 2001).

Table 1. RHBV resistance evaluations of T_1 transgenic plants derived from independent transgenic T_0 events *carrying the NS_4 sense or anti-sense gene.*

Genotype	Line	NS_4	Number Plants	% Plant Gus +	Leaf Area Affected (% Plants)	
					≤ 10	>10-100
Cica 8	pIC002-12	Sense del.	17	4	0	100
Cica 8	pIC007-4	Sense	16	ND	0	100
Cica 8	pIC007-5	Sense	16	ND	0	100
Cica 8	pIC009-16	Sense	16	ND	12	88
Cica 8	pIC009-4	Sense	16	ND	6	94
Cica 8	pIC008-8	@sense	16	ND	6	94
Cica 8	pIC008-9	@sense	16	ND	31	69
Cica 8	NT	None	16	-	0	100
Palmar	pIC007-1	Sense	15	ND	40	60
Palmar	pIC004-1	@sense	16	65	19	81
Palmar	pIC004-10	@sense	16	37	44	56
Palmar	pIC004-4	@sense	17	82	53	47
Palmar	pIC004-7	@sense	18	0	28	72
Palmar	pIC008-5	@sense	16	ND	37	62
Palmar	pIC008-6	@sense	16	ND	44	56
Palmar	NT	None	16	-	25	75

Cica 8 plants were inoculated at 13 days after planting in the greenhouse and Palmar plants at 15 days ND= Not determined, the plasmids: pIC007, pIC008 and pIC009 do not contain the gus gene. NT non-transformed

Future activities

- Molecular analyses of T2 generation derived from resistant T1 plants are currently in progress to identify plants containing and expressing the NS4 gene, and determine the gene inheritance
- Selected plants will be evaluated for RHBV resistance and resistant ones self cross to generated fixed lines
- Lines will be evaluated in the field

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2.1.8 Foreign genes as novel sources of resistance for rice fungal disease

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Introduction

The protection of crops against pathogens is of the utmost importance in modern agriculture. Major crop production losses are registered in rice each year due to various crop diseases. The fungi Rhizoctonia solani (causative agent for sheath blight), Helmithosporium, Rhincosporium, and Sarocladium have been implicated in causing important rice yield losses in the Southern cone of South America and increasing spreads have been reported in Colombia, Mexico and Venezuela. All rice varieties are susceptible to these diseases and there are no known sources of stable genetic resistance. An alternative approach for the management of the rice sheath blight disease is to introduce into the rice genome genes that encode proteins with antifungal activity. Datta et al. (2000) had reported the the introduction of chitinase gene in some indica rice cultivars, however transgenic plants were not stably resistant to sheath blight in advanced generations. The genus Phytolocca is the source of a number of proteins whose antiviral and antifungal properties have been analyzed. These antiviral proteins are designated PAP (pokeweed antiviral protein). PAPs inhibit the infection of a wide range of RNA and DNA viruses each representing a different plant virus group, such tobacco mosaic virus in plants (Chen et al., 1991) and animal viruses, including poliovirus (Ussery et al., 1977), herpes simplex virus in animals (Aron and Irvin, 1980) and human immunodeficiency virus (HIV) (Zarling et al., 1990). In recent years, there has been growing interest in using single-chain RIPs, such as PAP, as the cytotoxic component of immunotoxins targeted to cancer cells. The work by the Dr Tumer group of Rutgers University, USA shown that transgenic tobacco plants expressing PAP are resistant to virus from different groups (Wang, 1998). Mutated versions of PAP gene have potent antifungal activity (Zoubenko et al., 1997). Homozygous progeny of transgenic tobacco plants expressing these PAP genes displayed resistance to the fungal pathogen Rhizoctonia solani. Transgenic PAP potato showed protection against Phytophtora infestans, and transgenic PAP turf grass are resistant to various fungal pathogens. These results suggest the possibility of designing molecular strategies for incorporating fungal or viral resistance into rice. We are interested in constitutively expressing PAPy123 gene in transgenic plants in order to obtain sheath blight resistance in riceb. Last year we reported the generation several independent

transgenic events of indica varieties carrying the PAPy123 gene (Tabares et al., CIAT SB3 annual Report 2001), a new version of PAP gene with a deletion of 3 nucleotides. The southern blot analysis indicated the recovery of various transgenic lines showing the integration of at least one copy of the gene without rearrangements, and the Western analysis indicated that about 50% of the plants showed gene expression of PAP protein. This year we report the progress advancing plant generation with stable inheritance of gene expression of PAP and combining good agronomic traits, which will be evaluated for sheath blight resistance.

Materials and Methods

Molecular Analyses of PAPy123 Transgenic Rice Plants. Southern blot, PCR, RT-PCR, Northen and Western analyses were conducted as described in Tabares et al., (CIAT SB2 Annual Report 2001).

Evaluation of agronomic traits in the greenhouse. Agronomic traits were evaluated on plants grown to maturity in individual pots. The number of days to maturity was determined by scoring the number of days from sowing to flowering. At harvest time, the number of tillers and panicles per plant were counted. The percentage of fertility was determined. Plant height was measured from the base of the plant to the tip of the youngest fully expanded leaf. Non-trasngenic Palmar and Cica 8 varieties were used as controls.

Results and Discussion

Stability in inheritance and expression of PAPy123 gene in transgenic rice plants
Southern blot, PCR, RT-PCR, Northern, and Western analyses indicate the stable integration, inheritance and expression of the PAPy123 gene from T0 to T1 generation of some PAPy123 transgenic plants. Of the transgenic events evaluated, from 40% to 100% of the T1 plants inherited the PAPy123 gene (Table 1). Higher level of PAPy123 gene expression was detected in Palmar transgenic lines respect to the Cica 8 lines (Table 1).

Agronomic perfomance of PAPy123 transgenic T1 generation plants. No significant differences in flowering and plant height were noted between transgenic plants and the corresponding non-transformed controls (Table 2). Most transgenic plants showed a higher number of tillers than the non-transformed controls, especially those derived from Cica 8. Fertility was affected in some Palmar transformed lines, lines with high fertility level could be selected (Table 2). For example, PAPy123 transgenic Palmar line 31 and PAPy123 transgenic Cica 8 line 12 showed comparable or lower sterility percentages when compared with their respective controls, while showing relative increases in the number of tillers.

Table 1. hpt, npt II and PAPy 123 genes inheritance, and PAPy123 gene expression in T_0 and T_1 transgenic plants

Genotype	Plasmid	T_0 Plant	T_0 Plants					T_1 Plants		
			PCR [1]		PAPy123 gene[2]			PAPy123 gene[3]		
			Hpt	Npt II	PCR	S+	W+	% PCR+	RT-PCR	N+
Cica 8	NT-446	1	+	+	+	+	-	90	Nd	Nd
		3	+	+	+	+	-	90	Nd	Nd
		4	+	+	+	+	++	80	+	+
		8	+	+	+	+	Nd	89	Nd	-
		11	+	+	+	+	-	60	Nd	Nd
		12	-	-	-	Nd	-	40	Nd	Nd
		13	+	+	+	+	+	50	-	-
		14	+	+	+	+	+	50	-	-
		16	+	+	+	+	-	50	Nd	Nd
		25	+	+	+	+	Nd	60	Nd	Nd
		29	+	+	+	+	Nd	60	+	Nd
Cica 8	None	NT	-	-	-	-	-	-	-	-
Palmar	NT-446	4	+	+	+	+	++	40	-	-
		6	+	+	+	+	+	100	+	+
		8	+	+	+	+	++	90	++	+
		16	+	+	+	+	-	90	Nd	Nd
		22	+	+	+	+	++	50	++	+
		23	+	+	+	+	Nd	80	Nd	+
		27	+	+	+	+	++	67	++	+
		29	+	+	+	+	Nd	80	++	+
		31	+	+	-	+	Nd	100	Nd	Nd
		37	+	+	+	+	Nd	90	Nd	Nd
		38	+	+	+	+	Nd	80	Nd	Nd
		39	+	-	+	+	Nd	100	Nd	Nd
Palmar	None	NT	-	-	-	-	-	-	-	-

[1] PCR analysis to detect the transgenes in T_0 plants: hpt, hygromycin; nptII kanamicin.
[2] Evaluation of PAPy123 gene in T_0 plant, S = + Positive to Southern blot analysis. W= +positive to Western blot of PAPy123 gene.
[3]The PAP y123 gene was evaluated in 10 plant per each To mediate PCR. Some these plant were analyses mediate assay transcription RT-PCR and Northern blot, N= +positive to Northern blot of PAPy123 gene.
Northern blot and Western blot were scored based on the level of expression. (+) low; (++) intermediate; (+++) high. Nd= Not determined. NT = Non transgenic

Table 2. Agronomic traits of T_1 transgenic plants derived from different T_0 plants carrying PAPy123 gene in the glasshouse

Genotype	Plasmid	Plant[1]	DTH[2]	Plant Height (cm)	Number Tills	Number Panicle	% Sterility [3]
Cica 8	NT-446	1	104 bcde[4]	94 ab	24 ab	24 ab	11 ef
		3	103 bcde	94 abc	22 abcd	22 abc	11 ef
		4	105 b	87 abcde	21 abcd	20 bcd	10 ef
		8	105 bc	90 abcd	24 ab	23 abc	9 ef
		11	102 defg	92 abcd	21 abcd	20 bcd	15 ef
		12	102 cdefg	92 abcd	25 a	25 a	6 ef
		13	103 cdefg	90 abcd	24 ab	24 ab	7 ef
		14	103 bcde	86 cdefg	23 abc	23 abc	8 ef
		16	105 b	88 bcde	21 abcd	21 abc	8 ef
		25	110 a	80 hfg	22 abcd	22 abc	12 ef
		29	104 bcd	90 abcd	22 abcd	22 abc	15 ef
Cica 8	NT-446	Mean	104 bcd	90 e	23 a	22 a	10 ef
Cica 8	None	NT	103 bcd	96 e	17 d	17 cd	7 def
Palmar	NT-446	4	101 efg	80 hfg	22 abcd	22 abc	10 ef
		6	103 bcdef	66 I	17 cd	17 d	39 bc
		8	103 bcdef	79 hg	20 abcd	20 cd	66 a
		16	101 efg	79 h	25 a	23 abc	22 cde
		22	103 bcde	80 hfg	22 abcd	24 abc	31 bcd
		23	102 defg	90 abcd	21 abcd	20 bcd	13 ef
		27	100 fg	89 abcd	23 abc	23 abc	21 def
		29	102 defg	91 abcd	22 abcd	23 abc	49 b
		31	100 g	87 bcdef	23 abc	23 abc	7 ef
		37	100 fg	85 dfghe	23 ab	23 abc	12 ef
		38	102 defg	82 fghe	22 abcd	22 abc	35 bcd
		39	100 g	93 abc	18 bcd	17 d	5 f
Palmar	NT-446	Mean	101 bcde	83 bcde	22 abcd	21	26 cde
Palmar	None	NT	103 bcde	88 bcde	20 abcd	21 abcd	4 f

[1] Ten plants were evaluated per each T0 line, [2] Days to heading, [3] Percentage of sterility

3 Means followed by the same letter are not significantly different Ryan-Einot-Gabriel-Welsch multiple range test ($p>0.005$).

Future Activities

- Selection of T2 generation transgenic plants based of stable inheritance and gene expression, and good performance of agronomic traits.
- Sheath blight resistance evaluation with the collaboration of the IP4 pathology group (F. Correa) of the selected T2 plants.

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Activity 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base

Main Achievements

- A specific *Brachiaria decumbens* OMT gene involved in lignin biosynthesis was isolated from 10 day-old seedlings. This gene contains novel sequences in the coding region not yet reported for other monocots. Transgenic rice carrying this gene is being generated to evaluate the effect of this gene in then sense and anti-sense orientation.

- The growth rate of *in vitro* propagated soursop plants was doubled while the survival rate increased from 50 to 90% by using a combination of locally sourced materials as growth substrate. This substrate was made up of "cachaza" (filter cake), "carbonilla" (coal dust, both by-products of sugarcane processing), and pine bark chips, mixed in volumetric proportions of 3:1:1 parts, respectively. This improvement makes the propagation process more efficient and permits a faster production of clonal plants of this fruit tree species.

- The totality of the soursop trees propagated *in vitro* through micrografting that have been established in the field, several of which were already three years of age, continue to show a fast and healthy growth, and fruit production comparable in quality and quantity to trees propagated by conventional grafting methodologies. This indicates that the propagation process of *in vitro* micrografting is safe and do not produce off type plants with malformations attributable to genetic or epigenetic changes.

- The encapsulation-dehydration technology to cryopreserve the cassava core collection was developed

- The RITA system used for for cassava propagation was modified, adjusted and implementing with crops like *Brachiaria*, rice, tropical fruits and yams among others.

- A low-cost, in vitro propagation platform with different crops, integrating conventional, solid and liquid propagation systems was establishing.

- Results using Temporary Immersion System (RITA) suggest that the induction of embryogenic callus derived from zygotic embryos or anther culture is enhanced when a larger number of genotypes are tested, including recalcitrant *indica* types.

- Applying a pre-treatment of water stress or a high osmotic treatment during plant differentiation significantly enhanced plant regeneration from embryogenic rice callus. These results suggest an increased generation of plants from recalcitrant genotypes.

- This year a significan increased in plant regeneration from 20% to 60'% was achieved from naranjilla by optimization the *in vitro* culture conditions of donor plants. Comparative analysis of regenerated plants and in vitro propagated plants grown in the field indicated similar plant dvelopment for all traits. Plants derived from *in vitro* cultures flowered significantly earlier, at 90 days in contrast to 150 days, and formed fruits about 3 months earlier (at 160 days in contrast to 270 days) respect to standard crop. These results suggest that early flowering and fruits formation induced from *in vitro* derived plants could be an attractive advantage.

2.2.1 Induction of Friable Embryogenic callus (FEC) in cassava *Manihot esculenta* Crantz clones and plant regeneration using tecmporal immnersion

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Introduction

Although somatic embryos (SE) can be induced by the thousands, plant regeneration from either SE or Friable Embryogenic Callus (FEC) is quite low in cassava. Regenerating plants from FEC or organized embryogenic structures usually requires several media combinations, and efficiency may depend upon the clone used. Some authors report the use of semisolid and liquid media (Sofiari et al., 1998), or meM Bra ne rafts (Schöpke et al., 1997) to regenerate plants from FEC. More recently, temporal immersion systems such as RITA® have been adapted for massive, in vitro multiplication of cassava stem cuttings (Escobar et al., 2001), and SE of bananas (Alvard et al., 1993; Escalant et al., 1994), rubber (Etienne et al., 1997) and tea (Akula et al., 2000). Given the success of RITA in achieving high conversion rates of somatic embryos into plants in the last three of these species, it was decided to apply it to cassava plant regeneration from SE and FEC.

We report the establishment of FEC cell lines in cassava clones of commercial importance in Colombia. We also report improvements on embryo-to-plant conversion with the use of RITA in cassava plant regeneration from FEC. Improving plant regeneration from FEC is desirable for efficient genetic transformation of commercial cassava clones.

Materials and Methods

Plant material. We used ten cassava clones (M Col 2215, TMS 60444 or M Nig 11, M Tai 8, M Bra 383, CM 3306-4, CM 523-7, CM 4574-7, CM 6740-7, SM 909-25 and SM 1219-9) adapted to different edaphic and climatic zones in Colombia. Propagation on 4E medium was according to standard protocols (Roca, 1984). For the induction of FEC we used a modification of Taylor's method (Taylor et al., 1996). To improve plant regeneration we tested standard semisolid media and liquid media in RITA (Table 1).

Somatic embryo and FEC induction. Somatic embryos were induced from immature leaf lobes and/or axillary buds on semisolid MS4 medium. Then 30-day-old SE clusters were isolated from all clones and transferred to GD2-50Pi medium (Gresshoff & Doy, 1974). Clusters of SE were subcultured and purified every 20 days for 80 days on fresh medium. FEC induction was achieved in a growth chamber (Percival Scientific™, Model CU-32L; 28±2°C, 12-h photoperiod). The FEC obtained was alternatively purified by one growing cycle in SH-50Pi liquid medium (Schenk & Hildebrandt, 1972). After 30 days of shaking at 120 rpm, they were plated on GD2-50Pi semisolid medium to purify FEC.

Table 1. Composition of eight culture media used in FEC induction and plant regeneration of ten cassava clones.

	MS4	GD2-50Pi	SH-50Pi	MS2-NAA	MS2-AC	MS2-BAP[a]	MS2-GA_3[a]	4E
MS Salts	✓			✓	✓	✓	✓	✓
GD Salts		✓						
SH Salts			✓					
MS Vitamins			✓	✓	✓	✓		
GD Vitamins		✓						
B5 Vitamins	✓						✓	
$CuSO_4$ [2µM]	✓							✓
Sucrose	20 g/l	20 g/l	60 g/l	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l
2,4-D	4 mg/l							
Picloram		12.08 mg/l	12.08 mg/l					
NAA				1.86 mg/l				0.02 mg/l
GA_3							0.2 mg/l	0.05 mg/l
BAP						0.45 mg/l		0.04 mg/l
Hydrolyzed casein	50 mg/l						200 mg/l	
Activated charcoal					5 g/l			
Thiamine chlorhydrate								1 mg/l
M-inositol								100 mg/l
Gelrite™	2 g/l							
Agar		4.5 g/l						4.5 g/l
Agar:Gel-rite				3:1 (g/l)	3:1 (g/l)	3:1 (g/l)	3:1 (g/l)	

[a] Liquid media used in plant regeneration are the same as semisolid media, except they do not contain gelling agents and they were tested using RITA.

Plant regeneration. Plant regeneration from FEC was carried out on media MS2-NAA and MS2-BAP according to the scheme in Figure 1. FEC from clones M Col 2215, TMS 60444 and CM 3306-4 were selected to establish cell lines that provided enough material to make replicates for statistical analysis (χ^2 test, SAS version 6.0).

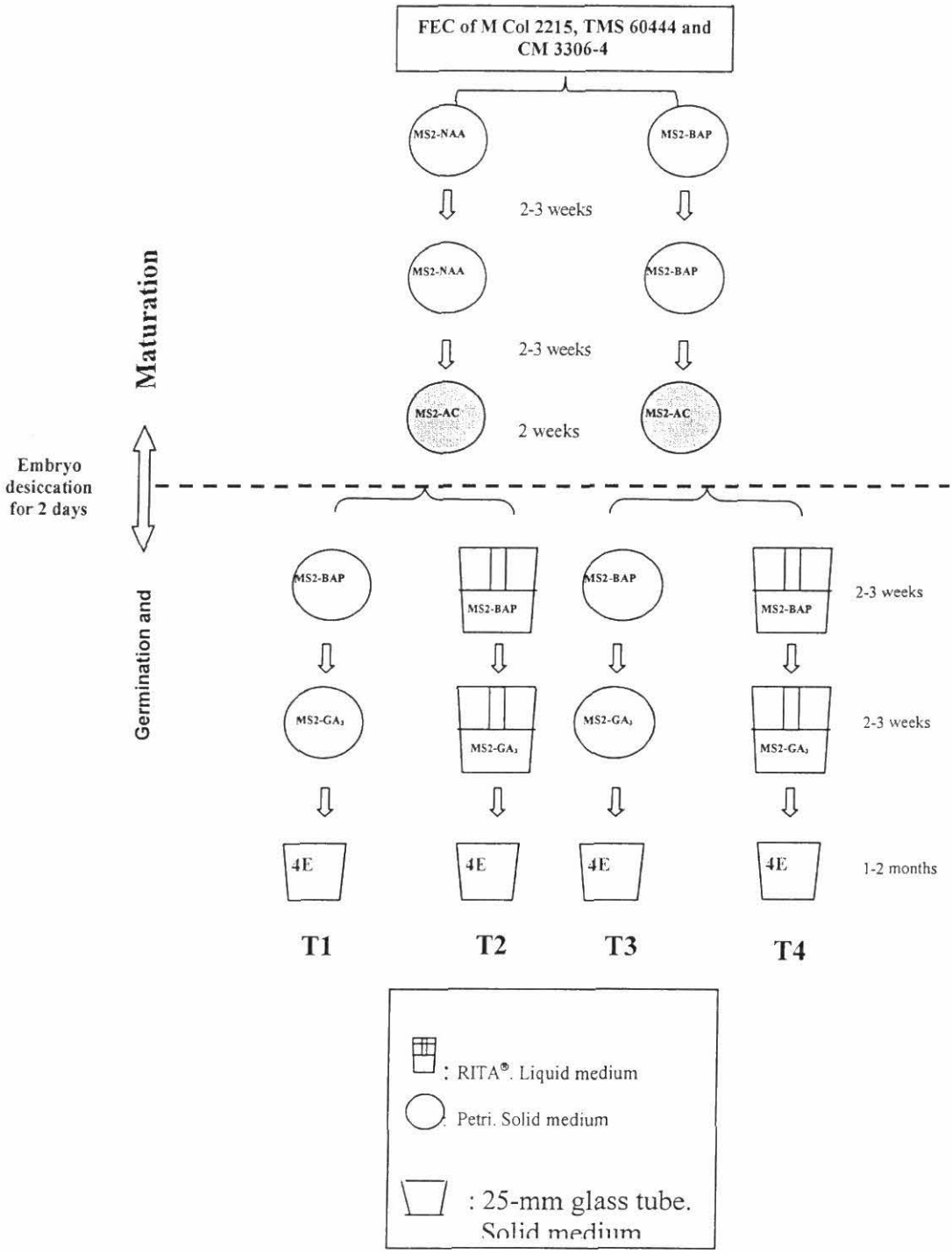

Figure 1. Maturation, germination and elongation of somatic embryos derived from FEC of three cassava cultivars. T1-T4 indicate different germination and elongation treatments using semisolid or liquid medium in RITA. For mediac conventions, refer to Table 1. Also shown is the culture duration (weeks or months) on each medium.

Three petri plates per clone, with nine FEC clusters each (130 mg FEC/petri; 14 mg/cluster of FEC) were set for embryo maturation. Embryos at the cotyledonary stage were placed on semisolid MS2-AC for 15-20 days to complete maturation. Mature embryos germinated and elongated on MS2-GA_3 medium, semisolid or liquid in RITA (Figure 1). Embryos contained in RITA vessels were left drying out (not inmersion) for two days before the bathing cycle started for 1 min every 6 h. Finally, the shoot apex of elongated embryos was excised and transferred to 4E medium for rooting and plant establishment. Culture conditions for plant regeneration on semisolid media were the same as for FEC induction and proliferation. For treatments using RITA, conditions were the same as for SE induction with a 12-h photoperiod ($44\mu mol^{-2}\ s^{-1}$ or $38\mu mol^{-2}\ s^{-1}$).

Results and Discussion

Somatic embryo induction. All cassava clones responded to the induction of SE, but to different degrees. Except for one genotype (M Bra 383), all clones produced SE from either leaves or axillary buds at percentages that ranged from 33.3-96% (Table 2). Leaves seemed to produce more embryos than axillary buds. Even when a clone produces very few SE, repetitive embryogenesis may be used to multiply SE and obtain sufficient explants for FEC induction; this was actually done when FEC was first reported in cassava.

Table 2. Induction of SE and FEC from ten cassava clones.

Cassava Clone	No. of leaves (l) or axillary buds (b) used for SE induction (left column), and avg % of explants producing SE		No. of SE clusters[1] (parentheses) used for FEC induction,and avg % of SE clusters producing FEC	Time (days) required for FEC appearance
	Number	%		
M Tai 8	(l)144	64.5	(135) 50.3[2]	24
	(b) 140	33.5		
CM 3306-4	(l) 55	83.6	(93) 37.6[3]	24
	(b) 82	50		
CM 523-7	(l) 87	55.1	(45) 40[2]	46
	(b) 64	62.5		
CM 4574-7	(l) 63	33.3	0	-
	(b) 111	54		
CM 6740-7	(l) 101	48.5	(41) 2.4[2]	25
	(b) 144	39.5		
M Bra 383	(l) 88	2.2	0	-
	(b) 84	13.1		
SM 909-25	(l) 42	62	0	-
	(b) 48	60.4		
SM 1219-9	(l) 69	33.3	(28) 53.5[3]	44
	(b) 47	6		
M Col 2215	(l) 60	96.0	(58) 13.7[3]	40
	(b) ND			
TMS 60444	(l) 60	38.0	(23) 4.7[3]	80
	(b) ND			

(1) SE clusters induced from leaves or axillary buds were mixed for FEC induction; (2) FEC was induced but did not proliferate; (3) FEC was induced and cell lines were established; (ND) not done.

FEC induction and proliferation. Induction of FEC also appeared to be genotype-dependant. It was possible to induce FEC in 7 out of the 10 clones studied. Although FEC induction was carried out at least twice for each clone and all clones produced SE, FEC could not be obtained in three clones (Table 2). Proliferation of FEC from clones M Tai 8, CM 523-7 and CM 6740-7 was hindered by tissue phenolization, which was not observed with other clones. It took 6-8 months to establish pure FEC lines—a period already long for in vitro culture, but within the range expected to establish lines (Taylor et al., 2000).

The development of FEC was asynchronous. Some clones produced FEC after only 24 days; whereas for others it took twice as long (Table 2). The amount of FEC obtained from each clone was also variable, from a few small clusters (case of TMS 60444) to abundant large clusters as in the case of M Col 2215 (Figure2). It was possible to establish FEC lines (actively growing in vitro, in liquid or semisolid medium) for clones TMS 60444, M Col 2215, CM 3306-4 and SM 1219-9. The most recent lines were initiated in Jan. 2002. These results suggest that, from the clones tested here and by other authors, FEC can be produced at different rates in a range of at least 21 cassava genotypes. Further research is needed however to fine tune the system for some genotypes, to reduce FEC in vitro culture time and to propagate and develop pure FEC lines that maintain their capability to regenerate abundant, normal-looking plants.

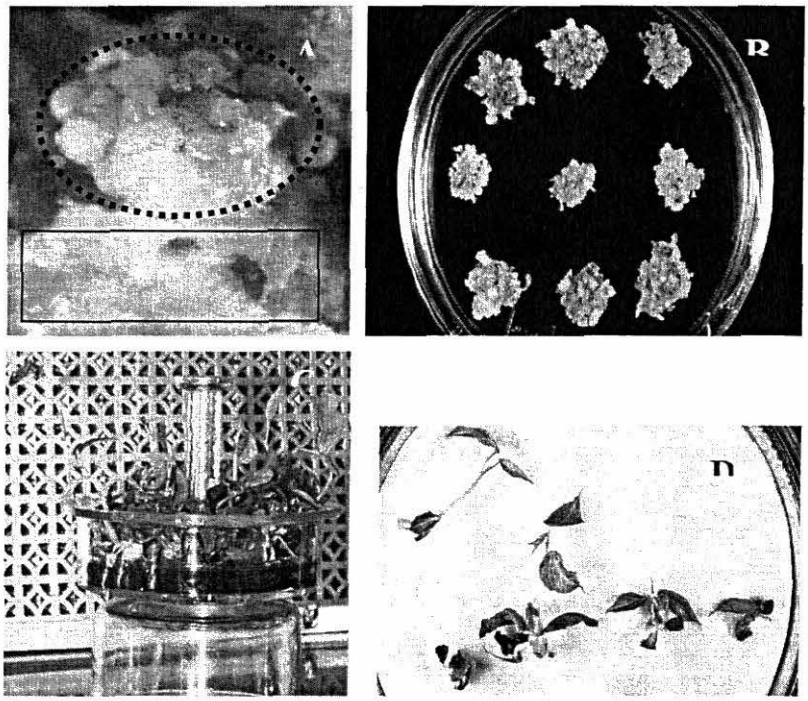

Figure 2. (A) Cluster of FEC from cassava clone M Col 2215 with several pro-embryogenic units (dashed oval) arising next to nonembryogenic callus (rectangle) on GD2-50Pi medium; (B) clusters of FEC maturing on medium MS2-AC; (C) RITA with germinated and elongated embryos from clone CM 3306-4 (MS2-GA_3 medium); (D) Plantlets obtained in RITA from cassava clone CM 3306-4.

Table 3. Response of FEC from three cassava clones to treatments for embryo-to-plant conversion (column 3) and *plant regeneration* (column 4) using hormones NAA and BAP in semisolid (S) medium or temporal immersion in liquid (L) medium with RITA. The total no. of FEC clusters per treatment (rows) was 27. Each FEC cluster weighs approx. 14 mg.

Cassava Clone	Media	Total No. Mature Embryos Recovered	Plants *Recovered*	Embryo-to-Plant Conversion Rate (%)	No.Plants Recovered/14 mg of FEC
CM 3306-4	ANA (L)	40	13	32.5000	0.4815
M Col 2215	ANA (L)	0	0	0.0000	0.0000
TMS 60444	ANA (L)	247	63	25.5061	2.3333
CM 3306-4	ANA (S)	19	0	0.0000	0.0000
M Col 2215	ANA (S)	9	1	11.1111	0.0370
TMS 60444	ANA (S)	190	24	12.6316	0.8889
CM 3306-4	BAP (L)	162	69	42.5926	2.5556
M Col 2215	BAP (L)	46	7	15.2174	0.2593
TMS 60444	BAP (L)	35	8	22.8571	0.2963
CM 3306-4	BAP (S)	148	12	8.1081	0.4444
M Col 2215	BAP (S)	43	4	9.3023	0.1481
TMS 60444	BAP (S)	29	2	6.8966	0.0741

Table 4. Effect of semisolid and liquid medium on the percentage of plants recovered from cassava clones M Nig 11, MCol2215 and CM 3306-4 after maturation, germination and elongation of somatic embryos obtained from FEC. Most plants, regardless of the clone or hormone, were recovered in RITA with liquid medium (highlighted).

	RITA with Liquid Medium		Semisolid Medium	Total Plants (%)
NAA	No. of plants	76	25	101 (49.75%)
	Row (%)	75.25	24.75	
	Column (%)	47.50	58.14	
BAP	No. of plants	84	18	102 (50.25%)
	Row (%)	82.35	17.65	
	Column (%)	52.50	41.86	
Total	No. of plants (%)	160 (78.82)	43 (21.18)	203 (100%)

Plant regeneration. From the genotypes tested for plant regeneration, TMS 60444 was the fastest in producing torpedo- and cotyledon-stage, green embryos, 8 days after being explanted onto medium with NAA. Clone CM 3306-4 followed (12-13 days after being explanted onto medium with BAP) and MCol2215 was the slowest (30 days after being explanted onto medium with BAP). Statistical analyses of the number of mature embryos and the number of plants regenerated from FEC clusters (Table 3) showed differences between clones, which depended upon the media (semisolid or liquid) and the hormonal treatment. However, if we compare the total number of plants obtained in liquid or semisolid medium (χ^2 test with 1 df, = 95%, p = 0,215), regardless of the clone or hormone, almost 79% of the plants regenerated from treatments T2 and T4 (Table 4 and Figure 2). In these treatments liquid media were used together with RITA to germinate and elongate embryos. RITA seemed to have a beneficial effect on plantlet development as they appeared more normal and vigorous than plantlets developed on semisolid media (Figure 2).

In summary, embryo-to-plant conversion from FEC in cassava, when compared with conventional systems on semisolid media, was significantly enhanced by the use of Temporal Immersion Systems such as RITA. We therefore recommend using RITA to test plant regeneration from somatic embryos in a wider range of cassava clones as an alternative to regenerating larger numbers of plants for genetic transformation experiments.

Future Activities

- Induce more FEC lines, at least four times a year, to replenish old cell lines and to establish new ones with new cultivars
- Implement cyropreservation for long-term storage of FEC lines

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2.2.2 Implementation of the encapsulation-dehydration method on the cassava core collection

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Introduction

Cryopresevation could be the safest way to maintain germplasm in the long term. In recent years CIAT developed the encapsulation-dehydration technique (Escobar et al., 2000) that made it possible to increase the percentage of recovered frozen material as compared with classical methods (Escobar et al., 1997). This new technique is not only less costly than the classical methods but also facilitates the handling of beads and drying steps.

To test the consistency of the methodology, we initiated activities to cover the entire core collection. This allowed us to have a general idea of how many cryopreservation response groups we have, and what kind of adjustments need to be implemented.

Materials and Methods

Methods have been described by Escobar et al. (2000) and Manrique (2000), and modifications have been implemented since then.

We have handled 245 clones from the core collection during the last 3 years. At present, we are still receiving more clones from the Genetic Resources Unit (GRU) to complete the collection. We initiated propagation schemes (3-4 cycles/clone) to complete at least 100-150 plants/clone.
Frozen and nonfrozen plants were planted in CIAT fields and harvested. Yield and root morphology (skin and pulp color) were compared. Leaves were also collected for isozyme and DNA analysis.

Results and Discussion

In 2002 we received 95 clones from the core collection of the GRU. We are maintaining 53% of this collection. Activities this year included a strong propagation scheme and cryopreservation methods (pre- and postfreezing management of tissues).

Preliminary data showed good shoot recovery across time, up to 9 mo, for clones M Cub 16, M Dom 4 and M Pan7. The maximum response observed on M Ven 90 was 10% after one month (Table 1). The initial response with control frozen tissues should be monitored in order to decide whether it is feasible keeping the tissues in liquid nitrogen for longer periods. We estimated that 30% should be the Minimum Shoot Recovery Percentage (MSRP) (Escobar et al., 2001). The lower initial MSRP observed in all clones was usually associated with the use of suboptimal tissues (Escobar et al., 2000). Only M Pan 7 showed an acceptable initial MSRP response after 1 h in liquid nitrogen.

Table 1. Preliminary observation of the response of 4 cassava clones under different conservation times and liquid nitrogen conditions.

Cassava Clones	M Cub 16		M Ven 90		M Dom 4		M Pan 7	
ConservationTime	% Viability	% Shoots	% Viability	% Shoots	% Viability	% Shoots	% Viability	% Shoots
CONTROL	0	0	0	0	23	0	89.26	30.3
1 month	83.8	32.8	60	10	92.9	82.9	90	60
3 months	70	56.6	16.6	6.6	80	65	93.3	37.7
6 months	92.6	41.1	32.5	6.25	93.9	39.09	93.6	61.1
9 months	87.5	75	16.65	0	92.5	63.3	93	40
First report	89.6	89.6	76.7	50	100	90	96.7	73.3

The procedure allowed us to standardize logistical aspects previous to the freezing step. Good-quality materials (proper age, appearance and shoot size) were used to initiate new experiments. That was why we used M Col 22 and M Per 436, which were in the high-response group as compared to M Ven 90 (the lowest response observed).

In all cases the materials frozen up to 12 months showed up to 80% recovery. The control was consistent in its behavior (Table 2). Differences observed between both experiments confirmed the importance of initial measurement and explant quality for continuing with freezing experiments. Despite the huge amount of labor involved in each experiment, it is better to discard those clones that do not reach at least 30% MSPR during the initial measurements. In Table 3, it can be observed that only 13.8% of the clones (9/65) did not reach 30% MSRP.

Table 2. Response of three cassava clones after four different conservation times.

Cassava Clone	M Col 22		M Per 436		M Ven 90	
Conservation Time	% Viability	% Shoots	% Viability	% Shoots	% Viability	% Shoots
CONTROL	88.1	88.1	100	87.5	88.85	88.85
1 month	100	100	91.65	78.75	95	95
6 months	96	96	100	83	95	95
12 months	100	100	96	92	92	80
First report	95	85.45	94.1	79.7	76.7	50

Table 3. Response of 65 cassava clones after being frozen in liquid nitrogen.

Cassava Clone	% Viability	% Shoot	Cassava Clones	% Viability	% Shoot
M Bra 356	96.3	92.6	M Col 474	100	56.25
CM 3306-9	55.5	18.15	M Col 590	100	57.7
M Arg 7	100	43.3	M Col 601	50	5
M Arg 9	100	85	M Col 912-B	100	70
M Arg 9	100	94.44	M Col 979	100	67.2
M Bol 3	100	25	M CR 1	81.25	62.5
M Bra 190	90.9	90.9	M CR 100	100	28
M Bra 315	100	88.8	M CR 18	92.9	64
M Bra 584	100	20.3	M CR 25	100	100
M Bra 674	100	58.56	M CR 65	100	90
M Bra 697	100	37.02	M CR 84	100	48.6
M Bra 730	94	46.1	M Cub 29	100	88.8
M Bra 781	100	80.6	M Cub 1	100	70
M Bra 435	93.3	45.2	M Cub 46	82.5	47.4
M Bra 534	100	20.8	M Cub 74	96.3	63.4
M Bra 73	93.3	49.1	M Cub 8	100	90
M Bra 77	100	100	M Ecu 144	100	43
M Bra 897	100	65.1	M Fji 6	93.9	81.9
M Col 1780	100	100	M Gua 44	91.9	55.2
M Col 1795	80.9	26.6	M Mex 49	100	100
M Col 638	93.3	33.3	M Mex 54	95.8	42.5
M Col 1055	67.7	36.6	M Pan 127	100	52.7
M Col 112	88.8	41.6	M Par 7	82.5	31.6
M Col 1186-A	96.6	96.6	M Per 184	100	58.1
M Col 1535	93.93	84.17	M Per 243	84.3	55.6
M Col 1736	76.38	47.2	M Per 333	100	60
M Col 198	100	15	M Ven 173	86.3	42
M Col 2212	96	7.8	M Ven 174	97.2	55
M Col 2409	100	44.24	M Ven 309	88.3	70.5
M Col 2493	37.2	86.9	M Ven 322	100	43.3
M Col 262	100	87.5	M Ven 61	100	42.7
M Col 317	95	65	SG 455-1	87.5	54.2
M Col 32	65.27	38.4			

Based on the skills and workforce capacity of our group, we did 8 repetitions per clone, with 10 shoots each. Treatments included one control (1 h under liquid nitrogen conditions), 3 different conservation times (1, 6 and 12 mo), and 4 extra cryo-tubes per clone for medium-term observations (24-36 mo). At present we are maintaining 200 clones under these conditions.

Eight isozyme systems (DIA, ACP, G6-PDH, GOT, IDH, MDH, SKDH and ß-EST) were used to compare cryopreserved and noncryopreserved clones (each clone had two lines in the gels, the first corresponding to *in vitro*, noncryopreserved plants and the second to cryopreserved clones, Figure 1). For M Bra 691 (first pair, line 1-2) and M Bra 542 (4th pair, line 7-8) the SKDH system showed differences in enzyme patterns, which were later accredited to human error (sample mislabeling or misplacing). The changes observed in both clones most probably did not correspond to the cryopreservation treatment. Ocampo and Hershey (1989) observed similar behavior due to mixing

materials in the field bank, using ß-EST system. We are now obtaining DNA for AFLP-fingerprint analyses.

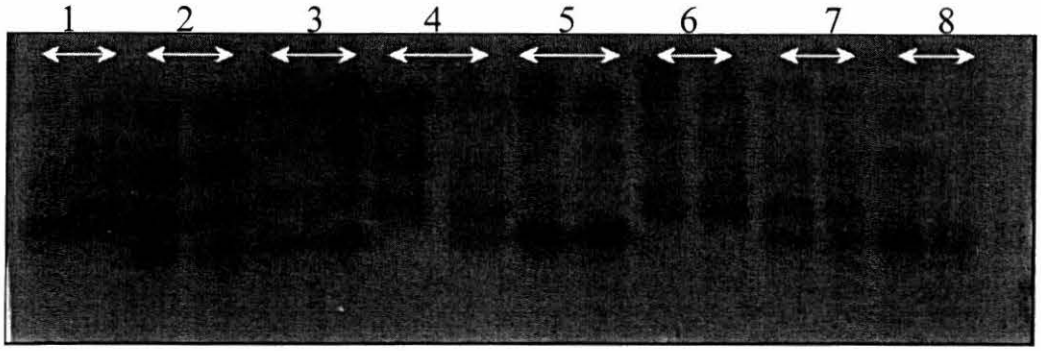

Figure 1. Comparison of eight cassava clones (in vitro and frozen) using SKDH. Sample: Young leaves of 6-month-old plants (Pair 1=Bra 691, 2=Bra 698, 3= Bra 759, 4= Bra 542, 5=Bra 769, 6= Bra 830, 7= Bra 881, 8= Bra 894).

Harvesting of the materials did not show yield differences between the in vitro and cryopreserved treatments. Morphological descriptors of the roots were consistent between treatments. As expected, mislabeled materials showed different color patterns (i.e., M Bra 542 and M Bra 691) (Figure 2).

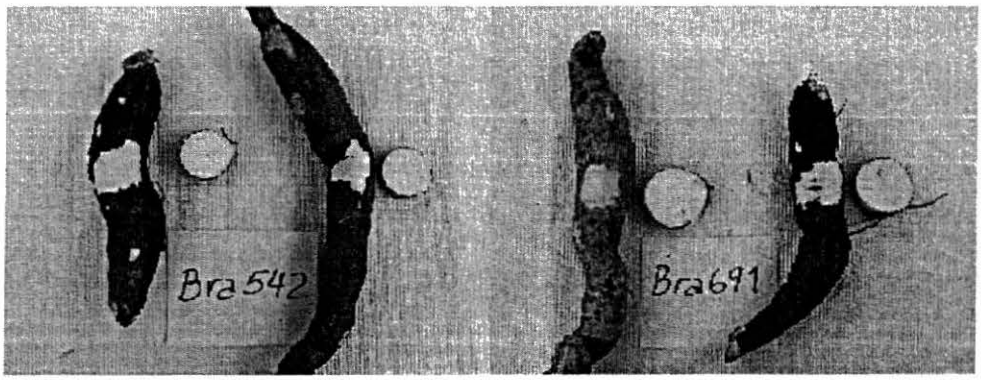

Figure 2. Morphological aspects of roots harvested from cryopreserved and non-cryopreserved cassava plants. The differences observed in the picture—for instance, skin color and root shape for clone M Bra 691—are most probably due to mislabeling of clones.

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2.2.3 Cassava propagation using low-cost *in vitro* propagation techniques and conservation of native varieties from Southwestern Colombia

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Introduction

In 2000, a group that includes a women farmer from Santa Ana (Cauca Province, Colombia), a local NGO (FIDAR) and CIAT developed a low-cost propagation system. Using local clone *Algodona*, we obtained a propagation rate (1:2-3) similar to that obtained with conventional solid media 4E (Roca, 1984).

Later on this method was implemented with other cassava clones. If farmers maintain their interest in cassava and the economic aspects are improved, the implementation of this method could support decentralized, local seed systems.

Materials and Methods

A total of 6061 plants from 6 clones (M Bra 383, CM 523-7, M Col 1522, HMC 1, CM 6740-7 and M Per 183) were produced at CIAT's pilot site and transplanted for the hardening phase to a rural greenhouse, in collaboration with local farmers from the community. These materials will be assessed in the field during the second semester of 2002 in order to collect data on plant development, yield and total management costs.

Different cassava clones were collected in several municipalities of Cauca, and data were gathered on them such as climate, soil conditions and topography of the collection sites; varietal characteristics and farmer uses. A total of 27 clones were identified, using the standardized morphological descriptors for cassava of the International Plant Genetics Resources Institute (IPGRI).

Two *in situ* seed banks were established with the stakes collected from the farmers' plots, using conservationist tillage practices and low levels of inputs. An additional set of these materials were used for an *in vitro* thermotherapy phase and for molecular analyses. The cassava clones sown in Perico Negro (Puerto Tejada, Cauca) during 2001 was harvested.

Results and Discussion

The very low prices of cassava roots over the last six months and the legal/illegal importation of cassava starch and derivatives have affected project activities because farmers do not consider cassava as an economic option. At present they cannot recover their investments.

When the greenhouse was built, the farm owner decided to improve her income growing pigs and chickens. These activities led to contamination, and all the material that the group had was lost. For that reason it was necessary to include other clones as well as implement a simple system to manage and control contamination; which involves periodic checks in LB culture medium. Different points and their coloring in the culture medium are used as indicators of the level of pollutants.

The key factor to ensure success is the management of *in vitro* plants during hardening. Different methods for ex vitro management have been published (Roca et al., 1984, Guo and Liu, 1994, Ng et al., 1994). We adapted a simple method that reduces costs and labor per activity. Based on the 6061 plants that the farmers received, the percentage of plant loss in the farmer-managed greenhouse hardening system is considered low (12.6%) because this was the first time that they had handled such a large lot of in vitro-produced materials.

Overall management of the *in vitro* cassava seed production project has allowed participating members to address issues such as education, health care (through food security efforts), community organization, improved agronomic practices and recovery of home vegetable gardens, among others, thus benefiting the entire community.

An international workshop was held at CIAT on in vitro cassava propagation and genetic transformation, with 38 participants including farmers, cassava program technicians from both the public and private sectors, and representatives of universities from Colombia, Brazil, Cuba, Ecuador and Venezuela. This activity was carried out with the collaboration of the Colombian consortium of researchers and small-scale farmers, Programa Colombiana de Biotecnologia Agricola (PBA), Cassava Biotechnology Network for Latin America and the Caribbean (CBN-LAC) and the system-wide Participatory Research and Gender Analysis (PRGA) as posthumous homage to Chusa Gines and Veronica Mera (Figure 1A). A local workshop on rapid propagation and pest management was carried out in Santa Ana.

Figure 1. (A) Participants in the pest management workshop and rapid propagation; (B) rapid propagation facilities in Santa Ana.

An experiment to compare *in vitro* material and stakes (CM 523-7 and M Bra 383) planted in the previous year in Puerto Tejada was harvested. When the control material (stakes) was harvested, strong FSD symptoms were observed. For that reason *in vitro* plants were not considered for the next cycle of rapid propagation. With support of the Virology group, we are initiating an indexing test using *Secundina*.

We are in touch with the Colombian Agency Servicio Nacional de Aprendizaje (SENA), Quindio and the regional cassava consortium, Consorcio Latino Americano de la Yuca (CLAYUCA) to obtain certified stakes to initiate the rapid propagation scheme (Figure 1B).

Materials that farmers had for field transfer could not be planted because a long dry season did not allow it. They could only use their water for household use, not for irrigation.

Conclusions

The workshop served as a space to share experiences in cassava propagation with different participating countries, analyze the problems encountered and discuss potential areas of collaboration.
It was possible to incorporate simple hardening systems that allow farmers to reduce planting losses.
Ideal propagation systems include low-cost and rapid propagation. With the former it was possible to certify material, with the latter, costs are reduced and farmers can maintain use of conventional stakes.
Despite the advantages of the *in vitro* system, it is necessary to introduce cultural practices and IPM activities. The Cauca area is under high pressure from the whitefly and viral diseases such as frog skin. This scenario means that the renewal of material needs to be done more frequently, and special care must be taken during the initial stake selection.
In view of the aforementioned situation, the group has shown interest in producing *in vitro* seed of other crops such as bananas and pineapple and reducing the work carried out with cassava until the market situation of cassava roots and starch improves.
Farmers have a reservoir of planting materials plot with six cassava clones that are important for the zone.

Future Activities

- Evaluate 6000 cassava plants produced *in vitro* in farmers' fields (San Rafael, Santa Ana Evaluate and Puerto Tejada) to produce high- quality planting materials and then increase stake production using the rapid propagation system (depends on water availability)
- Produce and return to farmers clean material of 27 local varieties
- Analyze costs and compare with other propagation systems
- Fingerprint the 27 clones collected using the AFLP molecular marker technique
- Write a paper that summarizes the project's experiences over two years in the use of *in vitro* techniques in farmer-managed cassava planting materials production
- Hold a workshop to diffuse experimental results on low-cost, rapid propagation and conservation activities using the farmer-farmer training technique

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2.2.4 Propagating commercial clones and transgenic cassava plants with RITA®

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Introduction

CIAT has developed a massive propagation system using RITA®. Vessels containing medium supplemented with TDZ were tested with 16 commercial clones (Escobar et al., 2000). We found that the propagation rate could increase 1:6-11 compared with 1:3 in conventional solid 4E medium (Roca 1984) and that it is clone dependent.

We also implemented RITA with transgenic lines. Once these plants pass the first small-scale trials and are ready for massive multiplication, it will be necessary to count on an efficient propagation system that guarantees high multiplication rates of desired planting material for field testing.

Materials and Methods

Ten cassava buds/container were grown on a medium supplemented with TDZ, with an immersion frequency of 1 min every 6 h and 5 min every 12 h.
Nine high-carotene content cassava clones were included in the propagation scheme.
The SB-1 project did meristem culture with 3 local clones. Initial proliferation on a solid 4E medium was implemented.
The Universidad de Sucre sent two *in vitro* yam materials. At the beginning, normal proliferation on solid media was implemented. Basal tissue used for the RITA treatment was used as the initial structure.

Results

We obtained the second highest propagation rate for the preferred local clone, M Col 1522 (known as *Algodona* in the Cauca area of Colombia). The clones, HMC 1 and CM 3306-19 had multiplication ratios of up to 8. (Table1). Indicator clone *Secundina* (M Col 2063) had a 1:8.7 propagation rate.

Table 1. Propagation rate of some commercial and local clones using the RITA system.

Clone	*Reps*	N	Tissue Recovered		Total	Propagation Rate
			Shoots	Buds		
HMC 1	1	10	51	59	110	11
HMC 1	2	10	57	43	100	10
HMC 1	3	10	80	97	177	17.7
CM 3306-19	1	10	36	51	87	8.7
CM 3306-19	2	10	38	40	78	7.8
M Col 1522	1	10	26	37	63	6.3
M Col 1522	2	10	80	83	163	16.3
M Col 1522	3	10	63	80	143	14.3

n = initial explants.

Local clones from the Colombian Northeast Coast were propagated using a medium supplemented with TDZ. Although the propagation rates did not different from solid conditions, there were numerous induced buds per cutting. This structure will enable us to increase the propagation rate in the next cycle (Table 2). Media with a high content of a stronger cytokinin induce bud activation, but do not allow their growth. Roca (1984) observed similar responses when his group established a cauliflower propagation scheme.

Some authors have referred to the potential use of high carotene-content cassava materials to reduce childhood blindness and Vitamin A deficiency. For that reason we introduced some experiments with nine clones with yellow roots. Some of them, especially CM 2772-3, could be released to the farmers in Putumayo. Clones M Bra-522 and M Bra-496 had the lowest response, similar to solid conditions (Table 2).

For transformation activities an efficient embryo-plant conversion system is necessary. RITA improved plant conversion up to 78.82% vs 21.18% on a solid medium. FEC conversion depends on the genotype and growth regulator used (Montoya, 2001). A medium supplemented with BAP gave a better response (41.38%) than when supplemented with NAA (37.44%).

Transformation events produce only a few plants, making it necessary to implement a propagation system to support small- to medium-scale trials in the greenhouse and then field testing. Three transformed lines were propagated on a solid medium and then put in RITA. Line 270400 gave the highest propagation rates (Table 3). These results should be considered as preliminary because no replications were done for lack of availability of initial stock.

Small farmers from the Colombian Atlantic Coast consider cassava, yams and plantains as their basic crops. In recent years the yam fields have been attacked by anthracnose, and some materials are being lost. The Colombian consortium of researchers and small-scale farmers, Programa Colombiana de Biotecnologia Agricola, (PBA) and the CORPOICA, the Colombian Agricultural Research Program organized an interdisciplinary team to focus research on this crop. Preliminary CIAT results showed propagation rates near 1:4-5. Further adjustments are necessary to achieve better results. Last month farmers sent us another 5 local yam clones for testing a new propagation system.

Two workshops to diffuse the method on in vitro propagation and the RITA system were held with PBA and CORPOICA staff.

Table 2. Propagation rate of three farmer-preferred cassava clones for the Northeast Coast of Colombia.

Common Name	n	Recovered Tissue		Total	Propagation Rate
		Shoots	Buds		
Por Encima	10	23	23	46	4.6
Por Encima	10	15	20	35	3.5
Por Encima	10	31	36	67	6.7
Por Encima	10	42	56	98	9.8
Yema de Huevo	10	25	25	50	5
Yema de Huevo	10	25	23	48	4.8
Ramirana	10	23	32	55	5.5
Ramirana	10	27	25	52	5.2

Table 3. Propagation rate of high-carotene content cassava materials (yellow-colored roots).

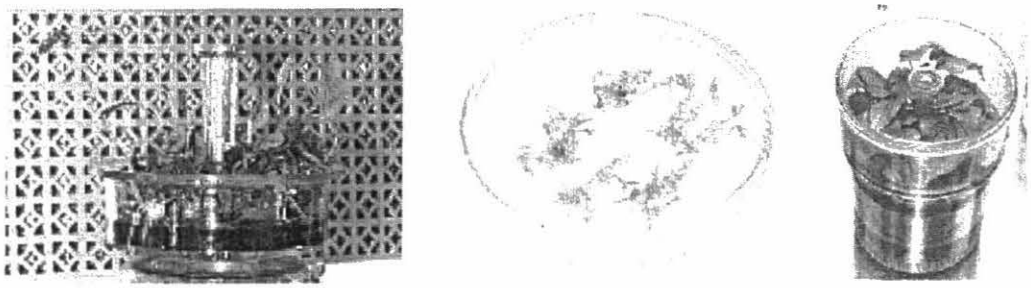
Clone	n	Recovered Tissue		Total	Propagation *Rate*
		Shoots	Buds		
M Col 2482	10	67	71	138	13.8
M Col 2482	10	50	9	59	5.9
M Bra 522	10	5	9	14	1.4
M Bra 522	10	18	19	37	3.7
M Bra 496	10	16	19	35	3.5
M Bra 496	10	23	15	38	3.8
M Col 2294	10	50	60	110	11
M Col 2294	10	59	59	118	11.8
M Bra 206	10	52	20	72	7.2
M Bra 206	10	34	40	74	7.4
M Bra 509	10	33	30	63	6.3
M Bra 509	10	60	60	120	12
M Col 2199	10	65	63	128	12.8
M Col 2318	10	56	70	126	12.6
CM 2772-3	10	61	41	102	10.2

Table 4. Preliminary results of propagation rates of transgenic lines.

Cell line	Reps.	n	*Tissue Recovered*		Total	Propagation Rate
			Shoot	Buds		
L.55	1	6	15	16	31	5.2
L.55	2	5	20	17	37	7.4
L.270400	1	5	21	35	56	11.2
L.5352	1	10	26	26	52	5.2

Table 5. Yam propagation rate using RITA.

Local Name	Medium	n	*Structure Recovered*
Pico de Botella	Normal	10	44
Pico de Botella	TDZ	10	54
Espino	Normal	10	39
Espino	TDZ	10	34



A B C

Figure 1. (A) Improved FEC conversion using RITA (M Nig 11); (B) response of Line-270400 tissue after propagation on RITA; (C) yam response to TDZ medium under RITA conditions.

Conclusions

RITA has been tested with 35 cassava clones (3 transgenic lines, 9 yellow, 18 commercial, 4 local and indicator clone *Secundina*), tropical fruits (lulo - *Solanum quitoense* Lam and tree tomatoes or tamarillos - *Cyphomandra betacea*), yams (2 local clones), sugarcane (2 clones), potatoes (one clone) rice anther culture and *Brachiaria.* In all cassava clones tested, there was a better response than on solid media.

Continuous cycles on RITA reduce the propagation rate and increase hyperhydricity; thus it will be necessary to introduce solid phases between the RITA cycles.
Materials produced with RITA increase their propagation rates on a solid medium due to the previously induced numerous tiny buds.

Future Activities

- Implement RITA in transgenic line management
- Adjust low-cost platform with different crops

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2.2.5 Embryo Axes of Immature and Mature Sexual Seeds of Genetic Stocks and Breeding Populations

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SB-2
Funding: The Rockefeller Foundation

Introduction

To safeguard some of the very valuable cassava genotypes infected by the frog skin disease (FSD) during the last growing season, the construction of a tissue culture facility for clean-up of the most valuable FSD infected lines via tissue culture and thermo-therapy was approved by management. The tissue culture facility, completed late August, consists of a growth room and a bio-safety hood room. It is available to the CIAT cassava community for tissue culture clean-up of FSD infected materials and propagation of clean planting materials.

Another use to which the facility has been put is embryo rescue of immature and mature sexual seeds. Interventions to control the escalating problems of white flies at the CIAT experimental station, namely a compulsory one-month "zero cassava" period at CIAT has meant that all plants other than plants in the hybridization block need to be harvested at 11 months after panting. This year, poor flowering of S_1 genotypes in the hybridization block and profuse flowering of S_1 plants in clonal observation trials (COT) implied that the COT plants had to be used for making genetic crosses to generate S_2 families. Because these plants must be removed in less than a year, the fruits were harvested less than 90 days after pollination, in other words before maturity. The embryos of the immature seeds from the S_2 families were rescued by embryo culture according to protocols developed at CIAT (Fregene 1999).

The facility was also used for establishment, from embryo axes, of mature seeds of breeding populations for CMD resistance. A key reason for CMD breeding at CIAT is to develop Latin America cassava gene pools adapted to the disease should in case it makes its debut in the region. A second important objective is to facilitate germplasm shipment of CIAT's elite cassava germplasm to regions, such as India and Sub Saharan Africa, where CMD is endemic, through the introgression of CMD resistance into CIAT's elite germplasm. To permit marker-assisted selection (MAS) of CMD resistance at CIAT for Latin America and at the same time fulfill plant quarantine conditions for the shipment of the CMD resistant CIAT germplasm to India and Africa, it is necessary to germinate and maintain in vitro breeding populations. This year more than 3000 controlled crosses, and >4000 open pollinated crosses were made involving CMD resistant parents introduced from IITA. The seeds were harvested as mature or immature seeds and are being germinated from embryo axes.

Objectives

To rescue embryos of immature seeds of S_2 families
To germinate from embryo axes of seeds from CMD breeding populations
To rescue embryos from crosses between high root protein content accessions of wild *Manihot* species and elite cassava parents

Methodology

Immature fruits, the fruits were cut open using a sharp knife and the seeds removed and tested for viability (ability to sink in water). A total of 446 viable S_2 seeds were obtained. Another 3600 matured seeds were obtained for crosses between CMD resistance and elite parents of CIAT cassava gene pools, wild accessions with high protein and high beta-carotene varieties. Another 450 immature seeds from crosses between high root protein content accessions of wild *Manihot* species and elite cassava parents also required embryo rescue.

Immature or mature seeds were removed and surface-sterilized by immersion in 70% alcohol for 1 min, followed by immersion in 0.5% sodium hypochlorite for 6 min, and then rinsed three times with sterile water. Under aseptic conditions, the seeds were split along the longitudinal axis utilizing sterile pliers and the embryonic axes were removed with sterile forceps and scapel. Excised embryonic axes were placed radicle down on 1/3 MS medium, supplemented with 0.01 mgl^{-1} NAA, 0.01 mgl^{-1} GA_3, 1.0 mgl^{-1} thiamine-HCL, 100 mgl^{-1} inositol, 2% sucrose, 0.7% agar (Sigma Co.) and 25 mgl^{-1} of a commercial fertilizer containing: N 10, P 52, K 10, pH 5.7-5.8 (Roca 1984). This medium is also known as 17N. The embryo cultures were incubated under an alternate temperature regime of 35°C for 16 h and 25°C for 8 h, in darkness for the first 5 days, to promote growth of the radicle, then under continuos illumination from a 40 W fluorescent bulb (5,000 $\mu mol\ m^{-2}\ s^{-1}$) for the next 5 days. The cultures were then transferred to a growth chamber with a 12h photoperiod (illumination, 5,000 $\mu mol\ m^{-2}\ s^{-1}$) at 27°C and grown for 40 to 45 days.

Over time, several modifications were made to the protocol to reduce the difficulty of opening up immature fruits, given the large number of fruits to be handled, and also to reduce damage to the embryos during removal. The first modification was to cut the fruit in half, remove the seed, disinfect and place directly on 17N media. The temperature and light regime was also modified for better germination as follows: a constant temperature regime of 28-30°C and a 12/12h photo-period and the use of a piece of gauze for 10 days to reduce influx of light, after which the cultures were fully exposed to light.

Problems with bacterial contamination were solved by adding the antibiotic rinfampicilin to the media at a concentration of 50mg/l. Fungal contamination on the other hand was eliminated increasing the concentration of sodium hypochlorite to 5% and extending incubation times up to 10 min. Due to continued problems with poor germination, a modification of the seed scarification and surface-sterilization was added for mature seeds. Seeds were soaked in 50% sulphuric acid for 30 minutes followed by immersion in sterile water for 30 min, then a 5 min wash in alcohol, and incubation in 5% sodium hypochlorite for 6 min, followed by 3 final rinses with sterile water. The softened testa was then scrapped off with a knife and the whole seed, cotyledons and embryos, was placed on the media.

Results

The 446 immature S_2 seeds were harvested the same day and stored at room temperature. Embryos were extracted and cultured over a period of 2 weeks, with an average of 40 seeds processed every day. During the first week, problems of fungal infection, apparently due to storage at room temperature, lead to a huge loss of seeds. This problem was eventually solved, but the 8-14 day storage of the immature fruits, reduced the viability of the seeds considerably. Previous experiences revealed that air drying excised immature seeds at room temperature drastically reduced germination rates (Fregene 1999). It appears that storage of immature fruits at room temperatures had the same debilitating effect as air drying immature seeds. Immature seeds from the inter-specific crosses were therefore harvested and embryos cultures on a daily basis. Only 67 plants or 15.02% could be recovered from the 446 embryos cultured. The S_1 genotypes from which S_1 plants cold be recovered include AM244-35, AM244-38, AM244-39, AM244-64, AM244-101, AM244-109, AM244-135, AM244-164, AM266-21, AM266-41, AM266-50, Y AM266-76. These S_1 plants have been multiplied in preparation for transfer to the green house and to the field the following year.

Results of more than 500 seeds processed so far have revealed more than 80% germination rates, confirming the damaging effects of storing immature seeds before embryo culture. So far, more than 100 mature seeds from the crosses generated for marker-assisted breeding of CMD resistance were cultured from embryo axes. Germination rates were higher than 90%. Results were much better here presumably due to the maturity of the seeds and the sulphuric acid treatment. No effect on germination and growth was observed of changing the temperature regime from the previous regimes described earlier for embryo rescue (Fregene et al 1997) to a constant 28-30°C day and night.

Future Perspectives

- Completion of the embryo culture of the CMD resistance breeding populations and the inter-specific hybrids for higher protein content.

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2.2.6 Tissue culture to support CIAT research agenda

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Introduction

In 1984 an *in vitro* tissue culture laboratory was established at CIAT, mainly for propagating cassava. Since then, this facility has played a significant role in plant virus elimination, propagation, conservation and safe international movement of certified germplasm. Nowadays, some CIAT projects and outside users have included in vitro plant handling in their research agendas for commercial or research purposes. The tissue culture lab has provided support to these initiatives by providing clonally propagated plants of specific cassava clones requested by them. A summary of this activity is presented here.

Materials and Methods

The Genetic Resources Unit provided the initial *in vitro* material, previous MTA sign. These materials were used for basic seed plots, but they could not support large-scale propagation.
A small propagation set was initiated in accordance with specific purposes such as the need for local or commercial clones, material with special characteristics, virus testing, etc. (Table 1).

Depending upon the research objectives, we adopted a solid (Roca et al., 1984) or liquid propagation system using RITA® (Escobar et al., 2001).
Hardening was done according to Roca et al. (1984).

Results

Ten different seed plots were established (Table 1).
Solid conditions were implemented when only a few plants/clone were requested.
Liquid systems (RITA) help increase materials when there are insufficient plants.
Propagation activities have been a key factor in meeting Cauca farmers' necessities. In order to sow at three different sites (Santa Ana, San Rafael and Perico Negro, Cauca), 6000 in vitro plants were produced and hardened.

Conclusions

Small propagation sets have been produced as an additional activity; but under special circumstances such as massive or rapid requirements, it will be necessary to build or strengthen present facilities. Laboratory space, supplies and the work force should be taken into consideration.
The propagation scheme must be designed according to the objectives and plant requirements.
Coordination among different users is critical to meet their requests. The initial meeting is a key factor to make a work plan based on the different research agendas.

Table 1. Materials involved in small propagation set and their potential users.

Characteristic of Interest	No. Clones	User or Partner	Materials Involved
High dry matter content	18	IP-3 project	SM 2546-52, SM 2546-54, SM 2603-9, SM 2618-16, SM 2619-1, SM 2619-5, SM 2619-6, SM 2621-4, SM 2621-28, SM 2622-1, SM 2623-1, SM 2772-2, SM 2772-7, SM 2772-8, SM 2773-46, SM 1438-2, SM 1411-5, SM 2545-20
Comparisons among in vitro vs. conventional stakes	5	IP-3 project	SM 1438-2, M Bra 383, M Col 1468, CM 3306-4, CM 523-7
Implementation of RITA system	8	PBA-CORPOICA	M Col 2215, M Col 1505, CG1141-1, CM 3306-19, CM 3306-4, SGB 765-2, SGB 765-4, CM 3555-6
Commercial-scale implementation	13	MADR	M Bra 383, CM 523-7, CM 4574-7, M Bra 507, M Cub 74, M Ecu 72, M Tai 8, M Ven 25, CM 6740-7, M Col 2063, CM 3306-4, M Col 1505, M Col 2215
Implementation of RITA with high carotene-content clones	9	SB-2	M Col 2482, M Bra 522, M Bra 496, M Col 2294, M Bra 206, M Bra 509, M Col 2299,M Col 2318, CM 2772-3
Local clones	3	PBA	*Ramirana, Por Encima, Yema de Huevo*
Wild materials	5	PE-1	*M. carthaginensis* 413-013, *M. flabelifolia* 444-002 *M. peruviana* 417-005, 417-003, 413-003
Material requested from Cauca farmers	6	ASOPROSA-FIDAR	HMC 1, M Bra 383, M Col 1522, CM 523-7, M Per 183, CM 6740-7
FSD testing	1	Clayuca	M Col 2063
Transgenic lines	3	SB-2	L-55, L-270400, L-5352

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2.2.7 Plant recovery from cryopreserved friable embryogenic callus lines

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Introduction

The Cassava Friable Embryonic Callus (FEC) system (Taylor et al., 1996) has been implemented as an alternative transformation technique (López, 2000) at CIAT since 1999. Despite the potential uses of this method, it is very labor intensive until sufficient good-quality FEC is recovered thereby justifying the need to have a preservation system for the FEC once obtained. In this way, the need to continually invest more time and resources in developing the FEC is eliminated as it can readily be thawed from the freezer and used when needed.

The cryopreservation of FEC may provide a means of ensuring genetic stability of cell lines and could provide a source of fresh tissue useful for genetic transformation. Cassava clones M Col 2215 and M Nig 11 were used to standardize desiccation and desiccation-vitrification techniques.

Materials and Methods

The cryopreservation technique established by Santos (2002) was implemented.
For initial plant recovery, the RITA® system was implemented, following Montoya (2002).
Hardening and greenhouse plant management were implemented as per Roca et al. (1984).

Results and Discussion

FEC from both cassava clones showed response after freezing when using desiccation and desiccation-vitrification.
Desiccation done with a 0.95% agar medium content showed better recovery after freezing than other levels of agar.
M Nig 11 had better plant conversion than M Col 2215.
The RITA system enhanced the conversion from FEC to plant.

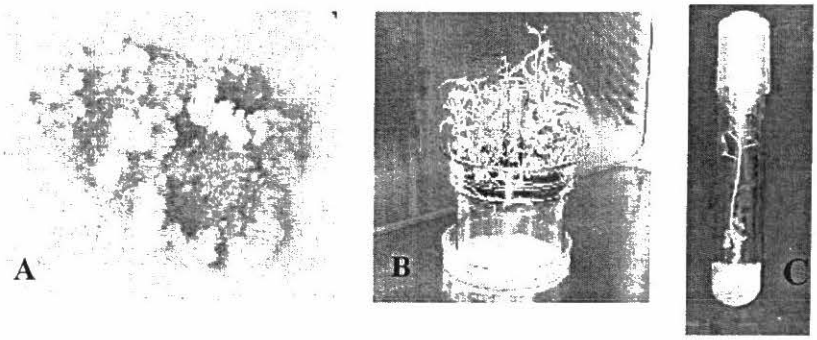

Figure 1. (A) FEC from M Col 2215 recovered after freezing, using a dehydration-vitrification technique; (B) plants from frozen FEC of clones M Nig 11 and M Col 2215; (C) regenerated in RITA.

Conclusions

Desiccation time and agar content were the key factors in FEC recovery after freezing.
Percent recovery is clone dependent.
Desiccation is less complex than desiccation-vitrification.

Future Activities

Adjust preliminary methodologies
Use other FEC lines established by the transformation group to standardize the cryopreservation procedure

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2.2.8 Temporary Immersion System (RITA) for Anther Culture of Rice

E. Tabares [1], G. Delgado [2], M. Quintero[2], R .Escobar [1], Z. Lentini[1,2]
[1]SB2, [2]IP4

Introduction

Plant *in vitro* culture using temporary immersion (RITA) offers all the advantages of a liquid medium system (automation, large scale production, easy changes of medium, filter sterilization, easy cleaning) without any of its drawbacks (reduce gas exchange, vitrification). Immersion time, i.e. duration or frequency, is the most decisive parameter for system efficiency. The optimization of the nutrient medium volume and the container volume also substantially improves efficacy, especially for shoot proliferation. Several reports confirmed large gains in efficacy from temporary immersion when using liquid medium for micro propagation. The main parameters involved reducing production costs are, firstly the drastic reduction of work labor, followed by a reduction in shelving area requirement and in the number of containers used. Scaling up the use of temporary immersions for embryogenesis and shoot proliferation procedures are currently taking place in order to commercialize this process (Berthouly & Etienne, 2002). This system has proved its efficacy for

somatic embryogenesis of banana (Alvard et al, 1993; Escalant et al, 1994), coffee (Berthouly et al, 1995; Etienne et al, 1997), citrus (Cabasson et al, 1997), oil palm and rubber plant (Etienne et al ,1997), and at CIAT for cassava (Escobar and Roca, 1999). High efficiency has also been demonstrated for clonal propagation through micro-cuttings of coffee, and sugar cane (Lorenzo et al, 1998); for proliferation of meristems of banana, and pineapple, and for micro-tuberization of potato (Teisson and Alvarad,1998).

We have previously reported preliminary results using RITA for the induction of embryogenic callus derived rice from mature zygotic embryos (Tabares et al., CIAT SB2 Report 2000) and from anther culture (Tabares et al., CIAT SB2 Report 2001). This year we report a comparative analysis including various indica and japonica rice genotypes.

Materials and methods

Mature zygotic embryos or anther culture of the indica rice Cica 8, Palmar, Fundarroz PN1, Cimarron, Fedearroz 2000, and CT 11275, and of the japonica breeding line CT 6241-17-1-5-1 were used. For anther culture, donor plants were grown in the field, panicles harvested, and anthers cultured according to Lentini et al., 1995. Tissues were either culture in liquid medium contained in RITA vessels or in liquid medium in baby food jars (for anthers, control) or on solid medium for zygotic embryos. Induced callus from each culture system, was then transfer onto solid plant regeneration medium according to Lentini et al. 1995.

Results and Discussion

Although there was a tendency for an earlier callus induction from zygotic embryos of the indica variety Cica 8 in RITA (5 to 10 days earlier respect to solid medium), this difference was not significant (Figure 1A). However, a significant higher number of healthy callus was induced in RITA respect to solid medium in petri dish (Figura 1B and C). A similar tendency was noted for the indica variety Palmar (Figure 2A and B). Although callus induction is enhanced in RITA, this system seems not to be appropriate for plant regeneration in rice. Green plant regeneration was highly inhibited when callus induced in RITA were also culture in liquid regeneration medium in the RITA system. In contrast, a high and similar green plant regeneration to the control was noted from callus induced in the RITA but when transferred onto solid medium in jars (Figure 3). Preliminary attempts also suggest that the induction of embryogenic callus is enhanced in RITA when a larger number of genotypes are tested, including recalcitrant indica types (Figure 4).

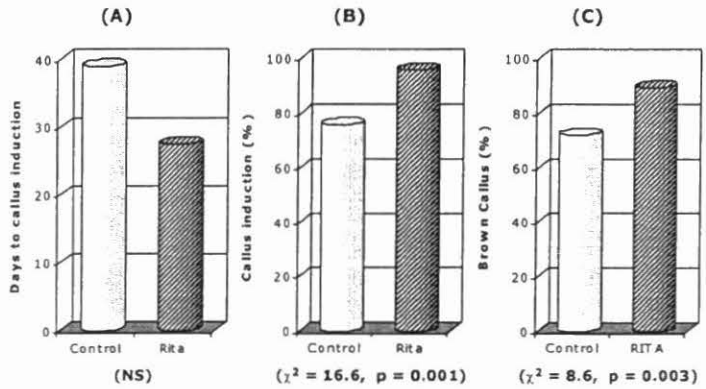

Figure 1. Callus induction in RITA system. (A) Days to callus induction. (B) Percentage of callus induced. (C) Percentage of brown (phenolized callus).

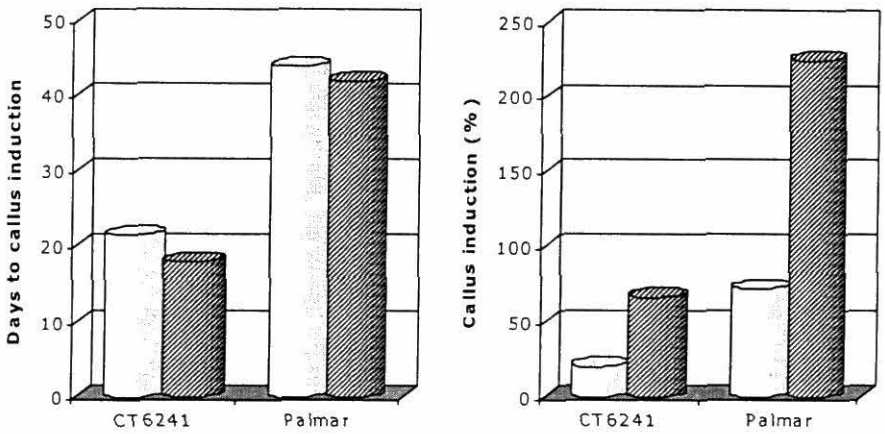

Figure 2. Callus induction in RITA system of indica (Palmar) and japonica (CT 6241-17-1-5-1) genotypes.

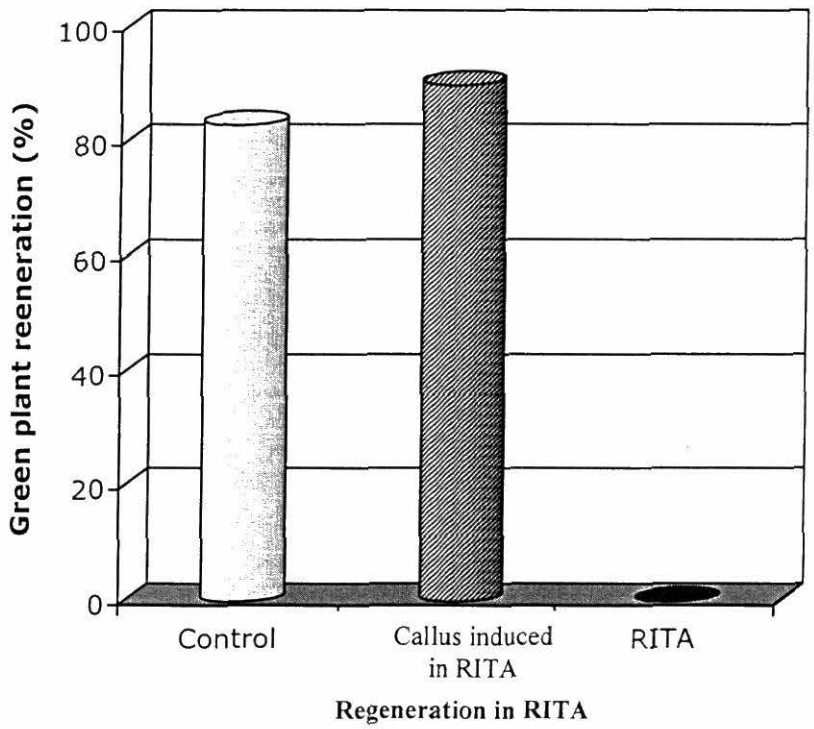

Figure 3. Green plant regeneration on solid medium in jars of callus induced in petri dish (control) or RITA, or callus induced and regenerated in RITA.

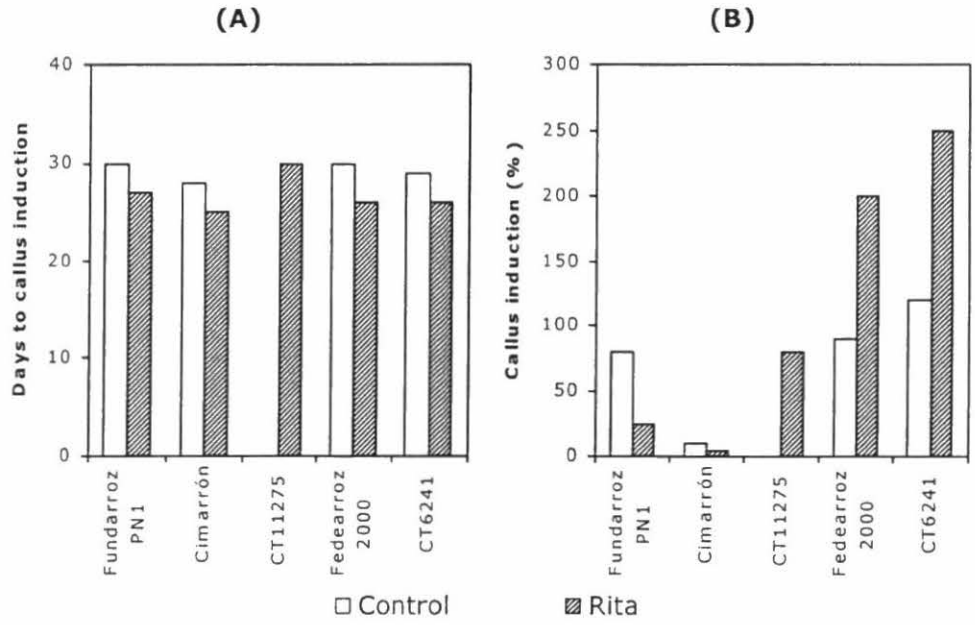

Figure 4. Callus induction in RITA of various indica and japonica genotypes

Future Plans

- Optimize conditions for the broad application of RITA for induction of embryogenic callus in a diverse number of rice genotypes commonly used in the breeding program.
- Standarize conditions for an efficient use of this technique for the generation of doubled haploids from anther culture.

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2.2.9 Osmotic stress as a tool to enhance plant regeneration in rice

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Introduction

Between the two rice types (indica and japonica) of the most widely cultivated rice species (Oryza sativa L.), indica rice cultivars have proved to be less amenable to the in vitro culture. Some of the factors that affect plantl regeneration frequency from rice callus are the concentrations of gelling agents, osmotic, and the specific combinations of plant growth regulators. So as to enhance green-plant regeneration, supplements such as tryptophan, proline, polyamines such as spermidine, changes in hormone composition, carbohydrate source, osmotic stress, partial desiccation and high concentration gelling agents (Khanna and Raina, 1998) have been used to improve the regenerability of rice callus culture. Partial desiccation has been reported to enhance somatic embryo differentiation and development in soybean (Hammatt and Davey, 1987), grape vines (Gray 1987), wheat (Carman 1988), spruce (Roberts et al .1991) and cassava (Mathews et al, 1993). Tsukahara and Hirosawa (1992) reported that this treatment was effective on japonica rice callus induced from cell suspension cultures. Plant regeneration frequency was considerably increased in most of the cultivars when the callus was treated with water stress, as compared with untreated controls (Lee et al., 1999). Kavi Kishor et al. (1986 and 1987) reported that the osmolarity of both growth and regeneration media was important for obtaining, retaining and reviving the high regeneration frequency of rice callus. Lai and Liu (1988) reported that the lower water content of callus cultured on a medium containing mannitol and a high concentration of agar was one of the elemental factors for efficient regeneration of rice callus. In the present study , a simple method was tested to reduce the water content of callus, thereby increasing the regeneration frequency of rice.

Materials and methods

Three separate experiments were conducted to test different treatments to increase the plant regeneration frequency from callus culture. Experiment 1(water stress treatment): six indica rice genotypes, (Palmar, Fedearroz 50, Fedearroz 2000, Cimarrón, Cica 8, CT-11275, and Fundarroz PN1) were tested. Scutellum-derived callus of 1-2 mm diameter, were induced on the NBA medium (Li et al ,1993) medium were transferred to MS (Murashige and Skkog,1992) semi-solid medium (0.4% agarose) or with 1% agarose (water stress), supplemented with 4mg/L Kinetin , 2mg/ l ANA, and 3 % maltosa. For water stress treatments, the cultures were incubated in the dark at 27°C to dehydrate callus. After two weeks of culture, stressed callus from 1% agarose-containing

medium were transferred to the corresponding 0.4% agarose solid medium for regeneration and incubated in light. The calli that were not treated with water stress were also cultured on the medium semi-solidified with 0.4% agarose Experiment two (concentration of gelling agent) after induction callus MS regeneration medium supplemented with 4mg/ l Kinetin, 2mg/l ANA, 3% sucrose, and 2g/l or 4g/l gelrite. Experiment 3 (osmotic stress), a set of callus were subcultured for 24 hr on callus induction medium +3% mannitol or 3% sorbitol, after partial desiccation treatment callus were transferred on regular plant regeneration medium. Another set of callus was transferred from callus induction medium without partial desiccation treatment to regular MS regeneration medium (control) or supplemented with either 3% mannitol or 3% sorbitol. For the three experiments a factorial experimental completely random design was used.

Results and discussion

Plant regeneration was significantly increased in all genotypes when callus were treated with water stress for 2 weeks on medium containing 1 % agarose (Figure 1). The most significant differences were noted in Palmar, CT11275, Fedearroz 50, Cica 8, Fedearroz 2000 and Fundarroz PN1, which plant regeneration was increased from 0%-15% to 60%-100% (Figure 1). Significant differences in plant regeneration was also noted when the gelling agent (gelrite) was increased from 2 gr/l to 4 gr/l (Figure 2). Although in this case the enhancement in plant regeneration was less pronounced (Figure 2) respect to the water stress treatment (Figure 1). Pre-osmotic treatment on callus induction medium inhits plant regeneration from those callus, whereas a highly significant increased was obtained with mannitol or sorbitol was included in the plant regeneration medium (Figure 3). The best response was noted when 3% sorbitol as used to increase the osmoticum of the medium (Figure 3).

Future Plans

- Comparative plant regeneration analyses of water stress treatment in callus induction and regeneration media with 1% agarose respect to increased osmoticum with 3% sorbitol in plant regeneration medium.
- Test best conditions with a wider range of genotypes used in rice breeding.

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2.2.10 Isolation and Characterization of a Caffeic Acid O-Metiltransferase (OMT) from *Brachiaria decumbens*, a lignin biosynthesis gene.

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[1]SB-2 Project, [2]Plant Biotechnology Centre, La Trobe University, AU.

Introduction

Lignin is the second most abundant organic compound on the earth, after cellulose. It is a complex aromatic polymer, which provides mechanical strength to plant cell walls and hydrophobicity to conducting vessels. In addition, lignin deposition is thought to represent a defense reaction against pathogen iscusió. However, lignin may have a negative impact on the utilization of biomass (Atanassova et al., 1995). In grasses and legumes a negative correlation between lignin content and in vitro dry matter digestibility has been reported. Lignin composition, particularly the relative proportion of syringyl (S) and guaiacyl (G) units in the lignin polymer, and the nature of the covalent linkages between lignin and other polymers are also important determinants of digestibility (Heath et al., 1998). COMT is a key enzyme in the lignin iscusión s pathway. In angiosperms, COMT is bispecific and methylates caffeic acid to produce ferulic isc using S-adenosul-L-methionine as a methyl donor. CDNA clones encoding putative COMTs have been isolated from several plant species including alfalfa (Medicago sativa) (Gowri et al., 1991), maize (Zea mays) (Collazo et al., 1992), tobacco (Nicotiana tabacum) (Pellegrini et al., 1994), barley (Hordeum vulgare) (Gregersen et al., 1994), Stylosonthes humilis (McIntyre et al., 1995), ryegrass (Lolium perenne) (Health et al., 1998) and sugar cane (Saccharum officinarum) (Selman-Housein et al., 1999). Therefore the modification of lignin content and/or composition by genetic manipulation would be of great economic interest. For these reason, CIAT jointly with The Plant Biotechnology Centre, La Trobe University, AU, are working in the isolation and characterization of genes from Brachiaria that are involved in the lignin discusións.

Material and Methods

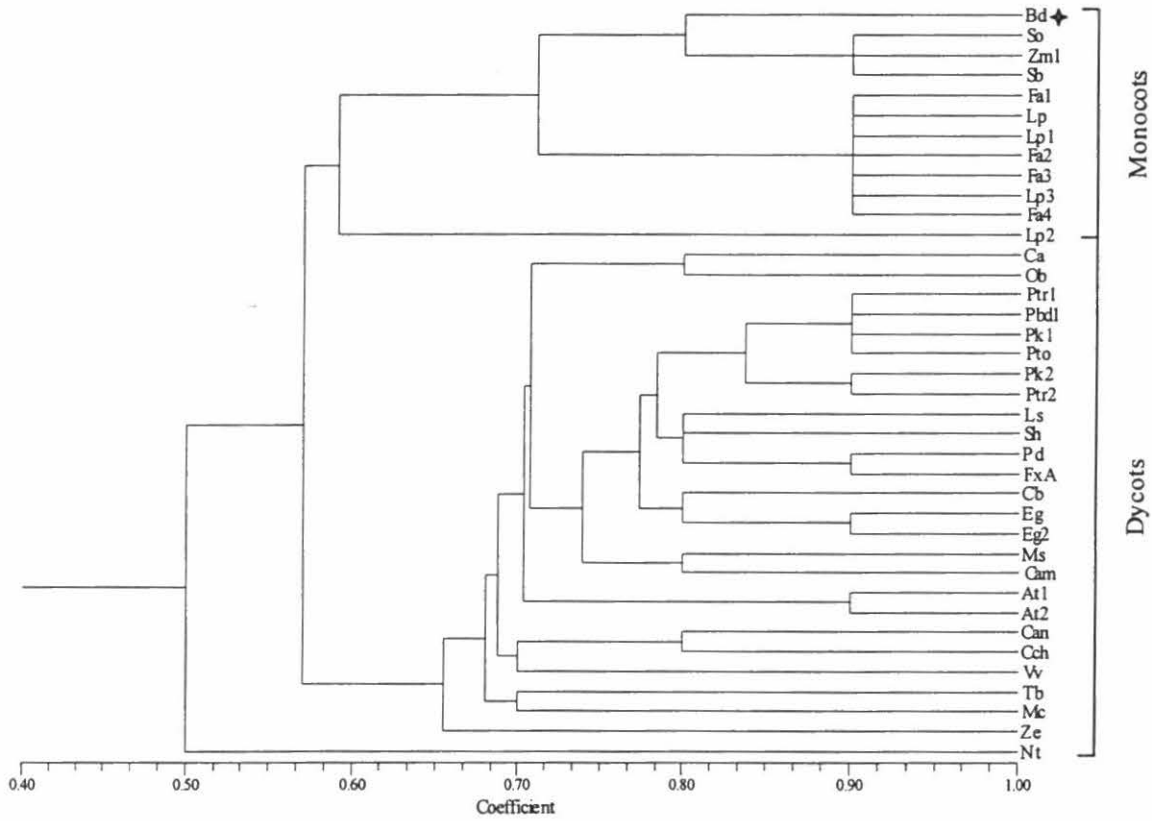
CDNA library screening. A Uni-Zap™ XR cDNA library (ZAP cDNA Synthesis Kit, Stratagene) was constructed from whole 10 day old Brachiaria seedlings. The library was screened with a [^{32}P] dCTP-labelled 1461 bp Xba I fragment of Lolium perenne OMT-1 (Accesion number AAD10253). CDNA inserts were excised and cloned using the ExAssist helper phage with SOLR strain as described by manufacturer (Stratagene).

DNA sequencing. High quality plasmid DNA (Maxi kit, Qiagen) was sequenced by the dideoxy chain termination method (BigDye™, ABI) on a ABI 373 automated sequencher. For sequencing the internal region of BdOMT, synthetic oligonucleotide primers were designed from the DNA sequences previously determined.

Southern Blot Hybridisation. Genomic DNA from Brachiaria leaves was isolated following the methodology McCouch et al. (1988). Twenty-five g of Brachiaria DNA, five g of bean DNA, 10 g of rice and cassava DNA, were digested with each of the restriction enzymes Bam H I, Xho I, Xba I and Eco R I. The fragments were separated on 1% agarose gels and transferred to Hybond N^+ membranes according to the manufacturer's instructions (Amershan). Probe consisted of the open reading frame of BdOMT prepared by PCR. DNA probes were random primer labeled using Megaprime DNA Labelling System kit (Amersham) and [^{32}P] dATP. The hybridization was carried out overnight at 60°C following the manufacturer's recommendations.

Results and Discusión

Isolation of a Brachiaria decumbens OMT cDNA. DNA library constructed from RNA of 10 day old Brachiaria seedlings was screened with a 1461 bp Xba I ryegrass OMT probe. A single highly represented cDNA was isolated and fully sequenced. BdOMT is a full length 1410 bp cDNA with an open reading frame (ORF) of 1,083 bp, a 5′ noncoding region of 62 bp and 3′ noncoding region of 256 bp including a poly(a) tail. The putative protein encoded by BdOMT cDNA consist of 360 amino acids. Within the coding region BdOMT has 89%, 88%, 87% and 77% sequence identity with OMT from sugar cane (Sc), sorghum (Sb), maize (Zm) and lolium (Lp1), respectively (Figure 1).



(Figure 1). These results suggest the presence of similar OMT genes in rice and cassava and confirm the OMT gene origin in three Brachiaria species.

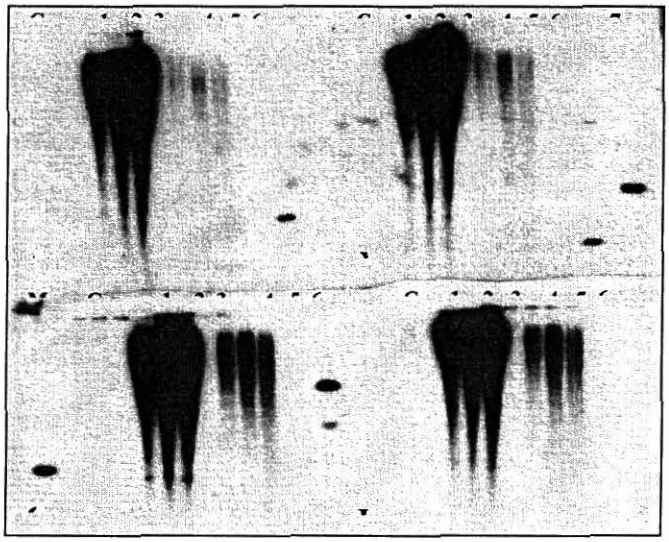


Figure 2. Genomic Southern Hybridization. Line 1. B. decumbens, 2. B. ruziziensis 3. B. brizantha, 4. rice var. CICA 8, 5. rice var CT02-2, 6. rice var. CT 11275. 7. bean and 8. cassava. M. 1 Kb, C. BdOMT. Digest with A. Xho I, B. Xba I, C. Bam H I. D. Eco R I.

Brachiaria, rice, bean and cassava DNA was digested with four restriction enzymes: Bam H I and Xho I (restriction sites inside of ORF of BdOMT) and Xba I and Eco R I (restriction sites for these enzymes are not present in the coding region of BdOMT).

A strong signal was observed in the three Brachiaria species analyzed (B. decumbens, B. ruziziensis and B. brizantha). At the same time, a light fragments were revealed in the lines of rice and cassava. Any signal was detected in bean

Future Plans

- To conclude BdOMT molecular characterization analysis
- To produce transgenic germplasm with manipulated lignin metabolism

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2.2.11 New Genetic Constructs with BdOMT gene

C.P. Flórez[1], L.F. Fory[1], E. Tabares[1], G. Delgado[1], M. Emmerling[2], G. Spangenberg[2], Z. Lentini[1]
[1]SB-2 Project, [2]Plant Biotechnology Centre, La Trobe University, AU.

Introduction

The modification of lignin content and/or composition by genetic manipulation would be of great economic interest particularly in forage species. Once the lignin gene OMT was identified is necessary to introduce this gene in transformation vectors. For this reason, the BdOMT gene in sense and anti-sense orientations driven by the 35S CaMV promoter were placed into the plasmid pCAMBIA 1301 and pCAMBIA 1305.2 both carrying the gus-intron and hygromycin resistance gene. At the same time, mature embryos derived callus of *indica* rice variety Palmar and *japonica* variety Nipponbare were transformed by *Agrobacterium.*

Material and Methods

Vector Construction. All DNA recombinant techniques were performed essentially as described by Sambrook et al., (1989). The Open Reading Frame of BdOMT was amplified by PCR from pBluescript SK (+) phagemid (Stratagene) containing the Brachiaria OMT cDNA full-length insert at the Eco RI/Xho I sites, with two specific primers design with the Xba I cut sites.

The 1,158 bp fragment generated by the digestion with Xba I of the PCR product was insert at the Xba I site of the binary vector pRT101. The orientation of the inserts was determined by restriction digestion of plasmid recovered from transformed Escherichia coli DH5 . The resulting vectors were pRTOMT-2 (sense orientation) and pTROMT-25 (antisense orientation).

A 1,857 bp Hind III fragment of pRTOMT vectors carrying the BdOMT gene flanked by the 473 bp CaMV 35 S promoter and 226 bp Nos PolyA terminator sequences, were inserted at the Hind III site of the polilinker of pCAMBIA 1301 and pCAMBIA 1305.5 vectors (Figure 1). Six new vectors were identify and their OMT gene integrity was verified by three ways (1) restriction patterns (2) Southern Blot of restriction patterns using a BdOMT gene as a probe and (3) sequencing all new clones.

All new vectors were transferred to Agrobacterium tumefaciens AGL-1 strain.

Genetic Transformation. *Mature embryos derived calli from rice varieties Palmar (indica) and Nipponbare (japonica) were used as a target. Agrobacterium transformation was made according Tabares et al., 1999.*

Results and discussion

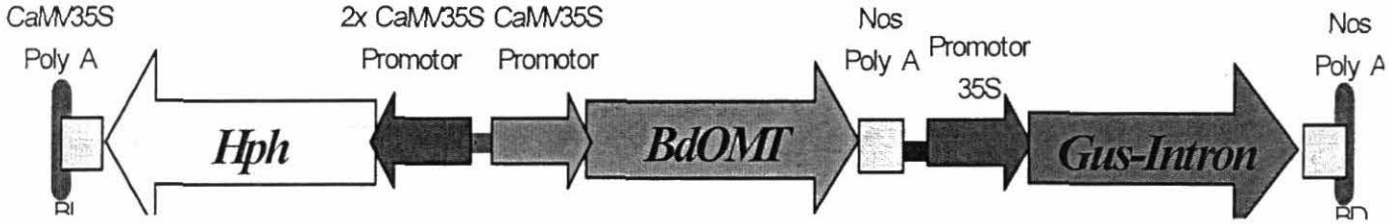
Vector Constructions. Eight new constructions were obtain (table 1, figure 1A, 1B). These constructions are accompanied by a complete identification card that include a restriction pattern (Figure 2). The restriction patterns were transferred and hybridized with the open reading frame of BdOMT (figure 3). Six of these new vectors were partially sequencing and their sequences coincide with the sequence previously established for BdOMT.

Table 1 Description of Bd MT constructs generated at CIAT

Name	Gene	Promoter	Vector / Orientation	Other Genes	Partial Sequencing
pRT MT2	Bd MT	35S CaMV	pRT101 / Sense	none	none
pRT MT25	Bd MT	35S CaMV	pRT101 / Antisense	none	none
pC01 MT 1	Bd MT	35S CaMV	pCAMBIA 1301 / Sense	**Hph** GUS – Intron	487
pC01 MT 2	Bd MT	35S CaMV	pCAMBIA 1301 / Antisense	*Hph* GUS – Intron	611
pC01 MT 3	Bd MT	35S CaMV	pCAMBIA 1305.2 / Sense	*Hph* GUS – Intron	677 bp
pC01 MT 4	Bd MT	35S CaMV	pCAMBIA 1305.2 / Sense	*Hph* GUS – Intron	1,089 bp
pC05.2 MT 1	Bd MT	35S CaMV	pCAMBIA 1305.2 / Antisense	*Hph* GUS Plus – Intron	846 bp
pC05.2 MT 2	Bd MT	35S CaMV	pCAMBIA 1305.2 / Antisense	*Hph* GUS Plus – Intron	none

pRTOMT-2

pRTOMT-25



pC01OMT-3

pC01OMT-4

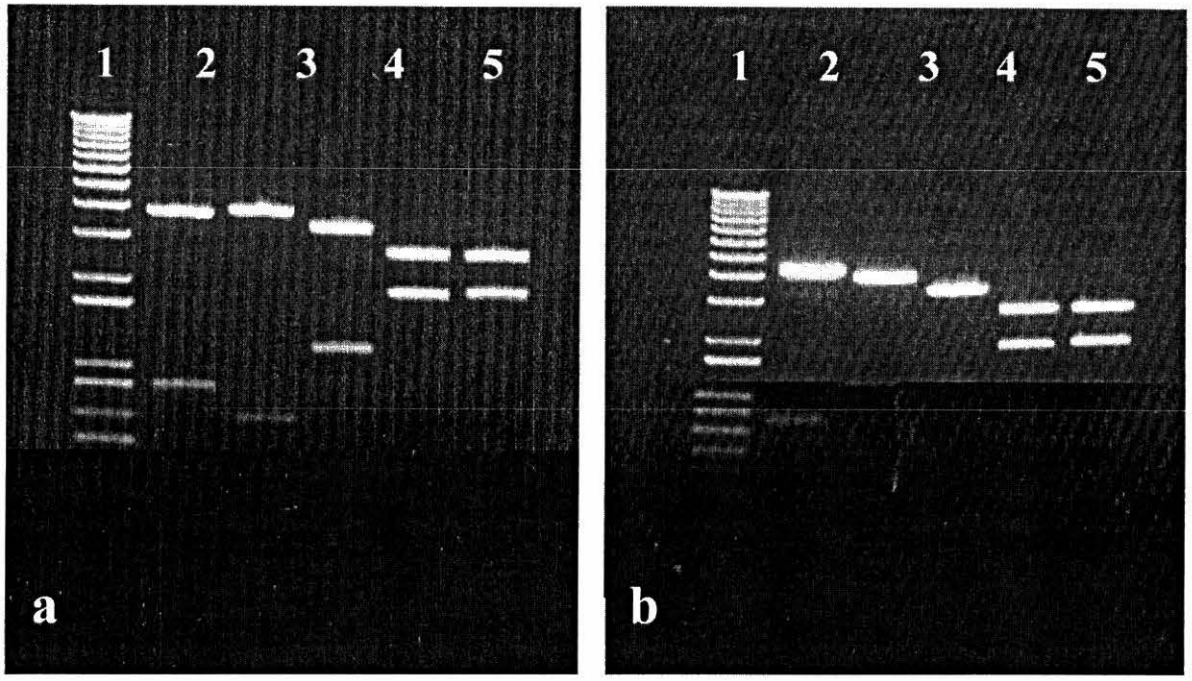


Figure 2. Restriction pattern. A. pRTOMT- 2 sense Orientation. B. pRTOMT-25 antisense orientation. Line 1. 1 Kb marker, 2. Bam H I, 3. Xho I, 4. Xba I, 5. Pst I and 6. Hind III

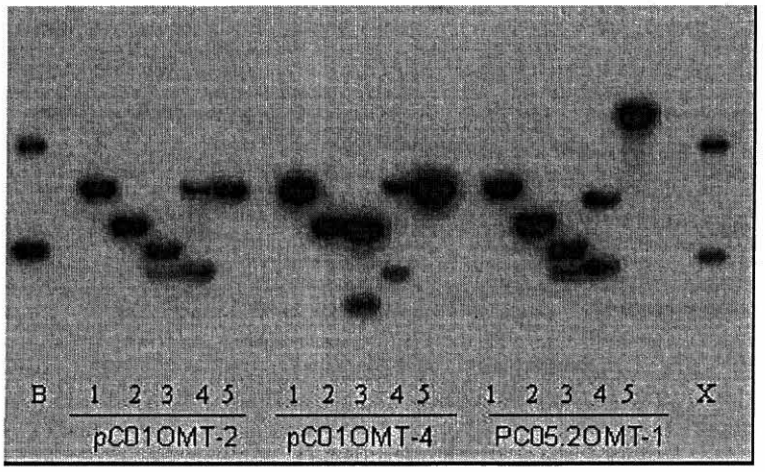


Figure 3. Southern hybridization of new constructions restriction patterns. B. BdOMT plasmid digested with Bam HI, X. BdOMT plasmid digested with Xho I. Line 1.Hind III, 2. Xba I, 3. Bam *H* I, 4. Xho I and 5. Sph I.

Genetic Transformation. Approximately 1800 callus derived from 304 Hyg^R callus were transferred to regeneration medium containing hygromycin as selection agent (Table 2). At the moment, GUS stable expression has been determined in some callus transformed with pC05.2OMT-2 and one GUS positive plant has been regenerated. Currently, regeneration phase is in progress.

Table 2. Summary of advances in genetic transformation with the new BdOMT vectors

Plasmid	Variety	C.C.	C.S.	R.C.	N.R.C.	% Hyg^R	R.P.C.
pC05.2OMT 1	Palmar	178	178	38	140	21.35	38
pC05.2OMT 1	Nipponbare	110	110	35	75	31.82	35
pC05.2OMT 2	Palmar	166	166	52	114	31.32	52
pC05.2OMT 2	Nipponbare	99	99	33	66	33.33	33
pC01OMT 2	Palmar	82	82	33	49	40.24	33
pC01OMT 2	Nipponbare	68	68	35	33	51.47	35
pC01OMT 4	Palmar	87	87	36	51	41.38	36
pC01OMT 4	Nipponbare	76	76	42	34	55.26	42

C.C. Cocultivated Calli
C.S. Selection Calli
R.C. Hygromicin resistant calli number
N.R.C. Hygromicin non resistant calli number
% Hyg^R Hygromicin resistant calli percentage
R.P.C. Regeneratio Phase Calli

Future Plans

- To conclude regeneration phase
- To obtain transgenic plants with OMT gene.
- To determine transgene presence mediated molecular and histological tests

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2.2.12 Development of methodologies for *in vitro* multiplication, plant regeneration, and genetic transformation of naranjilla (lulo)

V. Segovia, Z. Lentini

Introduction

A large number of fruits of Andean origin have great potential to become premium products for local and export markets with a high economic return for the farmers. Naranjilla (Solanum quitoense) is among these fruits. This species is native from Colombia and Ecuador, and it is normally cultivated between 700 and 2000 meters above sea level. Some of the main attributes of this fruit includes its high level of vitamin C, and the sub-shrubby perennial growth amenable for cultivation in hillsides and inter-cropping, aiding soil conservation practices. Recently in Colombia, naranjilla changed from being a fruit of local fresh consumption to become an important industrial fruit for juice and yogurt

products, increasing its market value. A major constraint for the rapid adoption of naranjilla by the local farmers is the limited availability of elite germplasm free of pathogens. The high level of trait segregation restrains its multiplication through seeds. Rapid multiplication of quality planting materials is of paramount importance. One of the main objectives of this project is to develop a protocol for in vitro propagation of naranjilla with application for conservation and rapid multiplication of clones free of pathogens. The expected results include the mass multiplication of elite clones that then can be distributed to farmers. Since breeding for this species is almost non-existing, paralelly to the in vitro propagation effort, it will be important to develop plant regeneration and transformation systems to aid the development of germplasm. Last year it was reported the advancement in establishing a system for maintenance of the in vitro germplasm collection, and the progress made identifying factors to increase the plant regeneration efficiency from elite naranjilla materials. This year it is presented the development of an efficient plant regeneration system, the evaluation of regenerated plants in the field and the comparison of the growth and development with in vitro propagated plants. The establishment of a protocol for introducing new material from plants growing in soil is also presented.

Materials and Methods

High quality and elite clones provided by the Andean Fruit Center (Centro Frutícola Andino – CEFA) were used. This collection includes naranjilla with or without thorns commonly grown by farmers. The effect of the in vitro propagation medium on the efficiency of plant regeneration was evaluated. Different propagation medium were tested using foam plugs to determine the interaction of the medium composition and free gas exchange. A protocol to readily incorporate material in vitro from the greenhouse or the field was optimized. A small scale field trial was conducted at 1700 m over sea level and a mean temperature of 22C was conducted to compare the growth and development of regenerated plants respect to in vitro propagated clones.

Results and Discussion

A randomized block design of four replicates each of 15 experimental units was used to determine the best medium composition and explant to induce a direct plant regeneration in naranjilla. A non-parametric chi-square analysis indicated that petioles showed showed from 3 to 9 times more more plant regeneration that the corresponding leaves from thorny and non-thorny clones respectively (Figure 1A). A significant higher response was also noted on medium originally develop for plant regeneration of tomato (Ultzen et al, 1995), consisting on MS salts, B5 vitamins, supplemented with sucrose 10 g/l, glucose 10 g/l, gelrite 1.5 g/l, zeatine 2 mg/l and IAA 0.02 mg/l (Figure 1A). On this medium petioles from thorny genotypes showed twice increase in plant regeneration respect to a medium reported for naranjila (Hendrix et al., 1987) composed by MS salts and vitamins and supplemented with sucrose 30 g/l, agar 7 g/l, IAA 0.01 mg/l, kinetin 5 mg/l, or with a modification consisting on gelrite 2 g/l and BAP 2 mg/l (modification suggested by Dr Richard Litz, Univeristy of Florida, laboratory which Hendrix work was conducted)(Figure 1A). Non-thorny genotypes did not regenerate any plant on medium developed by Hendrix (Figure 1A). The efficiency in response was highly affected by the medium composition on which the petiole donor plant were grown. Petioles of plants grown on medium A (MS basal salts and vitamins, and supplemented with calcium pantothenic acid 2.5 mg/l and gelrite 3.5 g/l) showed about 20% more plant regeneration respect to petioles from plant grown on medium ½ MS(½ MS basal salts supplemented with ANA 0.02 mg/l, BAP 0.04 mg/l, and GA_3 0.05 mg/l) (Figure 1B). Although plants developed better on media CEFA (MS salts with Tiamina 0.4 mg/L and Inositol 100 mg/L, gelrite 2 g/L y BAP 0.5 mg/L) or Corpoica (MS salts with Tiamina 0.4 mg/L and Inositol 100 mg/L, gelrite 2 g/L y BAP 0.5 mg/L) when foam plugs rather than plastic caps were used to seal the test tubes, the best development is obtained in medium A.

The re-establishment of an *in vitro* collection derived from greenhouse or field grown plants is under progress by using apical meristems instead of axillary's buds as the starting materials. Shoots derived from axillary's buds showed high levels of contamination. The current protocol used consist of sterilization with 70% ethanol for 1 min, followed a wash with water and then immersion in 1% sodium hipochlorite for 15 min. Then three washes with sterile bi-distil/ de-ionized sterile water. Apical meristems are cultured on medium A supplemented NAA 0.02 mg/L, BAP 0.04 mg/L and GA3 0.05 mg/L.

Field comparison of regenerated plants respect to in vitro propagated plants, indicate that naranjilla plant growth and development appears not to be affected by the organogenesis process (Figure 2). Plant height, leaf morphology, and plant type as well as days to flower formation, anthesis, and fruit development were similar in the two types of plants (Figure 4). In both cases, plants flowered 45 days after transplanting in the field (about 90 days after transfer from in vitro conditions to the soil in the greenhouse prior to the field), and fruit formation started at 65-70 days (Figure 3). These results indicate that in vitro derived naranjilla plants developed significantly earlier respect to standard crop, which flower at 150 days and fructify at 270 days.

Future plans

- Evaluate other factors affecting plant regeneration, especially those associated with ethylene effect(s)
- Develop a genetic transformation protocol
- Evaluate a medium scale field trial to complete evaluation of yield in regenerated plants and compare the growth and development with in vitro propagated
- Collaborate with farmers to compare the production of in vitro derived plants with those from standard crop conditions

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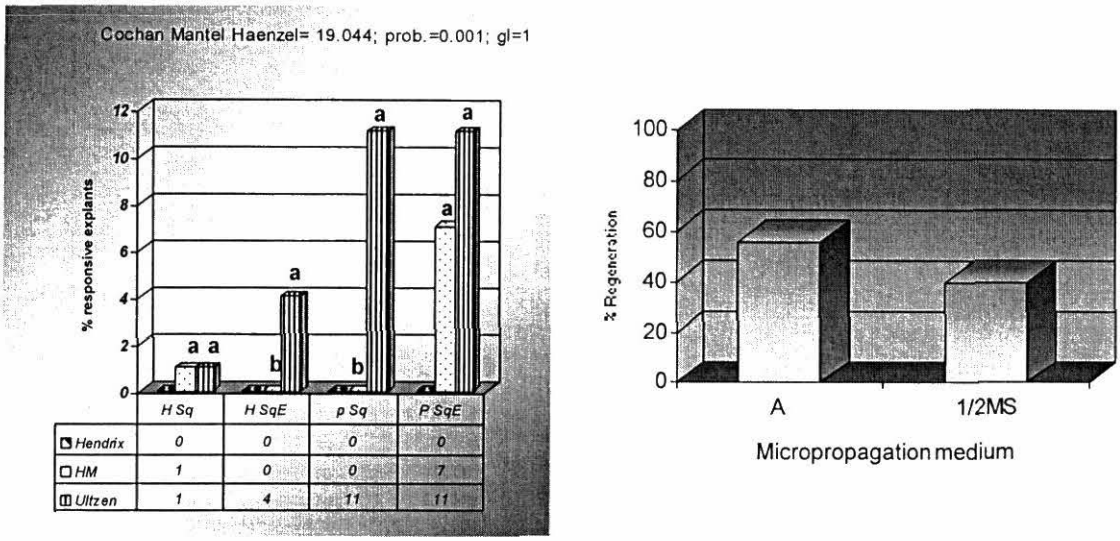

Figure 1. (A) Plant regeneration from petiole or leaf explants of genotypes with or without thorns using medium previously develop for tomato or reported by Hendrix for naranjilla. (B) Plant regeneration of petioles derived from plants micro propagated on medium A or 1/2MS.

Figura 2. Plant development in the field. Left, whole plant. Center , flower at anthesis. Right, fruits at 90 days.

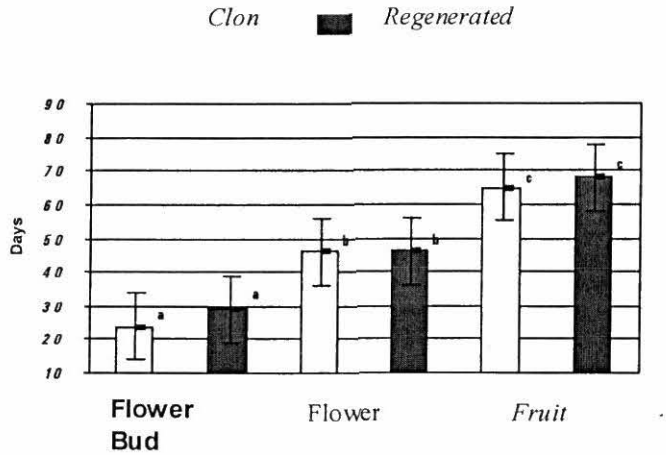

Figure 3. Development of field grown plants

2.2.13 Genetic transformation of tomato variety UNAPAL Arreboles for resistance to budworm (*Tuta absoluta*)

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Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most important crops in the fresh vegetable market as well as in the food processing industry (Rick and Yoder, 1988). Tomato is the major consumed vegetable crop in Colombia, with a planted area of 15.000 hectares yielding 450.000 tons per year (UNAL, 1997). In Colombia, this crop is highly affected by several pests and diseases, and abiotic stresses such as drought, high and low temperatures, and salinity. Since 1985, the vegetable breeding program at the Universidad Nacional de Colombia, Palmira Campus, has as main objective the development of varieties with resistance or tolerance to some of these traits. In 1997, this program released the tomato variety UNAPAL Arreboles, which has several traits attractive to tomato growers such as fruit firmness and good adaptability specially to the Valle del Cauca region. But this variety is susceptible to one of the major limitations to tomato production in this region: the budworm (*Tuta absoluta*), which eats the tomato buds and young leaves. It had been difficult to breed tomato resistant to this pest by standard breeding. The only sources of resistance genes is from wild tomato species which are incompatible with the cultivated tomato, and so far the attempts for an inter-specific breeding program has not been successful (Lourencao et al., 1985). The main objective of this work is to transform the tomato variety UNAPAL-Arreboles with the Bt gene cryIA(b), which had been used successfully to obtain resistance against Lepidoptera pests in various economical important crops (i.e. maize, cotton).

In previous reports it was described the evaluation of three protocols commonly used for tomato callus induction and plant regeneration (Fillatti et al., 1987, Narvaez, 1993, and Ultzen et al ., 1995). Results indicated that the highest response for callus induction and plant regeneration is noted on M3 medium sequence (Ultzen et al ., 1995). An increase in response of about 2-fold and 4-fold on callus induction and plant regeneration was noted on M3 media respect to the other media tested. The lowest response was obtained on M2 medium (Fillatti et al., 1987).

Two *Agrobacterium* mediated transformation protocols commonly used for tomato (McCormick et al., 1986; Fillatti et al., 1987), were tested using the tomato variety UNAPAL-Arreboles. Agrobacterium strains C58C1, Agl1 and LBA4404 containing the pBIGCry construct (L.I. Mancilla at CIAT) were used. This gene construct contains the *cry1Ab* gene driven by the 35s CaMV promoter, the nptII gene for kanamycin resistance as selection markers, and the gus-intron as a reporter gene. Transgenic plants were identified by southern blot analysis. Inheritance of gus expression and kanamicine resistance was evaluated from T0 to T1 generation. Clonallys propagated plants of the original T0 plants were evaluated for agronomic traits in the greenhouse.

A total of four hundred tomato explants (cotyledonary leaves of 7-10day-old plantlets) were infected with LBA4404/pBIGCry weekly. After co-cultivation for 48 hour, about 10% of the explants were analyzed for gus transient expression. Explants from cultures showing transient expression were transferred to selection media containing kanamycin for selection. After three weeks on selection media, regenerated plantlets were recovered. The number of transformed plantlets isolated varied among the different experiments. From 0 to ten plants were recovered per experiment.

A total of 59 putative transgenic plants were produced from 8 experiments (400 explants by experiment). This shows an efficiency of 1.84 % for recovering kanamycin resistant plants from the initial agro-infected explant. Of these plants 15 were transferred successfully to the greenhouse and 6 plants had shown stable gus expression throughout the vegetative and reproductive life cycle.

Last year it was carried out the morphological and molecular characterization of kanamycin-resistant and gus-expression P28, P33 and P47 clones at To and T1 generation. Selfed progeny derived from T0 plants (T1 clones) was evaluated for agronomic traits and resistance to kanamycin and Gus expression. No differences were noted neither between the T0 and T1 plants, nor between the transgenic plants and the tomato control plant for the various morphological traits evaluated (plant height, node lentgth, leaf type, presence of pubescence in stem, flower color and shape, fruit color and shape). These results indicated that no somaclonal variation is apparent in the transgenic plants. Preliminary results on the molecular characterization of clones T0 and T1 by PCR and Southern analysis suggested that nptII and gus-intron genes are inserted in the genome of these plants. The patterns of segregation for gus expression and kanamicine resistance of the selfed progeny of T0-28, T0-33 and T0-47 clones were 3:1 for both genes, indicating the insertion of an active locus for each of these genes. Variation was noted on the co-segregation of the two genes: 95% for selfed progeny of T0-28, 82% for T0-33 and 63% for T0-47.

This year it is reported the final biochemical molecular and insect-bioassay analysis of the UNAPAL-Arreboles transgenic materials.

Materials and methods

The T0 and T1 tomato transgenic clons were analyzed by PCR and Southern blot for the presence of nptII, gus-intron and cry1Ab transgenes, according to Sambrook et al., (1989) and Gonzalez et al., (1995) . The expression of the cry1Ab gene at the protein level in the transgenic plants was carried out using an ELISA kit (EnviroLogix Inc), according to the manufacturer protocol. And the insecticidal activity of the transgenic plants towards tomato larvae budworm (*Tuta absoluta*) was analyzed performing an insect-bioassay. Each of the T0, T1 and control plants were infected with 20 insect eggs and sixteen days after plant infection it was recorded leaf tissue damage, larva survival and larva weigh.

Results and Discussion

This year it was confirmed the presence of nptII and gus-intron genes by PCR and Southern analysis. A 0.7 Kb nptII and a 0.65 Kb gus-intron fragments, corresponding to the expected size, were amplified by PCR in all the transgenic plants, but these fragments were not present in the negative untransformed plants. These results were confirmed by Southern analysis. DNA from T0 and T1 transgenic tomato plants was digested with PstI and probed with the 0.65 Kb gus-intron or 0.7 Kb nptII-PCR fragments labeled with 32P. Gus-PCR probe hybridized a 1.7 Kb band, while nptII-PCR probe hybridized a 1.0 Kb band which were present in both the positive control and the transgenic plants, but were absent in the DNA of the not transgenic plants.
On the other hand, DNA digested with HindIII and BamHI probed with the 0.65 Kb gus-PCR fragment labeled with 32P, reveled that the transgenic plants present more than 10 gus-intron gene copies, with one gene insertion in T0-28 and T0-33 clones and two gene insertions in T0-47 clon. Similarly, DNA digested with XbaI and EcoRI probed with the 0.7 Kb nptII-PCR fragment labeled with 32P, reveled more than 10 nptII gene copies in the transgenic plants, with one gene insertion in T0-28 and two gene insertions in T0-33 and T0-47 clones.

PCR analysis for the presence of cry1Ab gene reveled a 1.2 Kb expected band, in both positive control and the transgenic plants, unfortunately this same band was also observed in the negative not transformed plants. This result indicate that cry1F and cry1R primers could be annealing unspecific regions in the tomato genome.
Genomic DNA digested with EcoRI probed with the 1.2 Kb cry-PCR2 (derived from pBT1291 plasmid), reveled a 3.2 Kb band in both transgenic plants and negative not transformed plants , while the positive (plasmid) control reveled a 0.5 Kb band. Similarly, genomic DNA digested with XbaI probed with the same 32P labeled probe (cry-PCR2) reveled a 0.4Kb band in both transformed and not transformed materials while the positive control showed a 3.5 Kb band. These results suggest possible changes in the cry1Ab gene sequence or possible rearragements in this region.
The inmunoassay analysis of these transgenic materials reveled that the amount of cry1Ab toxin was either too low to be detected by the ELISA test or the transgenic materials are not expressing the cry1Ab transgene.

An insect bioassay using T1-28 transgenic plants and not transformed plants indicated a *Tuta absoluta* larva survival of 92.5 and 95% respectively after 16d eggs plant infestation; while the percentaje of leaf tissue damage was 100% and the larva weigh average was 3.6 mg in both transformed and not transformed plants. These results are in concordance with the results obtained in the inmunoassay test.
To discard the possibility of insect resistance to cry1Ab toxin, it was evaluated the effect of the bioinsecticide Turicide (5 g/l) in larva survival. The results of this assay showed that the application of the bioinsecticide reduced significatively the three parameters evaluated: leaf tissue damage in 50% , larvae survival in 63.7% and larvae weigh in 61.5%. These results are indicating that this insect is not resistant to the cry1Ab toxin; for this reason the high level of larva survival in the tomato transgenic plants indicate that the cry1Ab gene is not expressed in these materials.

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2.2.14 Field evaluation of the agronomic performance of soursop (*Annona muricata* L.) clones propagated through *in vitro* micrografting

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[1]Project SB02; [2]Corporación BIOTEC; [3]Project supported by Pronatta; [5]Independent consultant; [8]Universidad Nacional de Palmira

Introduction

Between 1996 and 1999, a methodology was developed for *in vitro* propagation of selected clones of soursop (or guanábano in Spanish, *Annona muricata* L). This methodology consists in the *in vitro* micrografting of buds over rootstocks obtained from *in vitro* germitated seeds. The buds used are obtained either from shoots cultured *in vitro,* isolated from clones growing in the greenhouse or from micrografts produced previously (cyclic micrografting). *In vitro* propagation of plants offers many advantages over traditional methods of vegetative propagation, however some propagation methodologies have been shown to produce a variable proportion of abnormality in the growth of the plants, known as somaclonal variation. Somaclonal variants can be a real problem when *in vitro* propagation is used for supplying commercial plantations with planting material.

The occurrence of somaclonal variations in *in vitro* propagated plants has been attributed to genetic or epigenetic changes caused by the use of methodologies which involve cellular dedifferentiation, callus formation or direct production of adventitious buds.
Since in the soursop propagation process no adventitious formation of new buds is involved, and only the "natural" system of axilary bud re-growth is used for the production of new buds, no genetic changes are expected to occur in the propagated trees. However the induction of epigenetic changes such as pleiotropy or juvenility or simply root malformation (see George, 1993), can not be excluded.

In order to test if the developed propagation methodology is useful for producing true-to-type planting material, we started the evaluation of trees propagated through *in vitro* micrografting at CIAT and in farms located in different soursop producing zones of Colombia in February 2000.

Methodology

The evaluated plants were obtained from the clone Elita (Rios Castaño and Reyes, 1996) micrografted over rootstocks of the same variety. These were planted in January 1999 in farms belonging to experienced soursop growers located in Huila and Valle or later at CIAT in February of 2000. At CIAT besides the micrografted plants, other plants produced through the traditional grafting method (kindly provided by the Profrutales nursery) were planted. Micrografted plants were 8 months old while normally grafted plants were 10 months old at the time of planting at

CIAT. Preliminary data on parameters related to the vegetative growth of the trees, flowering, fruit set, production, and fruit quality have been collected while more detailed data collection is on going.

Results

Comparison of effects of *in vitro* micrografting and traditional grafting methods on the vegetative growth of trees

In order to study if plants produced through *in vitro* micrografting are different from those produced through traditional grafting methods, the vegetative growth, fruit production and fruit quality of trees from the same genetic background and propagated through both methodologies are being evaluated at CIAT.

Tree height, volume of the canopy and perimeter at the grafting site, were taken as indices of vegetative growth during the first 12 months. But because the micrografted plants have to be pruned starting from the 12th month after planting (MAP) in order to initiate tree formation, from this time onwards only the perimeter of the stem could be taken into account, in order to have a reliable estimate of tree growth.

Until 30 MAP, the growth rates of the tress propagated through both systems are the same in the field. (Fig. 1). The small differences in size of the stems shown by the different propagation methodologies can be attributed to the differences in age and size of the trees at the time they were planted in the field.

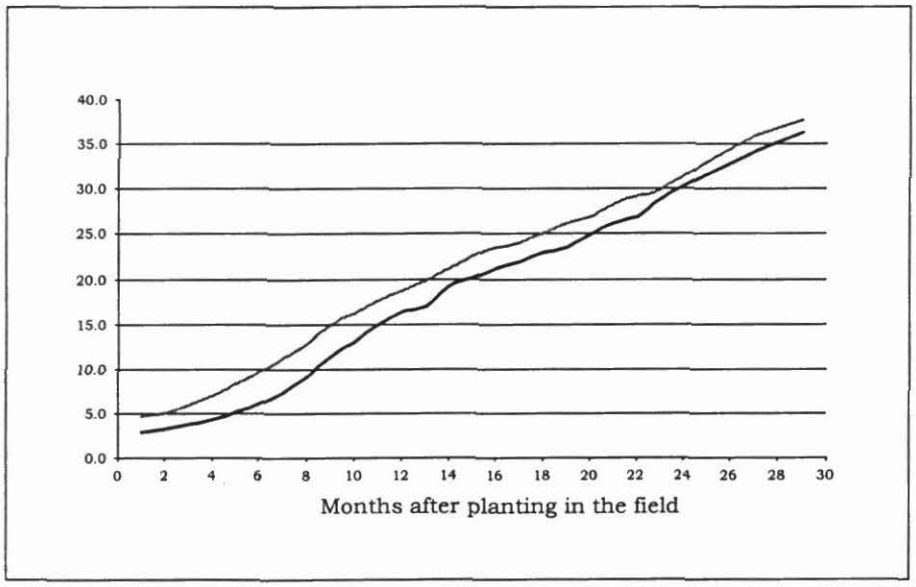

Figure 1 **Comparison of the vegetative growth of trees of the combination Elita/Elita propagated *in vitro* by micrografting (darker line) and through traditional grafting methodologies (lighter line), planted at CIAT in February 2000.**

Comparison of flower set and fruit production of trees propagated by *in vitro* micrografting and by traditional grafting methods

Just as was the case with trees propagated by traditional methods, micrografted trees initiated flowering at 11 MAP and produced the first fruits at 15 MAP in the field. Trees propagated by both methods showed a cyclic behavior in flowering and fruit production, which possibly coincided with the seasonal climate changes at CIAT (Figure 2).

Although these are preliminary results because soursop trees stabilize their production only after 7 years of planting, the measurements made so far, led to the conclusion that the plants propagated through *in vitro* micrografting suffered no delays in flowering and fruit production when compared to those propagated through the traditional methods.

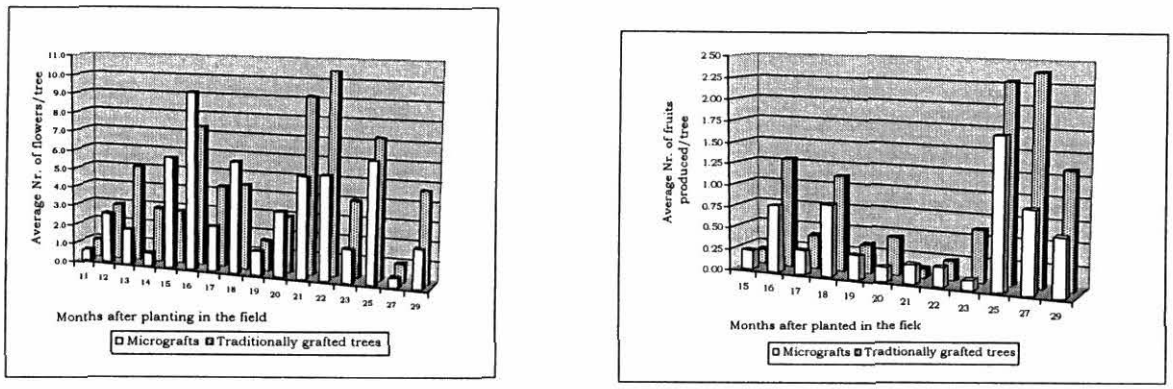

Fig. 2 Flower and fruit production per tree of Elita/Elita micrografted plants planted in the field in February 2000.

Field evaluation of new combinations of scions from selected clones and rootstocks of soursop and related species

Hitherto, only plants from one combination of scion and rootstock (Elita /Elita) have been evaluated under different field conditions. During the first half of 2002, a total of 11 new combinations were planted at Yaguará (Huila) and La Esneda (Valle; Table 1). Additionally, more than 1400 trees of similar combinations will be planted in other locations at the end the year 2002 and the beginning of 2003.

Conclusions

No genetic or epigenetic modification, which can be attributed to the propagation process, has been observed in any of the trees propagated through the *in vitro* micrografting method.

The *in vitro* micrografting propagation procedure is a safe method for producing genetically uniform and disease-free planting materials of this species.

Table 1 Micrografted plants of different combinations of scion and rootstocks planted for field evaluation during the year 2002 in farms belonging to experienced soursop growers

Scion	Rootstock	Quantity of plants
San Francisco, Yaguará - Huila		
Elita	Cristina	84
Elita	Rosa	68
Rosa	Elita	43
Rosa	Cristina	42
Elita	Elita	19
Cristina	Rosa	13
Cristina	Elita	9
Cristina	Cristina	8
Elita	*A. montana*	8
Rosa	Rosa	4
Rosa	*A. montana*	2
Total		300
La Esneda - Valle		
Cristina	Rosa	20
Cristina	Elita	20
Rosa	Elita	20
Elita	Rosa	20
Total		80

Future plans

- The project from which this field evaluation has been funded (PRONATTA project No. 981763225) comes to an end in December 2002. This activity will be continued if additional funds are received.

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2.2.15 Optimisation of the hardening process of *in vitro* micrografted plantlets of soursop (*Annona muricata* L.) under greenhouse conditions using different substrates

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[1]Project SB02 CIAT; [2]Corporación Biotec; [3]Project supported by Pronatta; [4]National University of Colombia, Palmira; [5]Independent Consultant.

Background

The hardening process of *in vitro* propagated plants in *ex vitro* conditions, normally carried out in a greenhouse, is one delicate step in any propagation process and is usually characterized by high mortality rates of the plantlets. In past years we have made important advances in the development of methodologies for *in vitro* clonal propagation of soursop (*Annona muricata* L.), but the hardening of micropropagated plantlets in the greenhouse has not received enough attention. During the year 2002 we continued with the optimisation of this process by the use of different combinations of substrates that are by-products of the sugar and paper industry in the Valle del Cauca region of Colombia. Some of the substrate components investigated were reported by Bruzon (1997) as having excellent properties for sustaining plant growth under greenhouse conditions. Additionally, the effect of microrrhization of the plants on their survival and vegetative growth is also being investigated.

Methodology

Two-month old micrografted plantlets of the clone Rosa over the rootstock Elita were used. They were transferred to the greenhouse and planted in different substrates that contained CIAT soil, sand, cachaza (filter cake), coal ash, rice shells or pine bark chips. Most of these components are by-products of the sugar or paper industry. Alternatively, the plants were inoculated with vesiculo-arbuscular (VA) micorrhizal fungi (Table 1) in D16 Deepot™ containers (Hummert International).

Table 1. Description of the vesiculo arbuscular micorrhizal fungi used (CIAT 2000)

No	Mycorrhiza	Altitude (m)	Host Plant	Collector	P (mg/kg)	pH	Al (meq/100g)	MO (%)	Cant (e/g)*
1	*Gomus. Desertícola*	200	Grasses	Sieverding	1	4.5	2.2	3.3	610
2	*Gigaspora margarita*	200	Grasses	Spain		4.5			42
3	*Gigaspora rosea*		Glycine max	D. Pellet	48	7.3	0	1.9	40

Results

Survival of *in vitro* micrografts planted on different substrate combinations in the greenhouse.

A very highly significant effect of the substrate type on the survival and growth of the micrografted plants in the greenhouse was found. While only less than 50% of the plants survived when planted on CIAT soil, substrates containing "cachaza" and coal ash, showed survival rates of over 70%. The highest survival rate was achieved with the substrate combination S2 (Table 1) which contained 3

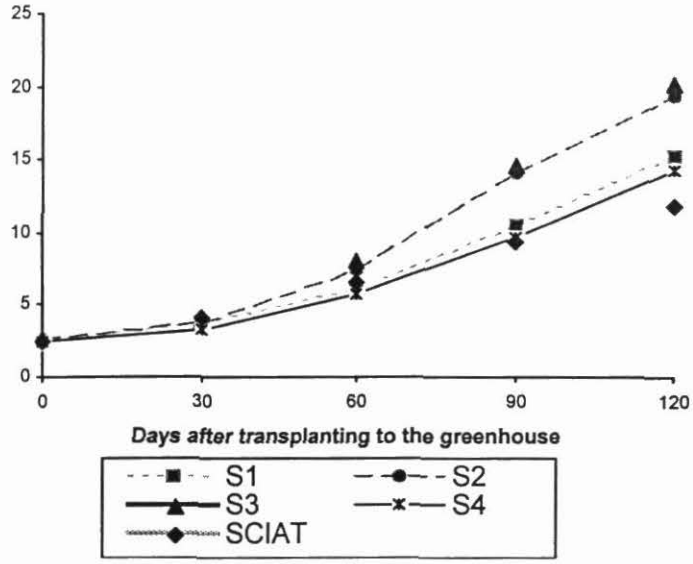

Figure. 1 ***Height reached by the scion*** **after 0, 30, 60, 90 and 120 days of culture of the micrografts on different substrates in the greenhouse. SCIAT = CIAT soil.**

parts "cachaza", one part of coal ash and one part *Pinus* bark chips. The highest growth however was shown by the micrografts planted on substrate S3 (Figure 1)

The high survival rate achieved on the S2 substrate can be attributed not only to the nutritional properties of the substrates (its high contents of phosphorus, nitrogen, calcium and organic matter; Table 2), but also to its better physical properties as compared to CIAT soil. These results confirm observations made by Bruzón (1997) with similar substrates.

Table 1. Survival rate of micrografts planted on different substrates.

Substrate	Component Proportion				Survival rate (%)
	CIAT Soil	"Cachaza" Filter cake	"Carbonilla" Coal Ash	Pinus Bark chips	
S1		3	1		71.43
S2		3	1	1	95
S3	1	2	1	1	76.67
S4	1	3	1		80
CIAT-Soil	*soil-sand 1:1*				48.89

Height, number of leaves, dry matter, leaf area and root volume of micrografts after 4 moths of culture on different substrates in the greenhouse:

Significant differences between substrates for plant height (Pr = <0.0001), but not on number of leaves (Pr = 0.5196 and 0.2398; Figure X) were found. Through Duncan Test we could identify S2 and S3 as the best combinations (Table 3). In contrast, through LSD test, a positive effect of substrates on dry matter, foliar area and root volume was found, with the substrates S2, S3 and S4 being the best in this regard.

Table 3. Mineral content, nutritional and physico-chemical properties of the components and the substrates used.

Substrate	N	P	K	Ca	Mg	MO	C.O	C/N	PH	Al
		%		g/kg		(%)				(meq)
Rice shells	0.45	0.14	0.337	0.81	0.61	67.90	33.95	75.87	-	-
Pinus Bark	0.26	0.02	0.005	2.97	0.44	76.00	38	145.04	3.96	3.17
Coal Ash	0.19	0.02	0.004	1.09	0.27	1.80	0.9	4.83	6.91	-
CIAT Soil	0.17	0.02	0.010	0.72	0.20	3.60	1.8	10.35	5.35	0.36
"Cachaza"	0.71	0.88	0.086	4.42	1.97	14.00	7	9.89	6.85	-
S1	0.51	0.69	0.063	3.81	1.54	9.80	4.9	9.63	7.04	-
S2	0.53	0.57	0.074	4.38	1.51	21.20	10.6	20.08	6.11	-
S3	0.31	0.32	0.043	2.97	0.85	12.00	6	19.23	6.51	-
S4	0.46	0.45	0.053	3.23	1.15	8.40	4.2	9.05	6.72	-

Table 4. Dry matter, leaf area, root volume, plantlet height and number of leaves of Rosa/Elita micrografts, after four months of culture in the greenhouse on different substrates.

Substrate	Volumetric Mixture (V/V)					Dry matter	Foliar area	Root volume	Height	Leaf number
	Soil	Sand	Cachaza	Coal Ash	*Pinus* bark chips	(%)	(cm^2)	(cm^3)	(cm)	
S1			3	1		19.39ab	168b	1.5b	6.76b	4.58a
S2			3	1	1	**20.30a**	**306.7a**	**3.5a**	**8.44a**	**4.68a**
S3	1		2	1		20.03ab	266.97a	3.12a	7.96a	4.64a
S4	1		3	1		18.35ab	318.72a	3.12a	6.75b	4.64a
CIAT soil	1	1				17.57b	120.55b	2.12b	5.66b	4.33a
Average						19.13	236.18	2.67	7.11	4.57
C.V.(%)						9.06	25.33	32.77	25.63	26.71
Pr>F						0.2014	0.0016	0.0376	<0.0001	0.5196

Values for each parameter within columns followed by a similar letter are not significantly different from each other ($Pr \leq 0.05$, Duncan's Multiple Range Test)

Based on these results, substrate S2 has been chosen for culturing all micrografts produced in the greenhouse. Micografts cultured on this substrate after 4 months attain sizes sufficient for surviving transplanting to the field.

Effect of the innoculation of micrografted plants with Vesiculo-Arbuscular Micorrhizal Fungi in the greenhouse

Four months after transfer to the greenhouse and inoculation, a low colonization of roots (between 12 and 25% of the evaluated rootsegments) was found. This colonization did not have any significant effect on micrograft height ($Pr=0.1973$). However its effect on the number of leaves was significant ($Pr=0.0184$).

Through microscopic observations, a high proportion of collapsed spores were found. Its presence as well as the low root colonization can possibly be caused by the high content of phosphate in the substrates or its high pH (Koide and Schereiner, 1992)

Conclusions

An improvement of survival and growth rate of soursop micrografted plants was achieved through the use of substrates containing "cachaza", coal ash and *Pinus* bark chips (substrates S2, and S3). These substrates also showed excellent physico-chemical properties.
No effects of VA-Micorrhizal fungi on micrograft growth were observed possibly due to the high phosphate contents of the substrates and their high pH.

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2.2.16 Optimization of the *in vitro* propagation methodology of selected clones of soursop (*Annona muricata* L.) and evaluation of the compatibility of different scion and rootstock combinations for *in vitro* micrografting

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[1]Project SB02; [2]Corporación BIOTEC; [4]Project supported by Pronatta; [5]Project supported by Fundación Banco de la República

Introduction

Between 1996 and 2001, we developed a methodology for the *in vitro* clonal propagation of elite selections of soursop (or guanábano in Spanish, *Annona muricata* L; Royero *et al.*, 1998), a fruit tree that is native to tropical America. The developed methodology allows a rapid clonal multiplication of selected trees and the production of disease-free plants. The plants produced through *in vitro* micrografting have been found to develop normal and healthy growing trees that produce the first fruits 15 months after planting (MAP) in the field.
The developed micrografting technology fulfills all the requirements for ridding planting materials of diseases. However before it can be applied for large-scale propagation, some critical steps in the propagation process have to be optimized.
In 2002 several steps involved in the propagation were reevaluated and media modifications were tested. Furthermore, the compatibility of scions from different selected clones of soursop micrografted over different rootstocks from the same and/or other related annonaceus species were studied at the laboratory and greenhouse levels.

Methodology

The general methodology for *in vitro* propagation of soursop through micrografting has been described in previous reports (Royero *et al.* 1998 and 1999).The composition of the media and solutions used in the whole process is presented in the tables 1 and 2.

Table 1. Composition of the culture media previously used and of the new improved media (in bold) for the propagation process of soursop through micrografting

Use	Medium denomination	Salts g/L	Sucrose g/L	B5 Vitamins g/L	Casein-Hydrol. g/L	BAP mg/L	GA_3 mg/L	PVP g/L	Agar[1] g/L
Culture of shoot pieces for axillary bud induction	M-I	WPM[2]	20			1			4.8
	RO-BAP 1	MS[3] 1/2	20	0.112	0.2	1			4.2
Culture of shoot pieces for axillary bud elongation	M-II	WPM	20			0.2			4.8
	RO-BAP 0.2	MS 1/2	20	0.112	0.2	0.2			4.2
Seed germination	M-III	B5[4]	20				0.5		4.8
	RO 1/2 GA3	MS 1/4	10	0.056	0.2		1		4.2
Micrograft culture and development	M-IV	WPM	20	0.112					4.8
	RO 1/2	MS 1/4	10	0.056					4.2
Micrografting solution	M-V	WPM	20					1	
	T3	WPM	20		0.2	0.2		1	

1 From Duchefa, Netherlands
2 Lloyd y McCown 1981, purchased from Duchefa, Netherlands
3 Murashige and Skoog 1962, purchased from Duchefa, Netherlands
4 Gamborg et al. 1968, purchased from Duchefa, Netherlands

Table 2 . Composition of the different antioxidant treatments evaluated during micrografting of combinations of scions of the clone Rosa with rootstocks of Rosa, *A. montana* and *A. glabra*.

Treatment	Basal medium	Antioxidant (g/l)	Additional compounds	Growth regulator mg/l
T0	Half concentrated MS[2] Salts	PVP[3] 1	-	-
T1	WPM[1] Salts	PVP 1	Sucrose 2%	-
T2	WPM Salts	PVP 1	Casein hydrolysate 200 mg/l Sucrose 2%	-
T3	WPM Salts	PVP 1	Casein hydrolysate 200 mg/l Sucrose 2%	BAP 0.2

1Lloyd and McCown, 1981
2 Murashige and Skoog, 1962
PVP = Polyvinylpyrrolidone

Results

Evaluation of different antioxidant solutions used during the process of *in vitro* micrografting
Cut explants of woody species cultivated *in vitro*, are characterized by the exudation of phenolic compounds that oxidize in contact with the air, forming a dark precipitation. This phenomenon is known as phenolization or oxidation and when it is severe, can cause the explants to die. Soursop as a woody tree is not an exception, and tissue phenolization greatly affect the success of micrografting. We have in the past years tested the effect of different antioxidants in reducing

phenolization during the micrografting process, and found polyvinylpyrrolidone (PVP) as being the best. In 2002 we investigated the effect of different modifications to the micrografting solution used on the efficiency of graft union, and in supporting growth of the micrografted scion. The results are shown in fig. 1. Compared to the antioxidant solution used before, rates of graft union and development of the scion after micrografting were improved by using micrografting solutions that in addition to the antioxidant were supplemented with WPM salts, sucrose and casein hydrolysate. But the best results regarding the scion development were obtained when the antioxidant solutions were supplemented additionally with the growth regulator BAP. This growth regulator or the combination of it with the other components of the solution also promoted a fast growth of the scion allowing the plantlets to be transferred to the greenhouse in less than 6 weeks after micrografting.

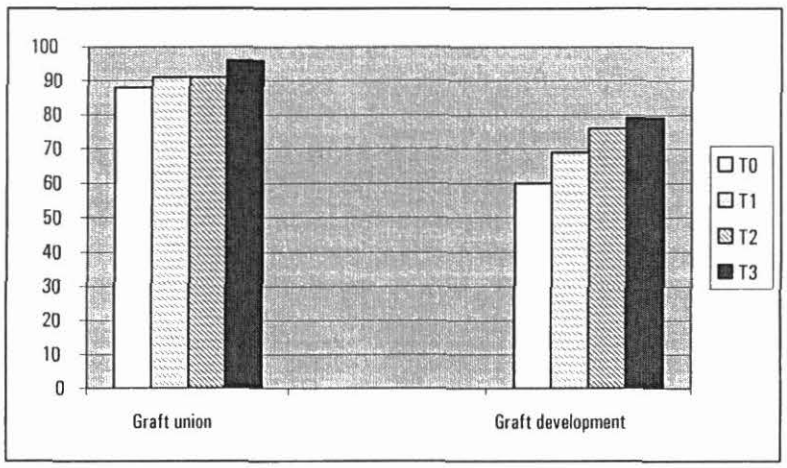

Figure 1. Evaluation of the effect of different antioxidant-solutions used during micrografting, on the graft union and on the development of the micrografted bud. Buds of the clone Rosa were micrografted on rootstocks of Rosa, A. montana and A. glabra. The composition of the antioxidant solution is explained on table 2.

Evaluation of the culture media used.

The *in vitro* phase of the propagation methodology of soursop through micrografting consists of many step: (1) seed germination *in vitro* (for rootstock production); (2) induction of growth of axillary buds, (3) elongation of axillary buds (for scion production), (4) culture of micrografts and (5) culture of mother micrografts (*in vitro* micrografts used as source of buds for the production of new micrografts). Each of these steps required a different culture medium of a different composition (Table 1). In 2002 these culture media were revised and their preparation simplified by using only one source of a ready to use salt mixture as the basal medium. The newly developed media can be prepared easier and faster. Also by using them an improvement on the success rate of every step involved in the propagation process was achieved (Fig. 2) , allowing the performance of the propagation with more efficiency than before. The most important modification of the culture media is the use of the MS-salt mixture in half or one-quarter concentration, instead of the complete WPM salts.

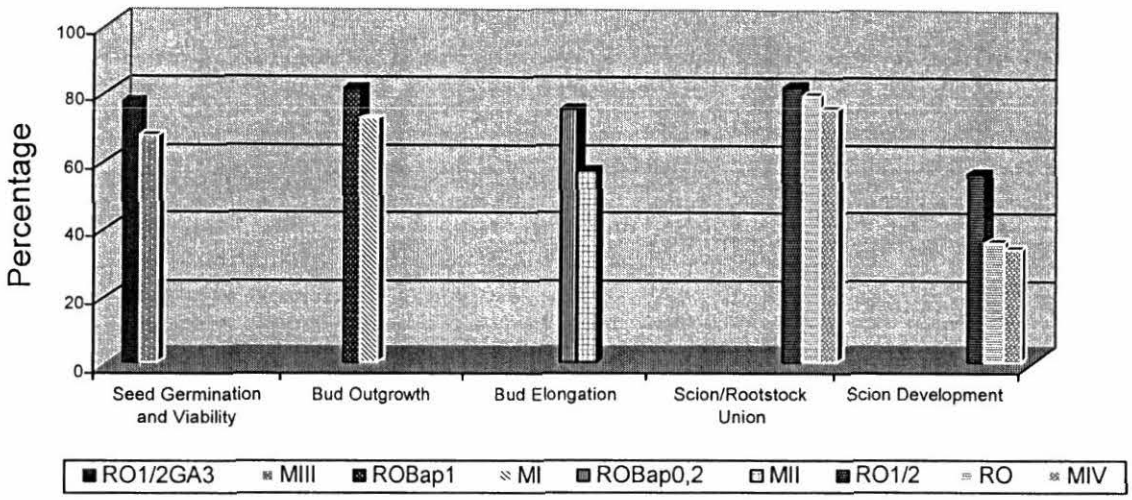

Fig.2 Comparison of the overall success rate achieved in the different steps of the propagation of soursop through *in vitro* micrografting with the old and the new culture media.

Use of rootstocks of other *Annona* species for the production of more vigorous, disease resistant and widely adapted soursop trees

The use of rootstocks of different genotypes or species from that of the scion for the production of vigorous, widely adapted or disease resistant plants is a common practice in fruit tree propagation. In soursop this avenue has been largely under-exploited.

We are investigating both at the laboratory and greenhouse levels, the compatibility of scions of different selected clones of soursop and different rootstocks of the same species and of the related species *A. montana* and *A. glabra*. The results of the micrografting experiments of rootstocks of these species with scions of the clone Rosa are presented in the figure 3. Regarding micrograft union (measured 15 days after micrografting) no significant differences were found between the different rootstocks. However regarding the development *in vitro* and in the greenhouse of the micrografted buds, significant differences were found. Surprisingly, it was the combination Rosa/A. montana, and not the micrografts of the scion of Rosa over its own rootstocks that was the combination that has shown the highest frequency of development. This combination is also the one that shows the highest growth rate in the greenhouse (data not shown).

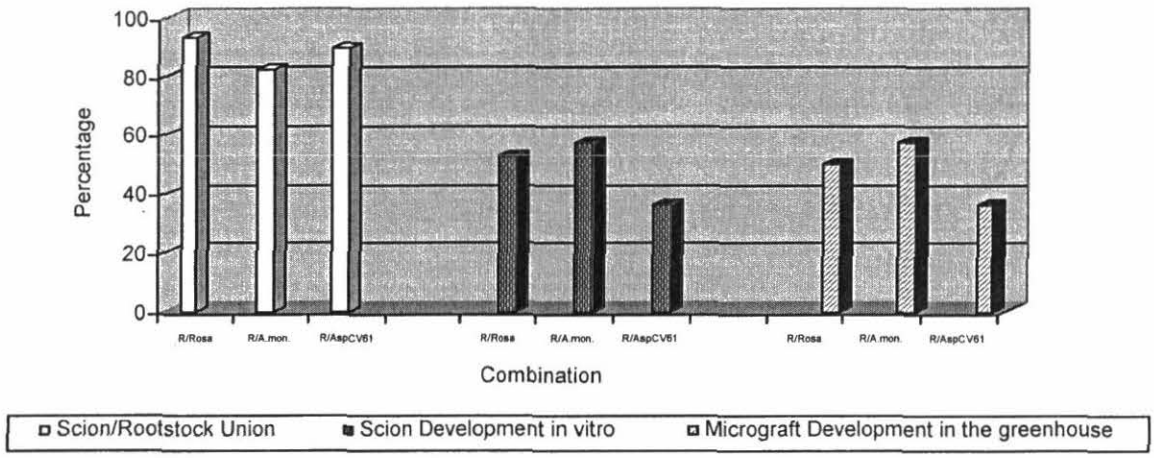

Fig.3 Percentage of union and development *in vitro* and in the greenhouse of scions from the clone Rosa of soursop micrografted over rootstocks of Rosa, *A. montana*, and *A. glabra*.

Total number of plants produced from different combinations of scion and rootstocks

In 2002, a total of 1871 micrografted plants were produced from different combinations of scion and rootstocks (Table 3). All of these will be planted in the field for further evaluation of their agronomic performance under different agroecological conditions.

Conclusions

With the newly evaluated media and solutions, an improvement on the success rate of every step of the propagation process was achieved

The survival of the micrografted plants in the greenhouse depends largely on the development achieved by them in the *in vitro* phase. With the combination Rosa/A. montana V54 the highest survival rate so far in the greenhouse, 68.4%, has been achieved.

The *in vitro* micrografting propagation methodology could be applied to 3 different soursop clones (all the clones evaluated) and 5 different rootstocks from the same and other annonaceus species (also all the rootstocks evaluated).

Efficiency of development of micrografted plantlets *in vitro* should be improved in order to apply the technology for massive propagation of soursop clones.

Table 3.Overall number of micrografts made, and micrograft development efficiency with different combinations of soursop scions and rootstocks of soursop or related annonaceus species between June 2001 and May 2002. Cristina, Elita, Rosa and Francia are selected clones of soursop (*Annona muricata* L.).

Scion/Rootstock Combination	Total of micrografts made	Micrografts showing graft union	Micrografts showing bud development	% of micrograft development
Rosa/*A. montana* V54	38	29	26	68.4
Elita/Rosa	260	207	157	60.4
Rosa/Rosa	289	268	174	60.2
Rosa/Elita	911	835	542	59.5
Cristina/Rosa	310	245	182	58.7
Francia/Cristina	178	150	99	55.6
Rosa/*A. glabra* CV61	282	258	154	54.6
Rosa/Cristina	100	90	54	54.0
Elita/Cristina	512	434	264	51.6
Cristina/Elita	273	234	136	49.8
Rosa/*A. montana* V51	124	93	55	44.3
Cristina/Cristina	100	39	28	28.0
TOTAL	3377	2882	1871	55.4

Future plans

- To improve the methodology of production of rootstocks *in vitro*, which is the most limiting step in the process of scaling up of the propagation methodology.
- To include more elite selected clones and more rootstocks in the propagation and evaluation process.

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Activity 2.3 Identification of points of genetic intervention and mechanism of plant stress interation

Main Achievements

- More than 1500 cassava root samples were analyzed for variation for carotene contents in cassava roots An HPLC pre-column and column, specifically designed for carotene quantification, was purchased and up to 100 root measurements were made using the HPLC system. Two relevant results were obtained. First, the more precise HPLC measurement showed, on average, a 26% increase in total carotenes compared with the colorimetric method. Second, about 93% of the total carotene measured was represented by β-carotene, which has the highest vitamin activity

- The nutritional and agronomic traits in roots and foliage of cassava clones from the germplasm bank and breeding project at CIAT were evaluated. Results observed in the large samples analyzed demonstrated that cassava roots and particularly the leaves are a valuable source of carotene, One additional advantage of the high carotene level was the reduction the physiological deterioration of roots.

- Brachiaria genotypes from the mapping population were identifying with contrasting aluminum resistance. 38 individuals were evaluated for several physiological traits. While the Al-resistant parent (B. decumbens) had the highest Al-resistance index, several genotypes had an almost similar AL resistance index. Evaluation of the lateral swelling of roots, demonstrated that roots of genotypes classified as Al resistant tended to swell less than those of Al-sensitive genotypes.

- The susceptibility of *Brachiaria decumbens* and *B. ruziziensis* to lanthanum (La^{3+}) and other lanthanide ions using relative root elongation (RRE) of seedlings as a measure of resistance was evaluated to study factor contributing to aluminum resistance of *Brachiaria decumbens* .The results obtained with La^{3+} show that Al-resistant *B. decumbens* is more resistant to La^{3+} ions than Al-sensitive *B. ruziziensis.*). In all other plant species tested thus far, genotypes differing in their resistance to Al^{3+} ions are equally sensitive to La^{3+}

2.3.1 Chracterizing B-carotene Pathways Genes in Cassava

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Introduction

The carotenoid pigments are essential components of photosynthetic organisms and have a great variety of functions in plants. The formal pathway of carotene biosynthesis is well know from chemical research and genetic and inhibitor experiments. Carotenoids derived from plant food sources are converted to vitamin A and other important compounds humans need for growth and development; moreover, certain carotenoids have been identified for their role in protection against cancer, in promoting immune responses and as antioxidants.

Vitamin A deficiency (VAD) is considered as the third most important nutritional disease for its high level of prevalence. In many countries where VAD is a serious problem, cassava is a basic staple; however its nutritional quality is poor because it lacks proteins and vitamins, and nearly 90% of its dry weight is represented by carbohydrates. In most clones, the edible portion of the root is white and devoid of any carotene, in contrast to the yellow-pigmented roots, which are rich in carotene but not widely cultivated.

Our work seeks to increase the knowledge of genes that code for specific steps in the metabolism of the carotenes, to improve the carotene content of cassava roots and enhance their nutritional value. In addition we are interested in isolating and characterizing tissue-specific promoters for cassava roots. In order to achive this goal we are characterizing the genes *phytoene synthase*, (Psyn) *phytoene desaturase* (PDes) *z-carotene desaturase* (CDes) and *b--lycopene ciclase* (Blyc), all of which are involved in the biosynthesis pathway of carotenes.

Methodology

Using Clustal X 1.62 software program (Intelligenetics, Mountain View, CA), the consensus sequences were identified for each of the aforementioned genes by sequence analysis of cDNA clones reported in GenBank NCBI (www.ncbi.hml). A set of primers for each of the consensus sequences was generated using Primer 3® software. The designed primers were used to amplify orthologous sequences in the cassava genome.

Genomic DNA was extracted (Dellaporta et al., 1983) from leaves of M Per 297 (high carotene content) and CM 523-7 (low carotene content), previously characterized by HPLC for carotene content within the cassava core collection at CIAT. Total RNA was extracted from fresh roots of each genotype by following the method developed by Relly et al (2001).

The PCR reaction from genomic DNA was carried out in a 25 ml of 1X PCR buffer containing 100 hg of DNA, 0.6 mM each of the reverse and forward primers, 2.5 mM $MgCl_2$, 0.24 mM dNTPs, 2 U *taq* polymerase, incubated at one cycle of 94°C (1 min), followed by 35 cycles of 94°C (45 s), 50°C (45 s), 72°C (45 s) and one cycle of 72°C (5 min).

cDNA obtained with the SMART™ cDNA Synthesis Kit (Clontech) was used as the template for PCR amplification of carotene genes. The same primer-pair combinations used with genomic DNA as the template were used when cDNA from cassava roots was used as the template in the PCR

reaction. Beside the same primer pair was used in the cDNA amplyfication, there were changes in the reaction conditions to optimize the PCR. All PCR reactions were carried out in a final volume of 25ml with 1X PCR buffer (Pelkin Elmer), 0.6 mM of each primer, 250 hg of cDNA, 0.24 mM of each dNTP and 1U *taq* polymerase (Pelkin Elmer). Depending on the primer combination and genotype, the concentration of $MgCl_2$ and annealing temperature differed from 2.0-4.0 mM and from 48-52°C, respectively. All the reactions were incubated at one cycle of 94°C (1 min) followed by 35 cycles of 94°C (45 s), annealing temperature (45 s), 72°C (45 s) and one cycle of 72°C (5 min).

PCR products were visualized by electrophoresis in agarose gel, the resulting bands were purified using a Qiaquick Gel Extraction® Kit (Qiagen) and cloned into pGEM-T cloning vector (Promega). The vector was used to transform *Eschlerichia coli* DH5a using the CELL PORATOR® system (GIBCO BRL). Screening of positive insert size clones was carried out by endonuclease digestion and PCR amplification, after DNA plasmid extraction was done (Sambroock et al., 1989)

Plasmid DNA of clones with the expected insert size were extracted by Quiaprep Spin Miniprep® Kit (Quiagen). These clones were sequenced using T7 and SP6 primers and Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems) in an ABI PRISM™ 377 DNA sequencer (Pelkin Elmer). The sequences were edited using Sequencher 3.0 (Gene Code Corp.) and compared with the Genbank databases (www.ncbi.htlm) using BLASTx.

Results

Consensus primers were successful in amplifying the orthologous sequences, both in genomic DNA and cDNA, (Figures 1-3). A single band was obtained with all the primer pairs used; only *CDEs* and *Blyc* amplified from cDNA of CM 523-7, as well as *Psyn* and *PDes* from M Per 297 amplified two different bands. PCR product sizes from DNA and cDNA were in accord with the expected sizes, based on consensus sequences.

Several genomic and cDNA clones from CM 523-7 and M Per 297 were sequenced , and significant homologies with genes reported for *phytoene synthase* have been found (Figure 4). The genomic sequences for *Cdes, PDes* and *BLyc* did not show homology with any of the expected genes (data not shown).

Conclusions

PCR products from cassava genomic DNA and cDNA for each one of the putative genes were obtained using primers designed from consensus sequences.

Significant homology with several clones for *phytoene synthase* in others plant species was found for genomic and cDNA clones obtained from cassava.

Conserved domains of families genes involved in the carotene biosynthesis pathway are useful to obtain orthologous sequences expressed in the cassava roots.

Future Plan

- The positive clones obtained will be use as probe in the isolation of full-length cDNA clones from two recently made cassava root cDNA libraries from M Per 297 (Creator™ SMART ™ cDNA library kit) and CM 523-7 (Lambda Zap II ™ Stratagene).

- A genomic library from M Per 297 and CM 523-7 will be made to isolate the complete genomic sequences, including their promotor region, for each one of the candidate genes.

- Sequencing clones. A set of candidate genomic and cDNA clones are pending to sequence and search for similarity.

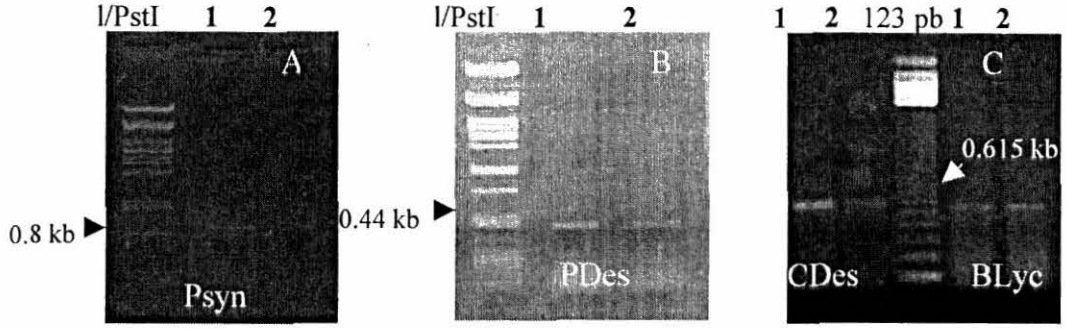


Figure 1. Electrophoresis in agarose gel 1.2% for PCR amplification from genomic DNA. **(A)** Phytoene synthase; **(B)** phytoene desaturase; **(C)** *z*-carotene desaturase and *b* -lycopene ciclase **1** = CM 523-7, **2** = M Per 297; weight marker l/PstI and 123 pb.

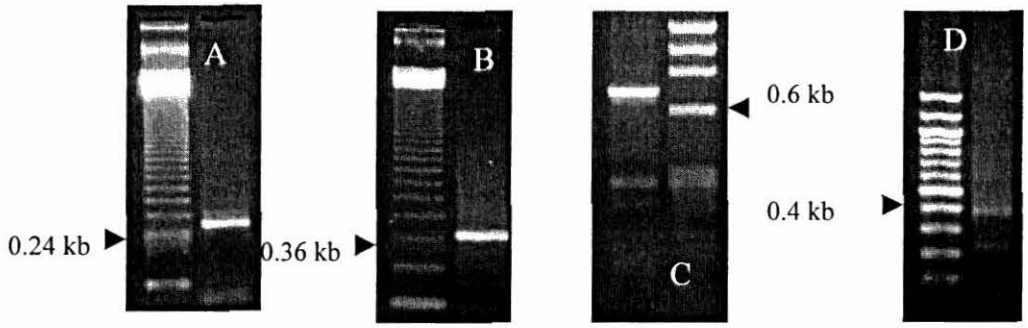


Figure 2. Eletrophoresis in agarose gel (1.5%) for PCR amplification from CM 523-7 cDNA. **(A)** Psyn, **(B)** Pdes, **(C)** Cdes, **(D)** BLyc.

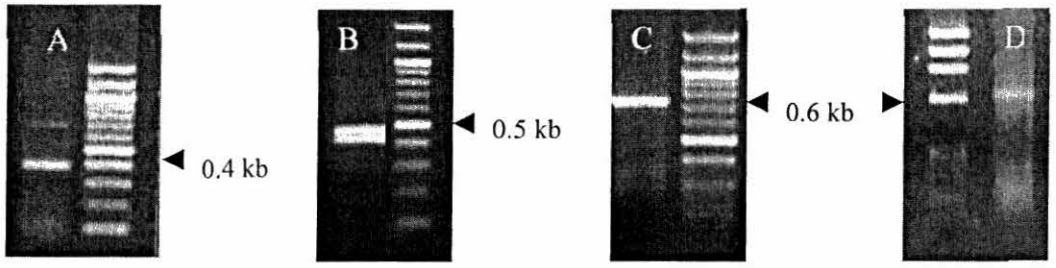
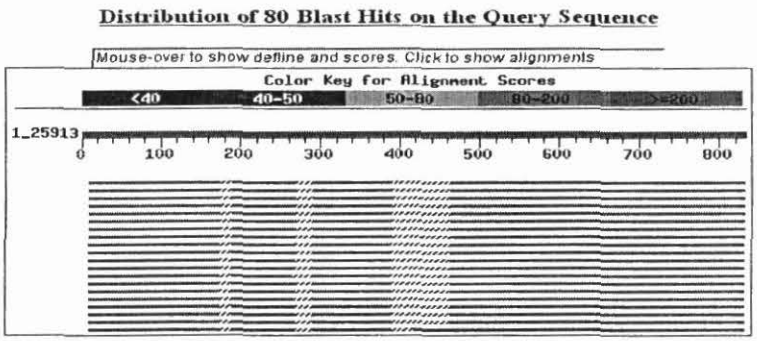


Figure 3. Eletrophoresis in agarose gel (1.5%) for PCR amplification from M Per 297 cDNA. **(A)** Psyn, **(B)** Pdes, **(C)** Cdes, **(D)** Blyc.



Sequences producing significant alignments:	Score (bits)	E Value
gi|13468821|sp|P49293|PSY_CUCME Phytoene synthase, chloropla...	243	2e-63
gi|6959960|gb|AAF33237.1|AF220218_1 (AF220218) phytoene syn...	243	3e-63
gi|13542332|gb|AAD38051.2| (AF152892) phytoene synthase [Ci...	241	8e-63
gi|585747|sp|P37273|PSY2_LYCES Phytoene synthase 2, chlorop...	240	1e-62
gi|585749|sp|P37272|PSY_CAPAN Phytoene synthase, chloroplas...	240	2e-62
gi|21360353|gb|AAM45379.1| (AY099482) phytoene synthase [Ta...	239	3e-62
gi|9971014|gb|AAG10427.1| (AF251015) phytoene synthase [Tag...	239	3e-62
gi|21553694|gb|AAM62787.1| (AY085565) phytoene synthase [Ar...	238	6e-62
gi|15237933|ref|NP_197225.1| (NM_121729) phytoene synthase ...	238	6e-62
gi|13584564|emb|CAC27383.1| (AJ308385) phytoene synthase [H...	238	8e-62

Figure 4 Homology and probabilities from BLASTx search in GeneBank for cDNA sequence of phytoene synthase from CM523-7.

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2.3.2 Carotenoids in cassava: Comparison of colorimetric and HPLC methods of analysis

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Introduction

Vitamin A deficiency (VAD) is a major public health problem in developing countries and is a cause of preventable blindness in extensive populations of young children who go blind every year (Combs, 1998).

Cassava is a primary staple for about 300 million people of those developing countries in which xerophthalmia caused by VAD is a serious problem. The main source of pre-ormed vitamin A is animal food, which is beyond the reach of many people in the developing world who, therefore, depend on those carotenoids (pro-vitamin A) present in plant foods that can be converted into vitamin A in the body The more widespread white-colored cassava roots contain only minor amounts of β-carotene (the carotenoid with the highest vitaminic activity, Bradbury et. al., 1988); but yellow roots contain considerable amounts (>1 mg/200 g of fresh tissue)

This study was designed to improve the analytical methodology for detecting and quantifying carotenoid compounds, estimate the proportion of carotenoids with and without vitaminic activity in cassava roots, and complement the results obtainable from the colorimetric methods with those from the HPLC methods. This is because while the colorimteric method gives only the estimate of total carotenoids, the HPLC method has the capability of compartmentalizing the values into the individual components of β–carotene, α-carotene, lutein, lycopein, etc.

Materials and Methods

Colorimetric method. The extraction procedure outlined by Safo-Katanga et al. (1984) was adjusted by extracting root parenchyma with petroleum ether. The extraction protocol for leaves had to be modified due to the presence of tannins and chlorophylls. The adjusted protocol included several extractions with petroleum ether at 35-65°C and washing steps with methanol in order to minimize the interference from the other pigments. A 5-g sample was taken out of the root or leaves at random, 10-11 months after planting. The quantification of total carotenes was done by ultraviolet spectrophotometry using a Shimadzu UV-VIS 160A recording spectrophotometer. UV detection was done at λ = 455nm for root extracts and λ= 490 nm for leaf extracts.

HPLC method. Starting with the method used for the colorimetric quantification of total carotenes, aliquots (20 ml) of petroleum extract were completely dried by rota-evaporation. Then the dry extract was dissolved in 1 ml of HPLC mobile phase (methanol: methyl-terbutyl-ether: water, 80:15:4 v/v), centrifuged at 14000 rpm, and 10 µl were injected in the HPLC system using a YMC carotenoid 5 µm, 250 mm x 4.6mm column. Separation was done by isocratic elution with a mixture of acetonitrile:methylene chloride: methanol, 70:20:10 v/v) as mobile phase at 1 ml min^{-1} and 30°C. β-carotene was detected by monitoring absorption at 450 nm. Identification and quantification was done by comparing retention times and UV-VIS spectra with a β-carotene standard (Sigma C-0126).

Root samples of 99 clones from the germplasm bank or the cassava breeding project at CIAT were used for this study. Harvesting took place at 9-10 months of age (normal harvesting time for cassava at CIAT), and commercial size, disease-free roots were taken to represent each clone.

Results and Discussion

Root samples from the 99 clones were analyzed using the colorimetric method for total carotenes, and then the same sample was analyzed using the HPLC procedure described above. Results of the two different methods are presented in Table 1.

Table 1. Mean, maximum and minimum values for total carotenes measured with the colorimetric method and for β-carotene and α-carotene quantified by the HPLC methodology. Roots from a sample of 99 clones were analyzed.

	Colorimetric Method	HPLC Method	
Parameter	Total Carotenes (mg/100 g FT)	β-carotene (mg/100 g FT)	α-carotene (mg/100 g FT)
Mean	0.42	0.52	0.04
Maximum	1.07	1.69	0.19
Minimum	0.14	0.08	0.00

The more recent HPLC methodology, using pre-columns and columns specifically designed for carotenoids, is considered to be more precise than the standard methodology employed for quantifying carotenoids in cassava roots and leaves. The results presented in Table 1 are very relevant for two reasons:

A large proportion of total carotenes have been found to be β-carotene, which has twice as much vitaminic activity as α-carotene

Total carotenes have been found to be considerably higher ($0.52 + 0.04 = 0.56$ mg) when using HPLC than with the colorimetric method (0.42 mg).

Despite the foregoing differences, it is clear that there is a high, positive correlation (0.88) between the two methodologies for measuring carotenes. The degree of association between total carotenes (colorimetry) and β-carotene (HPLC) is further illustrated in Figure 1.

Surprisingly, the results at the top right corner of the plot in Figure 1 correspond to a pinkish rather than a yellow cassava root. As this root produced the highest level of β-carotene among the roots evaluated, greater emphasis will be placed on analyzing roots with pink parenchyma.

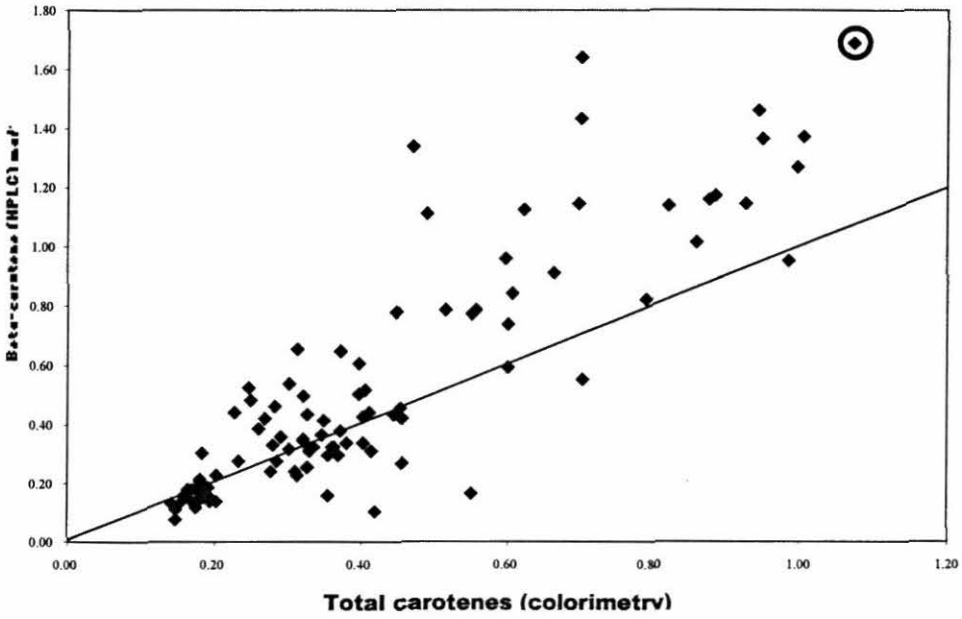

Figure 1. Carotene contents measured by the colorimetric and HPLC methods.

Conclusions

Several important conclusions can be obtained from this work:

The amount of total carotenes in colored cassava roots is higher than suggested by the colorimetric method.
A large proportion of total carotenes is β-carotene, which has higher vitaminic activity.
Cassava has a high potential in helping overcoming vitamin A deficiency in human populations affected by this problem.
Greater emphasis should be given to pinkish roots.

Future Plan

- Currently other studies are being carried out to measure the stability of carotene content in the roots across environments (genotype x environment interactions) and with different processing methodologies (boiling, sun- and oven-drying, and gari production). The carotenes are measured not only in the fresh roots, but also after freezing at –80 and –20°C or lyophilization.

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2.3.3 Production of Waxy Cassava Starch via the Down Regulation of GBSSI Gene

Gina Jazbleidi, Paul Chavariagga, Chikelu Mba, Martin Fregene
SB-2
Funding: Ministerio de Agricultura y Desarollo Rural de Colombia.

Introduction

Higher incomes from cassava in marginal areas of the developing world where the crop is generally found requires the industrialization of the crop and the development of novel industrial products for cassava with the aid of modern biotechnology. There are several novel products that can be produced from cassava. They include modified starches, such as 100% amylopectin or 100% amylose starches, from the down regulation of the granule bound starch synthethase (GBSS) gene, or the starch branching enzyme (SBE) gene. The industrial applications of either pure amylopectin or pure amylose starches, such as the production of high value biodegradable polymers from pure amylose starches or the use of 100% amylopectin in thickeners, pastes, and glues, is a market with unlimited growth potential.

With funds from the Ministerio de Agricultura y Desarollo Rural of Colombia a project has been initiated to genetically engineer industrial varieties with an anti-sense construct of the GBSSI. The granule bound synthethase (GBSS), is the predominant starch synthase gene, and catalyses the conversion of ADP-glucose to amylose through the linkage of a ADP glucose to a pre-existing glucan chain. Anti-sense disruption of the GBSSI gene has been employed to create potato transformants with 70-100% amylopectin via the down-regulation of the GBSSI gene Salehuzzaman et. al. (1993).

Methodology

Isolation of a cassava GBSS cDNA clone. More than 87, 000 clones of a cassava root and leaf cDNA library cloned in the vector pCMV SPORT (GIBCO BRL inc. USA) has been gridded onto high density filters (Mba et al. 2000, unpublished data). The library was screened using a potato GBSS cDNA clones, a kind gift of Dr Christine Gebhardt, Max Planck Institute, Cologne, Germany. The potato GBSS gene was labeled with [^{32}P] dATP by random primer labelling and hybridized overnight to the cDNA filters according to standard protocols for Southern hybridization used in cassava (Fregene et al. 1997). The filters were washed 2 times with 2XSSC + 0.1%SDS for 5 minutes at 60°C and autoradiography was at –80°C using 2 intensifying screens.

Construction of transformation cassettes. Primers were designed from published sequences of a full length cassava cDNAs of the GBSSI gene (Salehuzzaman et. al. 1993) that incorporate *BamHI* and *XbaI* restriction enzyme recognition sites to enable sub-cloning of the cDNA clone in the anti-sense orientation into the multiple cloning site (MCS) of the vector pRT101.). The primers were used to amplify the cDNA clone obtained above and the PCR product was cleaned using the QIAGEN PCR clean up kit (QIAGEN Inc., Los Angeles, California), digested with the appropriate enzymes. A 2.1kb *BamHI/XbaI* fragment was subcloned in the sense and anti-sense between the 35S promoter and the 35S poly adenylated terminator region of vector pRT101, a kind gift of Dr Ryohei Terauchi, Iwate Biotechnology Research Center, Kitakami, Japan. The 35S promoter, GBSS gene, in anti-sense orientation, and the termintor region were excised using the restriction enzyme Hind *III*, separated on a special gel, symergel (Diversified Biotech Inc., USA), eluted and cloned into the

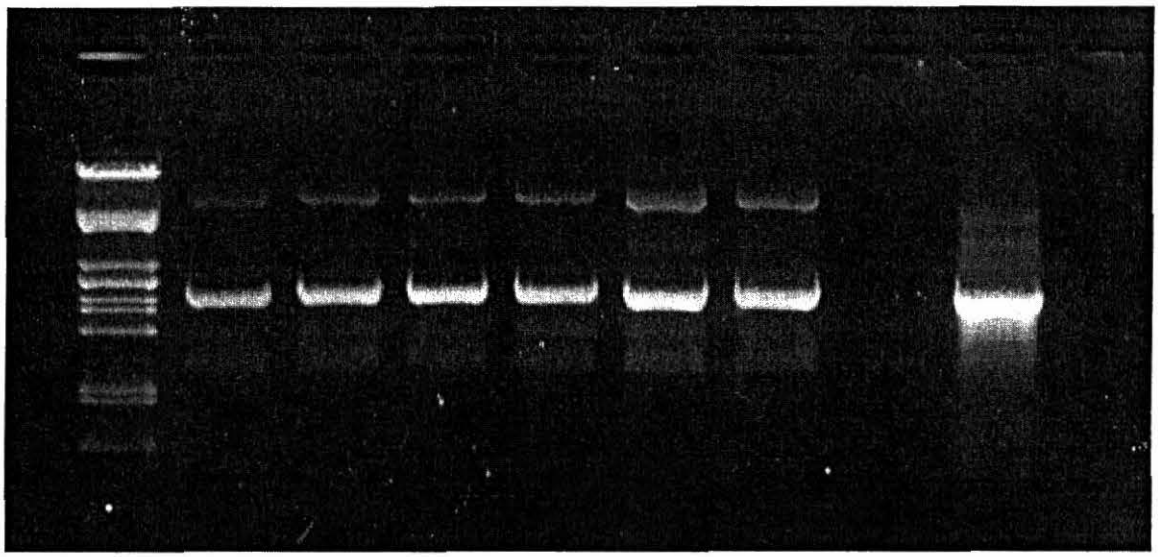
Hind *III* site of the binary vector pBIG101 having the GUS-intron and *nptII* reporter genes, a gift of Dr Richard Sayre, Ohio State University, Columbus Ohio.

*Genetic Transformation.*Genetic transformation was by particle bombardment and *Agrobacterium* transformation of friable embryo callus (FEC) cultures. About 20μg of the pBIG101 constructs plasmid was coated onto gold particles and used in the helium gun bombardment of new FEC suspensions of the model variety for cassava transformation TMS60444 according to standard protocols established for cassava at CIAT (CIAT2001). For *Agrobacterium* transformation, the pBIG101 construct was transformed into strain EHA105 according by electroporation and transformed clones were selected on LB media plates plus Kanamycin (50ug/ml final). After 2 days, white colonies were picked and incubated in 10ml LB + Kanamycin (50ug/ml final) for another two days at 28°C. Bacteria was collected by centrifugation at 3000rpm for 20 min in a table top centrifuge and re-suspended in 500ul of solution containing 10mM MgCl2, 1omM MES, and 100uM Acetosyringone. Cassava FEC was co-cultured with the *Agrobacterium* suspension according to standard protocols at CIAT (CIAT 2001)

Results

Three GBSS cDNA clone obtained from screening the cassava library were sequenced and one clone was found to be a complete cDNA clone. The cDNA clone has the ATG start codon 81 base pairs down stream from the beginning of the cDNA sequence and a stop codon about 100 base pairs from the poly A tail. PCR amplification with the designed primers yielded a fragment about 2.1kb in size that corresponds to the full length GBSS cDNA clone (Fig1).

Fig 1. PCR amplification of the GBSS cDNA clone using primers designed to introduce restriction enzyme sites at the ends of the gene. The first lane by the right is molecular weight marker Lambda DNA digested with *HindIII* , the next six lanes are PCR amplification of the GBSS gene, the last lane is a control, PCR product of the GBSS potato gene.



The resulting PCR fragment was digested with *BamHI* and *XbaI* restriction enzyme digestion and cloned into the MCS of pRT101. The GBSS gene, promoter and terminator sequences were excised with *Hind III*, and the two resulting fragments of sizes 2.7 and 2.6 kb were separated by electrophoresis (Fig2). The bigger fragment was eluted and cloned into the *HindIII site* of

pBIG101. This is the construct that was used in the particle gun and *Agrobacterium* mediated transformation.

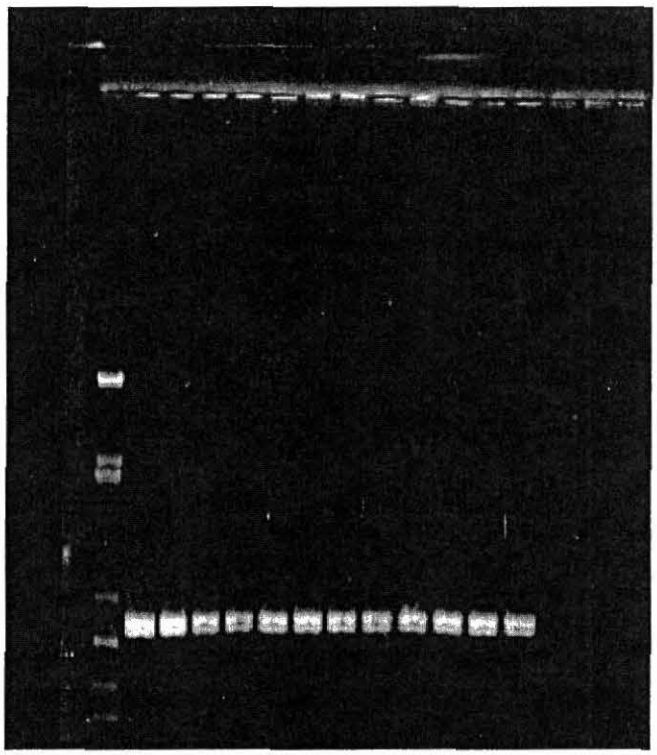


Fig 2. *HindIII* digested pRT101 plasmid containing the cassava GBSS gene in anti-sense orientation. The fragments of about 2.7kb and 2.6kb in size respectively represent the GBSS gene flanked by the 35S promoter and the polyadenylated terminator sequence and the rest of the pRT101 plasmid.

The transformation experiments are ongoing and conclusive results of reporter gene assays are expected at the end of December. Once the transformed calli has been revealed to have stable incorporation of the construct, regeneration of the transgenic calli will be initiated.

Future Perspectives

- *Agrobacterium* transformation and regeneration of the industrial cassava variety "Reina" with the anti-sense construct of GBSSI cloned in pBIG101

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2.3.4 Transient Transformation Assay of a Cassava Mosaic Disease (CMD) Candidate Resistance Gene Differentially Expressed During Host Plant Disease Resistance Response

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[1]IBRC, Kitakami, Japan; [2]CIAT

Funding: The Rockefeller Foundation.

Introduction

High levels of resistance to the cassava mosaic disease (CMD), the crop's most important production constraint in Africa, is mediated by a single dominant genes designated *CMD2*. The serial analysis of gene expression (SAGE) has been employed to identify many genes differentially expressed in resistant genotypes in response to heavy disease pressure of the African Cassava Mosaic Virus (ACMV) and the East African Cassava Mosaic Virus. The most differentially expressed gene was a beta-tubulin gene found to be expressed 12 times in CMD resistant genotypes compared to susceptible genotypes. Tubulin are the building block of microtubules the main component of celluar cytoskeleton and they have been implicated in cell-to-cell progression and cytoplasm-to-nucleus movement of viruses.

To further understand the role beta-tubulin plays in the molecular basis of resistance, an experiment was designed to transiently co-transform a CMD susceptible cassava genotype and other host of the virus, for example *Nicotiana benthamiana* and *Nicotiana tabacum*, with infectious viral clones and the beta-tubulin gene. Infectious DNA clones of two strains of the virus were obtained from Drs John Stanley and Rob Briddon, John Innes Center, Norwich UK, and a license to work with them was obtained by the Iwate Biotech Research Center (IBRC). Method of transient transformation was by agro-infiltration, *N. benthamiana* and *N. tabaccum*, or biolistic inoculation, cassava. A CMD susceptible cassava variety, TMS30555, and the cassava land race that is the original source of CMD2, the single dominant CMD resistance gene, TME3, were used for the experiments.

Methodology

Plant materials were the CMD resistant variety TME3 and the susceptible variety TMS30555. Both genotypes were obtained as tissue culture plantlets from IITA and transferred to pots with soil for the experiments. A full length beta-tubulin cDNA clone was PCR amplified using primers that contained the recognition site for the appropriate restriction enzymes and cloned into PRT101 (35S promoter), for biolistic inoculation, or into the XVE inducible binary expression vector or into the binary vector pBIN m-gfp-ER, for agro-inoculation. The PCR fragment was cleaned and digested with the appropriate enzyme and eluted from a 1.5 % agarose gel. The purified fragment was then cloned into appropriate vector and transformed by electroporation into *E.Coli* strain HB101. A 1:50 dilution of plasmid preparations from an overnight culture of a single *E.Coli* colony was analyzed by PCR, using the original beta-tubulin primers, to confirm success of the cloning experiment. To that ensure that a full length beta-tubulin protein will be expressed in the transient assay, 3 clones from each cloning experiment was sequenced with 4 primers that covers the entire length of the gene.

Infectious DNA clones from two virus strains were employed in the transient assay experiment, partial repeats of the A and B genome of the Sri-Lanka Cassava Mosaic Virus (SLCMV A and SLCMVB) cloned into the binary vector pBIN PLUS, and full length clones of the African Cassava Mosaic Virus (ACMV) A and B genome cloned into pUC19 (ACMV A and ACMVB). Infectivity of cassava by virus partial repeats in a binary vector clones and agro-inoculation has not been demonstrated, therefore all agro-inoculation experiments were with *N. benthamiana* and *N. tabaccum*. But cassava has been successfully infected by biolistics, therefore the biolistic experiments were with cassava and *N. tabaccum*, as control.

A preliminary biolistic experiment was conducted to standardize the conditions for the BIO-RAD particle gun bombardment and to ensure expression of the beta-tubulin gene cloned into PRT101. Leaf discs of about $5cm^2$ from the cassava genotype TME3 and *N. tabaccum* were used for the preliminary experiment. There were three treatments: bombardment with the PRT101 plasmid containing the luciferase gene alone, the luciferase construct combined with a PRT101 plasmid in which the luciferase gene has been replaced with a beta-tubulin gene having the 11bp truncation and the non-truncated beta-tubulin gene. Three leaf discs were used for each treatment and per crop. Particle gun bombardment was according to standard procedures established at the IBRC (Terauchi, 2002, personal communication). One 1 month-old live plant each was included for both cassava and *N. tabaccum* in the luciferase treatment as control. Leaf discs were bombarded once with 2.5ug of DNA from the respective clones, while whole plants were bombarded 3 times. After bombardment, leaves were placed on water in a petri dish an incubated at 25°C for two days.

Two days later, total proteins were isolated from leaf discs transiently transformed with the beta-tubulin gen, to confirm the expression of the beta-tubulin gene using standard procedures established at the IBRC (Saitoh, 2002 personal communication). Exactly 3.6ug of total protein lysate from all leaf discs from treatments 2 and 3, and 2 non-bombarded controls were separated on a SDS-PAGE gel. Western blot analysis, using an anti-body raised to purified rat brain beta-tubulin, was carried out as described in the manufacturer's product sheet (SIGMA Inc, St. Louis, USA).

Following the preliminary experiment, a biolistic experiment was designed to inoculate the CMD resistance genotype TME3 and the CMD susceptible variety TMS30555 with the ACMV A and B infectious clones. A second treatment was co-bombardment of the ACMV clones and the beta-tubulin gene into the CMD susceptible genotype TMS30555. Three one-month old plants of TME 3 were used in the first treatment, while 2 one-month old plants of TMS30555 were each used in the first and second treatments. Plants were bombarded twice with 2.5ug of DNA from the respective clones as described earlier. The inoculated plants were transferred to a virus containment area in a secure green house.

For the agro-inoculation by infiltration experiment, the SLCMV A and SLCMV B clones were transformed into agro-bacterium strains MOG and EHA105. Similarly the beta-tubulin gene in the binary vectors XVE and pBIN m-gfp-ER were transformed into agro-bacterium strains MOG and EHA105. Agro-infiltration was according to standard methods established at the IBRC (Saitoh 2002, personal communication). There were 4 treatments: the virus alone (a mixture of SLCMV A and SLCMV B), the virus and the beta-tubulin gene (in XVE), the beta-tubulin gene alone, and a dilution of the beta-tubulin gene to reflect the concentration in the mixture with viral clones. Three plants of *N. benthamiana* and *N. tabaccum* each were used per treatment. Agro-infiltrated plants were transferred to a virus containment area in a secure green house. After two days, the beta-tubulin gene will be induced by treatment of agro-infiltrated plants with the animal steriod, estradiol. Plant tissue will be collected from all plants and stored for Western analysis of gene expression using a conjugated mouse beta-tubulin antibody or antibodies raised to the coat protein

of ACMV. Agro-inoculation of cassava could not be achieved due to poor infiltration, cassava leaves are covered with a thick cuticle.

Results

The full length of the beta-tubulin gene is 1667bp, the translated portion of the gene corresponds to 1341bp or 447 amino acid motifs. The beta tubulin protein showed more than 80% homology with similar genes from rice and arabidopsis. Comparison of the complete sequence of the beta-tubulin gene with ESTs generated for tag annotation during the SAGE experiment revealed two beta-tubulin molecules, one transcript had an 11bp truncation in the 3' untranslated end of the cDNA molecule. This molecule was also the most abundant from the EST project, 4 as against 1 of the non-truncated. It is not clear if this molecule plays any role in the over-expression of beta-tubulin, therefore the molecules were separated in at least one of the transient assay experiments, the biolistic inoculation.

Results of the biolistic experiment to standardize the conditions for the BIO-RAD particle gun bombardment for expression of the beta-tubulin gene particle gun revealed good expression of the full length beta-tubulin gene in all leaf disc by western blot (Fig 1). Similar results were also obtained with non-bombarded controls suggesting the detection of endogenous beta-tubulin. There is therefore a need to separate the confounding influence of endogenous tubulin and transient expression via the use of a non-plant secondary antibody fused to the tubulin protein. The luciferase assay was conducted on leaf discs from all treatment using a rapid enzyme substrate assay and a flourescence reader according to standard methods in use at the IBRC (Terauchi et al. 2002, personal communication). Results revealed good enzyme activity on the average for all samples, although samples from the co-inoculation (luciferase and beta-tubulin) had a 5 to 10 magnitude reduction. This may be an effect of the quantity of leaf samples, as more than half of the leaf disc had already been used for the Western blot analysis. The luciferase activity of the bombarded cassava and *N. tabaccum* whole plants were the highest for all samples.
The western blot analysis of the tobacco plants agro-infiltrated with the infectious virus clones or beta-tubulin gene or both remain to be carried out. The plant tissue is stored at –80oC pending a trip to Japan to conclude the above studies.

Future Perspectives

- Western blots of agro-bacterium and particle gun infected plans to assess expression of the beta-tubulin gene and the infectious viral clones
- Additional co-transformation experiments to better synchronize expression of the beta-tubulin gene and infection by viral clones.

2.3.5 Evaluation of nutritional and agronomic traits in roots and foliage of cassava clones from the germplasm bank and breeding project at CIAT

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Introduction

Cassava is one of the most important sources of food energy in many tropical countries. There are an estimated 70 million people who obtain more that 500 cal/day from cassava, particularly in Africa and NE Brazil (Cock, 1985), as well as in Asia (Kawano, 1998).

Vitamin A (VAD), iron and zinc deficiencies are widespread in sub-Saharan Africa and in many tropical areas where the diets of poor populations are mainly plant-based and the intake of animal products is low. Improving the vitamin A status of children can reduce mortality rates by 23-30% (Beaton et al., 1993).

Given that cassava is a staple in regions where there are severe deficiencies of micronutrients, the crop can be used as a vehicle to deliver vitamins and minerals in higher concentrations. A total of 2457 cassava clones have were sourced from the CIAT Germplasm bank and Breeding Program and used during the different stages of this study. They were analyzed for their nutritional quality and a few key agronomic traits. There were two types of clones: those produced from breeding projects (CIAT, IITA and Thailand) or from the CIAT cassava germplasm bank. Because of limitation in the number of samples that can be analyzed at any given time, the evaluations were carried out over a four-year period (1998-2001)..

One of the main purposes of this study was to evaluate the nutritional properties of cassava, for both humans and animals. It was of particular interest to determine the potential of cassava to provide carotene through the diet as a contributing factor for alleviating VAD in human populations. This initiative, which is referred to as "*biofortification,*" is envisioned to supplement other sources of vitamin A such as supplementation and fortification of processed foods.

Materials and Methods

Carotene concentration. Peviously reported (CIAT, 2001).
Postharvest physiological deterioration (PPD). Peviously reported (CIAT, 2001).
Mineral concentration. Peviously reported (CIAT, 2001).
Dry matter content. Dry matter content was estimated using the specific gravity methodology (Kawano et al.,1987).

Root coloration. A 1-9 scale for the visual estimation of root coloration was developed and printed for a uniform estimation of color intensity. The color of root parenchyma can vary from white, cream and yellow, to orange. Pinkish roots (score 9) have also been observed in cassava.

Results and Discussion

Table 1 presents a summary of measurements for dry matter, HCN, total carotene for roots and leaves, as well as color, PPD and sugars in the roots.

Carotene content in the roots ranged from 0.102-1.040 mg/100 g FT, demonstrating the potential of cassava clones with yellow roots to contribute to overcoming VAD in regions of the world where this malady is a chronic problem. As expected, carotene in the leaves was much higher, ranging from 12.05-96.42 mg/100 g FT. Concentration of carotene in the leaves correlated poorly with the same trait when measured in the roots ($\rho = -0.074$).

Cassava therefore offers a dual approach for solving VAD: introducing and promoting yellow-rooted varieties and/or favoring foliage consumption. The phenotypic correlation between total carotene content in the roots and root color score (n=788) based on the visual scale was very high and positive ($\rho = 0.860$).

Table 1. Descriptive parameters for traits of industrial relevance in accessions from the cassava germplasm bank and the Breeding Project at CIAT.

Variable	Sample Size (No.)	Minimum	Maximum	Average	Standard Deviation
Root dry matter (%)	2022	10.72	57.23	34.27	6.95
Foliage dry matter (%)	1404	13.27	43.68	30.44	4.04
HCN in roots (ppm)	2022	13.9	2561.7	263.7	324.2
HCN in foliage (ppm)	1404	n.d.	3103.9	729.9	383.9
Carotene in roots (mg/100 g FT)	1789	0.102	1.040	0.2457	0.1351
Carotene in foliage (mg/100 g FT)	1719	12.05	96.42	47.71	10.65
Root color (1-9)	788	1	8	2.26	1.46
PPD (%)	1374	0	100	24.47	19.63
Total sugars (%)	1755	0.2	15	2.876	2.028
Reducing sugars (%)	1755	0.0	12.9	0.753	0.957

Mineral concentrations in the leaves were much higher than in roots (Table 2). A high positive correlation between minerals content in roots and leaves was observed for K (0.49), Mn (0.43), N (0.38) and P (0.36). Mineral concentrations in leaves further illustrate the nutritional value of this tissue for animal as well as human consumption. The leaves, in addition to high carotene and protein contents, also showed excellent levels for key minerals. Regarding protein content in the roots (estimated through N measurements), the mean crude content of 3.06% agrees with reports in the literature (Onuma, 1987). The average crude protein content in the foliage (30.6 %) was much higher than in the roots.

Table 2. Simple descriptive statistics (minimum, maximum, mean and standard deviation) for mineral concentrations of 600 genotypes of cassava and equivalent data from alfalfa.

Mineral	Leaves				Roots[§]				Alfalfa [¶]
	Min	Max	Mean	S.D.	Min	Max	Mean	S.D.	Mean
Measurements in mg/kg									
Fe	119	2600	339.0	202.4	6.0	230.0	17.1	15.2	73-82
Mn	16.7	200.0	58.9	23.2	0.45	5.0	1.4	0.6	77-88
B	4.02	74.32	20.6	12.5	1.14	9.91	2.0	0.6	36-37
Cu	2.81	12.36	7.2	1.5	0.79	40.31	5.8	5.4	7-13
Zn	15.14	150.47	47.7	21.7	2.63	37.52	7.5	3.6	45-51
Na	10.3	157.8	34.1	19.2	18.6	1230.0	129.2	147.3	-.-
Al	60	2800	277.6	224.0	4.4	330	11.5	20.4	53
Measurements in %									
Ca	0.630	3.900	1.522	0.493	0.031	0.250	0.076	0.032	2.2-2.8
Mg	0.260	1.500	0.544	0.186	0.052	0.240	0.105	0.028	0.48-0.74
K	0.540	2.300	1.394	0.326	0.410	2.500	1.172	0.321	0.67-1.09
P	0.149	0.760	0.362	0.079	0.071	0.320	0.165	0.036	0.28-0.33
S	0.220	0.520	0.318	0.045	0.012	0.055	0.027	0.008	-.-

[§] Sample sizes for root evaluation of B = 580; for Cu = 599 and for Al = 460.
[¶] Extracted from Bickoff et al. (1972).

Postharvest physiological deterioration (PPD) is a characteristic that limits the marketability of the fresh roots and necessitates either consumption or processing shortly after harvesting. The average for PPD was 24.47%, with individual values ranging from 0-100%. The correlation coefficient (ρ = 0.348) indicates that there is a positive association between dry matter content and PPD. HCN does not seem to have a strong effect on PPD, whereas carotene did reduce and/or delay the onset of PPD (ρ = -0.123). Figure 1 illustrates the relationship between PPD and carotene, suggesting the occurrence of a threshold effect with carotene values lower that 50 mg having gradually lesser effect in reducing or delaying PPD.

Results observed in the large samples analyzed demonstrate that cassava roots and particularly the leaves are a valuable source of carotene, which can help alleviate chronic VAD in human populations suffering from it. The additional advantage of carotene's reducing the physiological deterioration of roots somewhat could encourage farmers to grow cassava clones with yellow roots. Consumption of foliage by humans is to be promoted whenever possible.

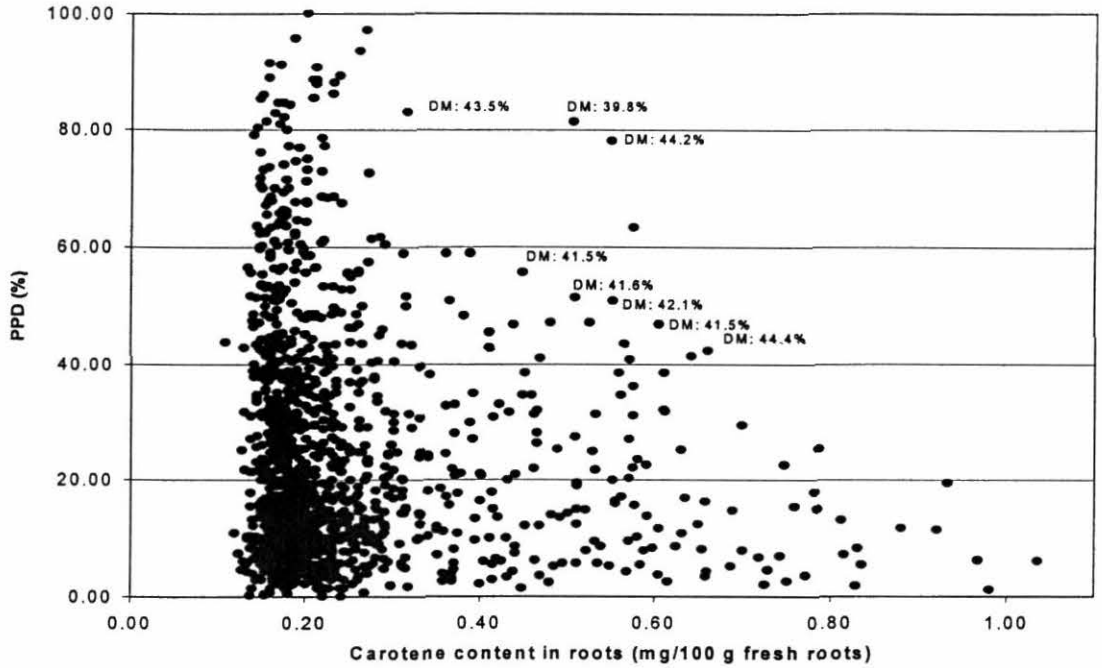

Figure 1. Relationship between carotene content (mg/100 g FT) and PPD (%) analyzed in a sample of 1315 cassava roots. Most data points in the upper periphery of the distribution came from root samples with dry matter content (%) considerably higher than the average for the sample analyzed.

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2.3.6 Evaluating Traits Contributing to Acid-Soil Adaptation in a Population of 274 Brachiaria ruziziensis × Brachiaria decumbens hybrids

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Introduction

In a pilot experiment we found that hybrids of a *Brachiaria ruziziensis* × *Brachiaria decumbens* population segregated for root-related characters that are relevant for the adaptation to acid soils. These include 'root growth potential' (the ability to initiate and elongate roots in the absence of externally supplied nutrients) and aluminum (Al) resistance (see "Identifying individuals with contrasting aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrid population" in this report). We therefore extended the ongoing phenotypic evaluation to the complete hybrid population available at CIAT.

The results of this experiment are going to be useful in two ways:

They will enable us to refine a single-step screening procedure to discard rapidly genotypes not adapted to acid soils.
The phenotypic data will provide a basis for the mapping of QTLs controlling physiological and morphological characters associated with acid-soil adaptation. The latter should provide an avenue towards implementing marker-assisted selection for acid-soil adaptation in the future.

Materials and Methods

Stem cuttings from hybrids of a *Brachiaria ruziziensis* × *Brachiaria decumbens* population were rooted in a low ionic strength nutrient solution in the glasshouse during 9 days. Rooted stem cuttings from each genotype were grouped into homogeneous pairs. One cutting from each pair was used to measure root growth in the absence of Al (in 200 M $CaCl_2$; pH 4.2), while the other was used to measure growth in an identical solution, to which 200 M $AlCl_3$ had been added. Treatment solutions were changed every second day to minimize pH drifts. At harvest on day 21 after transfer to treatment solutions, stems were separated from roots and dried to determine their dry weight. Roots were put into a solution containing neutral red and methylene blue and left to stain for at least 24 h. After rinsing with water, they were scanned on a flatbed scanner. The resulting images were analyzed with WinRHIZO image analysis software to determine root length and average root diameter. Thus far, three growth cycles, each comprising up to three pairs of stem cuttings per genotype (more for the two parents) have been fully analyzed in this experiment, which is still ongoing.

Results and Discussion

An extensive root system that explores a large soil volume can take up immobile nutrients such as phosphorus more efficiently. It is presumably an important trait that permits plants to grow on nutrient-deficient, acid soils. Adapted genotypes should therefore have long thin roots and cluster in the lower right corner of a plot of root diameter (Y axis) versus root length (X axis). To produce such a plot, root length and thickness data were log-transformed and adjusted for the effect of stem cutting dry weight as described in the following report on "Identifying individuals with contrasting

aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrid population." The two sets of data were then plotted against each other, for stem cuttings grown in both the absence and presence of Al (Figure 1).

In the absence of Al, stem cuttings of hybrids had root lengths ranging from 0.6-7.0 m. While the poorly adapted parent (*B. ruziziensis*) was at the low end of the range, a significant number of hybrids produced longer roots than the well-adapted *B. decumbens* (Figure 1, left panel). The two parents were close to the two extremes of the range of root diameters, however (Figure 1, left panel). As expected, root elongation was inhibited in the presence of Al, and roots became thicker (note differences in population means in right vs. left panel of Figure 1).

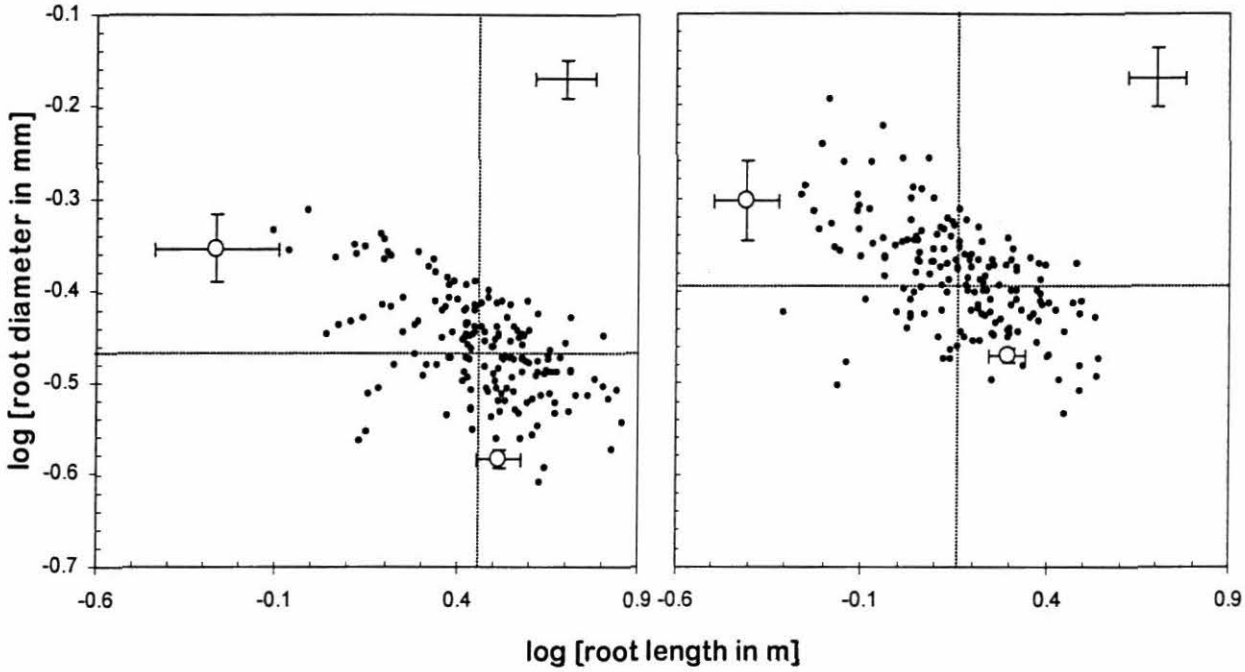

Figure 1. Comparison of root length and root diameter of rooted stem cuttings from a *B. ruziziensis* x *B. decumbens* hybrid population. The values of the two parents are shown with large symbols (upper left, *B. ruziziensis*; lower right, *B. decumbens*). The values of the hybrids are shown with small symbols. The error bars in the upper right corner designate average standard errors for the two variables. Only hybrids for which at least two independent measurements had been taken were included in these graphs.

Growing rooted stem cuttings in the Al treatment and choosing genotypes with long and thin roots (Figure 1, right panel) could be an effective screen to discard nonadapted segregants in the *Brachiaria* breeding program. It is important however to keep in mind that such a test simultaneously screens for several physiological characters (root growth potential in the absence of external nutrients + Al resistance + inherently thin roots). The genetic components controlling these characters may however be scattered throughout different genotypes and could be lost if the test is applied too stringently.

For these reasons it would be desirable to isolate "physiological components" contributing to root growth in the Al treatment. Performing the same screen in the absence of Al provides access to the component that allows plants to initiate and elongate roots in the absence of all nutrients but Ca in the growth medium and to the component that is responsible for inherently thin roots (Figure 1, left panel). A comparison of root length for each genotype, in the presence and absence of Al, provides indirect access to the physiological component that governs Al resistance (see "Identifying

individuals with contrasting aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrid population" in this report).

Future Plan

- We continue to collect data from additional growth cycles to increase the precision with which we will be able to "dissect" root growth into these physiological components. Subsequently, the set of physiological data will be combined with genetic data to identify QTLs associated with these traits.

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2.3.7 Identifying individuals with contrasting aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrid population

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Introduction

Previous experiments have established that there is a significant difference in aluminum (Al) resistance between *B. decumbens* (resistant) and *B. ruziziensis* (sensitive) (Wenzl et al., 2001). Given that apomictically reproduced plants tend to be highly heterozygous, alleles conferring Al resistance in apomictic *B. decumbens* are expected to segregate in a *B. ruziziensis* × *B. decumbens* hybrid population. The main objective of this work was to use this population to isolate genes whose expression is associated with Al resistance.

Here we report on a physiological evaluation of 38 individuals of this hybrid population selected on the basis of data from preliminary experiments to include both Al-resistant and sensitive hybrids. Based on the results of this evaluation, two groups of contrasting individuals will be formed to compare gene expression patterns in root apices, the presumed site of action of the Al-resistance mechanisms.

Materials and Methods

Rooted stem cuttings of each genotype were grouped into homogeneous pairs. One stem cutting from each pair was used to measure the root-elongation potential in a solution containing 200 M $CaCl_2$ (pH 4.2); the other was used to measure growth in an identical solution, to which 200 M $AlCl_3$ had been added (see "Evaluating acid-soil adaptation of 274 *Brachiaria ruziziensis* ×

Brachiaria decumbens hybrids" in this report). The experiment consisted of ten independent growth cycles, each with up to nine pairs of stem cuttings per genotype.

Results and Discussion

Inhibition of root elongation is a well-known symptom of Al toxicity. Addition of Al to the basal solution containing only $CaCl_2$ had a pronounced effect on root elongation in most genotypes. Initial analyses, however, indicated that the effect of Al was highly variable. This was true both for within-growth-cycle replicates and the average effect of Al in different growth cycles.

Several factors may have contributed to this variability:

Root elongation in a solution lacking all nutrients but Ca largely depends on reserves (sugars, nutrients) present in stem cuttings. Those that have a greater biomass are therefore expected to show superior root elongation in both treatments.
Previous results suggest that a good nutritional status increases the Al-resistance level of *Brachiaria* spp. (Wenzl et al., 2002). This may enhance the positive effect of the stem cutting's biomass on root elongation in the Al treatment.
Stem cuttings taken from plants with a good nutritional status are likely to contain more nutrients per unit of biomass, thereby potentially amplifying the effect of the stem cutting's biomass on root elongation in both treatments.

These considerations highlight the importance of adjusting root elongation data for stem cutting biomass and the nutritional status of the donor plants—the latter presumably varying in an unpredictable manner from one growth cycle to another.

All data were log-transformed because growth data tend to be log-normally distributed (Causton & Venus, 1981). This improved normality in most cases as determined by the Kolmogorov-Smirnov test. Linear regression of root length and diameter on the dry weight of stem cuttings indicated a highly significant effect for the former ($P < 10^{-25}$), but not the latter. At least 13% of the variance of root length data in the basal treatment and 20% in the Al treatment could be accounted for by the variable dry weight of the stem cuttings. Root length data were therefore adjusted for the effect of the dry weight of the stem cuttings (using separate linear regressions for individual growth cycles, which slightly increased the percentage of explained variance).

Adjusted root lengths of most genotypes fell within the range of 2.5-6.3 m per stem cutting (log values from 0.4-0.8; Figure 1). Only *B. ruziziensis* (1.0 m) and hybrid No. 114 (0.7 m) had much shorter roots. Interestingly, about half the hybrids had longer roots than the acid-soil adapted parent *B. decumbens*. Given this inherent variability in root elongation potential, root elongation in the presence of Al is expected to be the result of a superimposition of two physiological components: The ability to elongate roots in the absence of externally supplied nutrients plus Al resistance. While this might be advantageous for rapidly discarding nonadapted genotypes in a breeding program, it makes it necessary to isolate the component of overall performance in the Al treatment that is attributable to Al resistance. This is because the experiments to isolate genes rely on the assumption that sensitive genotypes do not express genes that confer Al resistance. Not dissecting the two components, however, could result in a misclassification of genotypes expressing Al-resistance genes as "sensitive" because they lack the (presumably unrelated) ability to elongate roots in the absence of nutrients.

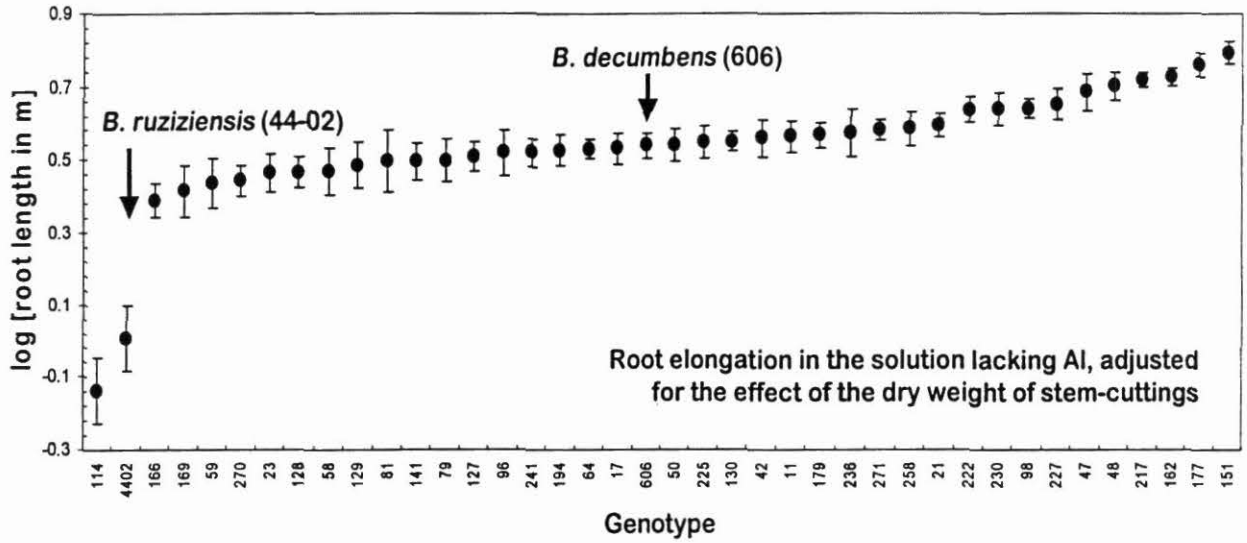

Figure 1. Log-transformed root lengths adjusted for the variable dry weight of stem cuttings.

Therefore Al resistance was quantified by comparing root elongation in the Al treatment with that in the basal treatment. For each pair of stem cuttings that had been split between the two treatments, the difference between the log-transformed root lengths in the two treatments was calculated (equivalent to computing the ratio of untransformed root lengths). The two poorly elongating genotypes (114, 44-02) were excluded from this analysis because too large a fraction of their final root length had already been present at the time of transferring stem cuttings into the treatment solutions. The average effect of Al on root elongation varied among growth cycles (33-60% inhibition), perhaps because of the differences in the nutrient status of the stem cuttings (see discussion above). An adjustment for growth cycle-specific effects, which accounted for 13% of the variance of the difference between the log-transformed root lengths, was achieved by subtracting the average difference in a particular growth cycle (i.e., the average effect of Al) from the individual values computed for pairs of stem cuttings. The resulting value was called "Al-resistance index."

If Al resistance and the ability to elongate roots in the absence of external nutrients are separate physiological components that segregate independently, then there should be no correlation between Al-resistance indices and the root elongation potential (the log-transformed root lengths in the absence of Al). Figure 2 appears to confirm this assumption ($r^2 = 0.02$) and underscores the need to break down the two components for the purpose of associating gene expression patterns with either of the two.

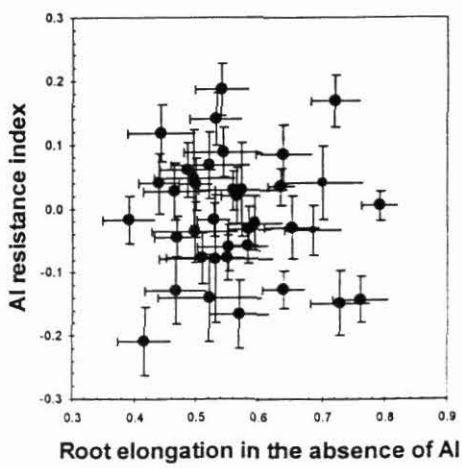

Figure 2. Lack of relationship between Al resistance and root elongation potential. Al-resistance index = [log(root length in Al solution) – log(root length in basal solution)] – (average difference for a particular growth cycle). Root elongation in the absence of Al = log(root length in basal treatment). All log-transformed root lengths were adjusted for the effect of stem cutting dry weight.

Figure 3. genotypes were organized according to their Al-resistance index. Two independent findings seem to confirm the validity of our approach to quantify Al resistance in the stem cuttings:

The Al-resistant parent (*B. decumbens*) had the highest Al-resistance index (the Al-sensitive parent could not be scored due to its extremely poor root elongation in the basal treatment).
Evaluation of a different Al-toxicity symptom—the lateral swelling of roots—demonstrated that roots of genotypes classified as Al resistant tended to swell less than those of Al-sensitive genotypes ($r^2 = -0.78$; Figure 3).

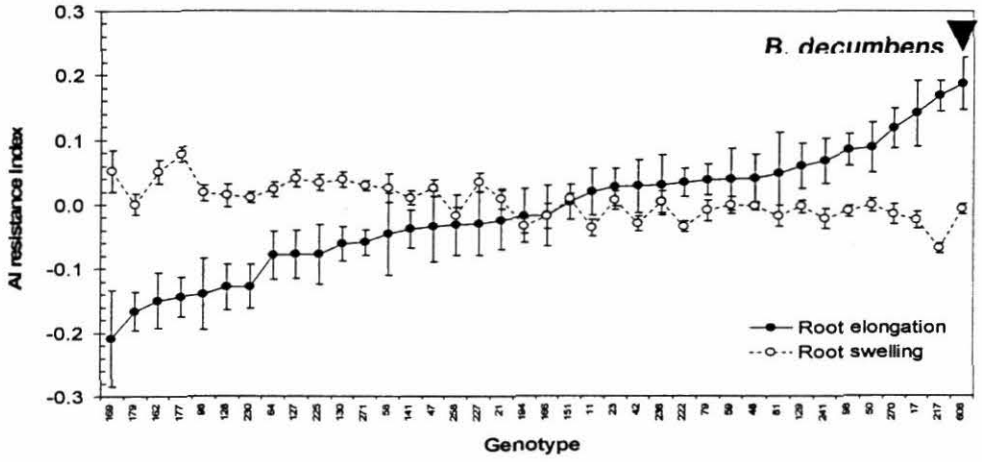

Figure 3. Genotypes ordered according to their Al-resistance index computed from root length data. A similar index that was computed using root thickness data is included for comparison.

Future Plans

- Two groups of contrasting genotypes (4–6 each) will be chosen from the upper and lower end of the range of Al-resistance indices for subsequent experiments to associate gene expression patterns with an Al-resistant phenotype.

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2.3.8 Exploring surface charge density of root apices as a potential factor contributing to aluminum resistance of *Brachiaria decumbens*

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[1]SB-2 Project, CIAT;

Introduction

There is a pronounced difference in aluminum (Al) resistance between *B. decumbens* and *B. ruziziensis*. Previous research has indicated that *B. decumbens* might exclude phytotoxic Al ions from root apices, using a mechanism that does not rely on external detoxification by organic acids (Wenzl et al., 2001). Based on these results we hypothesized that a less negative (or even positive) surface-charge density of root apices could be a highly effective mechanism of nonmetabolic Al exclusion. According to this hypothesis, *B. decumbens* should also be more resistant to other toxicants with different physicochemical properties (ion radius, etc.) as long as they are cations. Here we report on experiments to evaluate the susceptibility of the two *Brachiaria* species to lanthanum (La^{3+}) and other lanthanide ions using relative root elongation (RRE) of seedlings as a measure of resistance.

Materials and Methods

Seeds of *B. decumbens* and *B. ruziziensis* were germinated in 200 M $CaCl_2$ (pH 4.2) for 4–5 days. Homogeneous seedlings with roots 2-3 cm long were transferred to continuously aerated solutions containing 200 M $CaCl_2$ (pH 4.2) plus a lanthanide salt (chlorides of lanthanum, cerium or ytterbium). The seedlings were left to grow in the glasshouse for 3 days. At harvest, root lengths were measured, and root elongation was calculated by subtracting the root length at transfer.

Results and Discussion

The results obtained with La^{3+} show that Al-resistant *B. decumbens* is more resistant to La^{3+} ions than Al-sensitive *B. ruziziensis* (Figure 1). In all other plant species tested thus far, genotypes differing in their resistance to Al^{3+} ions are equally sensitive to La^{3+}. Other lanthanide ions such as Yb^{3+} and Ce^{3+} triggered a similar differential response (Figure 2).

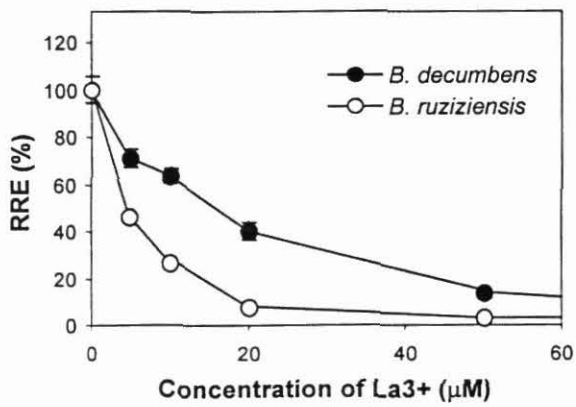


Figure 1. Relative root elongation (RRE) of *B. decumbens* and *B. ruziziensis* as affected by La^{3+} in the nutrient solution. The values shown are means ± SE of 12 seedlings

This is the first time that a general resistance to trivalent cationic toxicants has been observed in plants. These findings are consistent with the possibility that the surface-charge density of root apices—the principal site of Al injury—acts as a generic mechanism to avoid uptake of phytotoxic cationic toxicants. Yet the possibility remains that this "cross resistance" is due to other factors. Further work is in progress to evaluate the significance of a surface-charge-based Al-resistance mechanism compared with other mechanisms.

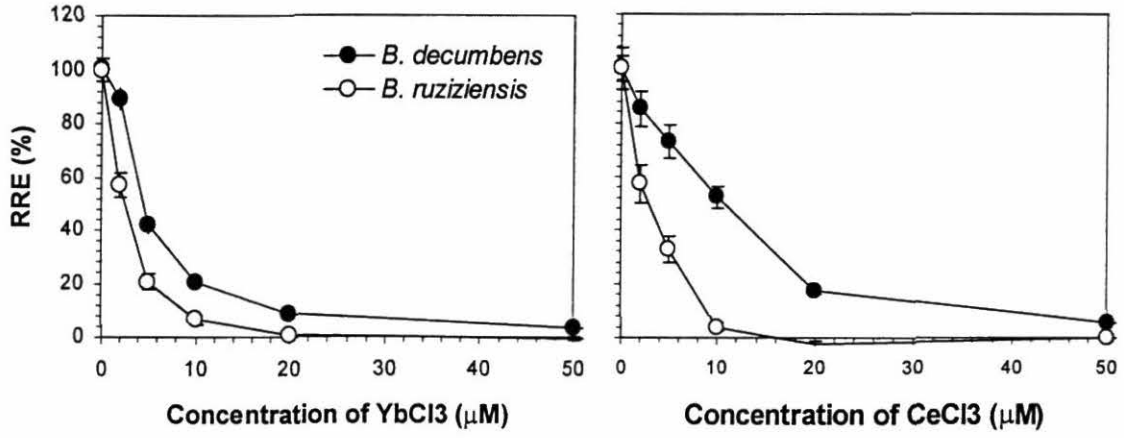


Figure 2. Relative root elongation (RRE) of *B. decumbens* and *B. ruziziensis* as affected by two different lanthanide cations in the nutrient solution. The values shown are means ± SE of 12 seedlings.

Results from these experiments may provide insights into breaking down *B. decumbens* resistance to Al into physiological mechanisms. This may prove to be particularly useful if genetic control of the trait turns out to be complex (which may indeed be the case; see "Identifying individuals with contrasting aluminum resistance in a *Brachiaria ruziziensis* × *Brachiaria decumbens* hybrid population" in this report).

References

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

OUTPUT 3. Collaboration with public and private partners enhanced

Activity 3.1 New Collaborative Arrangements, Networks, Databases, Training and Workshops

Main Achievements

- The Cassava Biotechnology Network for Latin America and the Caribbean (CBN-LAC), now in its second year of operation, is a network of cassava researchers and end-users united by the goal of mobilizing the development and application of biotechnological tools for the enhancement of the value of cassava for food security and economic development in the poorest rural areas of the LAC. Three functional Pilot Sites in Brazil, Colombia and Ecuador were established while that of Cuba is yet to take off. The network carried activities at the pilot. CBN also undertook activities in the areas of fundraising, communication and creation of awareness and staffing

- Guidelines policy on the use of genetic transformation was prepared for management approval. The document "Pact with society"outline. CIAT policy on transgenic research at the center

- A database containing functional categories of ESTs and their application in the construction of cDNA microarrays for the analysis of a plant-pathogen interaction ((cassava – *Xanthomonas axonopodis pv. manihotis)* was implemented. The data set contains 4800 sequences distributed in fifteen functional groups. Among genes with an assigned function, about 8 % were involved in energy utilization; 8 % in protein synthesis and degradation; 4% in disease and defense. 96 clones have been sequenced and compared with GenBank databases. Significant homologies with known genes or putative genes have been found, 8 sequences showed homology with plant resistance or defense related proteins. Among the homologies, we found a catalase, a translation initiation factor 2B, a calmodulin, a glutaredoxin and a UDP-glucose dehydrogenase

- A database was developed in Filemaker Pro for expressed sequence tag information that has been used to search for microsatellites in common bean.

- A set of biotechnology lectures was prepared for public schools in Cali. A guidebook covering basic theory and lab experiments was developed for use in regular science courses. Training of the high school science teachers was also carried to familiarize them with the new teaching tools.

- 13 Genomic and cDNA libraries were constructed in beans, cassava, rice and *Brachiaria*

- During the period of Oct 2001-Sept 2002 a total of more than 70presons from 18 national and international institutions received training with Sb-2 project staff. Approx 318 persons visited the facilities of the project, which amount to 25 percent pf the total of CIAT visitors. A workshop on biosafety was given to 16 staff from the Colombian ministry of the Environment and the Colombian Ministry of Agriculture.

- An international experts meeting on the use of molecular tools for germplassm conservation and use was organized by SB-2 for FAO. Staff members also participated in the organization of an international biotechnology Cassava planning workshop in Bellagio at the Rockefeller Center with funding from RF.

- In the period of Oct 2001-Sept 2002, SB-2 team members published 17 articles in referees journals, two book chapters, 14 articles in Conference proceedings and made 28 presentations at scientific meetings and 7 articles were submitted,

- Assistant from the genome lab received trainings on Mircoarray at the core facilities of Vanderbilt University; on the use of the high through put liquid handling at Tecan in Switzerland, on the use of SNP detection at Luminex in Texas.

- Staff of SB-2 played a major role in the planning and development of the biofortification challenge program and co-organized with IFRPI technical and stakeholder meetings.

- Staff of SB-2 continued the contact with the private sector with Colombian subsidiary or the International branch of several companies to obtain freedom to operate on relevant technologies and to access genes and gene constructs. Similar contact took place with GO in the Andean regions.

- 13 proposals were approved and a total of 24 organizations contributed to the funding of SB-2.

3.1.1 Highlights from the Cassava Biotechnology Network' Activities for 2002

Chikelu Mba
SB-2 Project

Introduction

The Cassava Biotechnology Network for Latin America and the Caribbean, hereafter referred to by its acronym of CBN-LAC, is a network of cassava researchers and end-users united by the goal of mobilizing the development and application of biotechnological tools for the enhancement of the value of cassava for food security and economic development in the poorest rural areas of the LAC. The network is supported by generous funds provided by the Special Program on Biotechnology and Development Co-operation (DGIS/BIOTECH) of the government of the Netherlands and the Canadian International Development Research Center (IDRC) for the funding of a project titled "*The Cassava Biotechnology Network in Latin America: Strategies for Integrating Small-Scale End-Users in Research Agenda-Setting, Testing and Evaluation*". The current regional CBN-LAC started activities in 2001 as an offshoot of the erstwhile global CBN (1992-1998), which was also funded by the DGIS. The regionalization was as a result of reviews of the parent CBN

CBN operates along three main complementary thrusts:

I- Priority setting and evaluation through the strategic use of social science strategically to ensure that cassava end users have a real voice in decision-making in the development and implementation of biotechnologies
II- Technology diffusion by further adapting key biotechnologies together with small farmers by public sector research.
III- Information to promote awareness building/dialogue among scientists and end-users of the opportunities and constraints inherent to biotechnology

The first year of CBN-LAC (2001) was devoted to the establishment of contacts and building collaborations with the responsible entities at the proposed pilot sites at Brazil, Colombia, Cuba, and Ecuador. Proposals were developed and funded for the pilot sites at Brazil and Colombia thus:

Country	Institution	Project
Brazil	EMBRAPA - Mandioca y Frutas Tropicales (CNPMF) Empresa Baiana de Desarollo Agropecuária (EBDA) Farmer communities	Farmer participatory *in vitro* cleaning and multiplication of local and improved cassava varieties
Colombia	Fundación para la Investigación y Desarrollo Agrícola, FIDAR CIAT, PRGA Farmer Association	Application of low-cost *in vitro* propagation techniques to conserve native varieties and produce quality cassava seed in southwestern Colombia

In the case of Ecuador, on account of the peculiarity of not having any agency assigned the role of cassava R&D, and considering all the prior investments into cassava R&D, a multi-stakeholder analysis was initiated leading eventually to the commissioning of a diagnostic study on the Status of Cassava Production and Use. Data collection from the Manabí province, the cassava-producing

region has been completed. An immense body of data was collected and has been commissioned to a local Ecuadorian Consultant for analysis.

While activities are yet to commence in Cuba, partners have been identified in the country, a proposal received and it is hoped that a project will get funded before the end of 2002.

Tragically, the year 2002 was heralded with very unfortunate circumstances in the tragic deaths in an airline accident of Dr. Maria Jesus (Chusa) Gines and Ms Veronica Mera, CBN Coordinator and Social Scientist, respectively in January. This tragic occurrence effectively wiped out the institutional memory of the network. Stop gap measures had to be taken and in May, Dr. Chikelu Mba was appointed interim Coordinator with the task of recovering what had been done to date, continuing with them and thereby laying the groundwork for a Regional Coordinator to be appointed in early 2003.

This report prepared by the Interim Coordination is therefore an update on the status of funded activities at the 3 pilot sites. The report also highlights several other activities undertaken by the Network in the areas of fundraising, communication and creation of awareness, staffing, etc.

Status Report on Activities at Pilot Sites

We present below a synthesis of activities carried out at the individual Pilot Sites at Brazil, Colombia and Ecuador.

Brazil

Project Title: Farmer participatory *in vitro* cleaning and multiplication of local and improved cassava varieties.
Institution: EMBRAPA Mandioca y Frutas Tropicales
Address : Cx. Postal 007, Cruz das Almas- BA, 44380-000, Brazil
E-Mail: wfukuda@embrapa.cnpmf.br

- Collaborating Institution:

Empresa Baiana de Desarollo Agropecuária - EBDA
Farming Communities of Caetite (South-East of Bahia), Brazil

Achievements and constraints. The project took off effectively in March 2002 with the general objective of introducing and implementing participatory biotechnology methodologies with small-scale cassava farmers in the cleaning and multiplication of cassava varieties. This is to be done using the low-cost rapid *in vitro* multiplication techniques developed at CIAT. The project site is the Maniaçu region, Caetite, Bahia, Brazil.

The following activities were carried out. Plans are underway for a technician from *EMBRAPA Mandioca y Fruticultura* who had earlier been trained in the low cost cassava *in vitro* rapid multiplication techniques in the Biotechnology Research Unit of CIAT to understudy the transfer of this technology as is on-going in a similar CBN-PRGA project in Colombia. Adapting this technology for use by the farmers remains a bottleneck for the project in Brazil.

Meeting with the farmer communities from the Caetite Municipality and the Extension Agents to discuss the project and select a pilot site in the region for the farmer managed rapid multiplication laboratory.

Meeting of staff of EMBRAPA, EBDA, CBN Coordination, farmer communities, women representatives, farmers' trade union members and reperesentatives from the Municipal government

Selection of the communities, farmers and Extension Agents for training in EMBRAPA in low cost meristem culture. One Extension staff of EBDA and 2 farmers from one community in Maniaçu were selected.

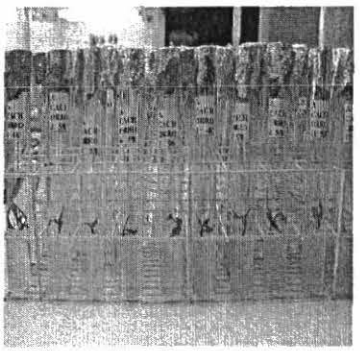
Field trip for site selection in the Maniaçu region

Participatory diagnostic studies involving the farmers and extension agents for the selection of the farmer preferred local varieties *in vitro* cleaning and multiplication. The 2 varieties most widely cultivated by the farmers, Lazam and Aipim Cachorro, were selected.

Five improved cassava clones, 005, 003, BGM1318, BGM 1393 and BGM 1389S, were selected from farmer participatory breeding trials on the basis of resistance to bacteriosis and drought tolerance. These were also the varieties most preferred by farmers in the Caetité region.

Planting materials (stakes) from these selected clones have been submitted to the CNPMF-EMBRAPA Biotechnology Unit for cleaning and rapid multiplication.

Arising from the rapid *in vitro* multiplication of these 5 selected varieties, a total of 432 plantlets have been produced in the Biotechnology Laboratory of CNPMF-EMBRAPA. The multiplication of these clones is on going.



Plantlets from the 5 selected cassava clones in the growth chamber of the Biotechnology Laboratory of CNPMF-EMBRAPA

Difficulties with the work plan. The initial work plan was delayed on account of personnel changes at CNPMF-EMBRAPA leading to the late take off of the project, effectively March 2002. Since this date however, the pace of work has moved really fast. There is ever indication that the project will expand to other regions from Caetite. This prognosis is based on the numerous requests for the sighting of the pilot site in several other places and in the general enthusiasm observed amongst the farmers, extension agents and community leaders from other regions.

Communication and dissemination of information. The project has received a lot of publicity through the radio e.g. the local radio station, Radio Educadora de Caetité, meeting with farming communities, leaders of rural associations, Caetité Municipality, women associations, and the exchange of information between the farmers

Colombia

Project Title: Application of low-cost in vitro propagation techniques to conserve native varieties and produce quality cassava seed in southwestern Colombia
Institution: Fundación para la Investigación y Desarrollo Agrícola, FIDAR
Address: Carrera 42a # 5C-44, Cali, Colombia , Phone: 5134944 Fax: 5536299
e-mail: fidar@cali.cetcol.net.co

Achievements and Constraints

Objective 1

The project has continued to use the multiplication system based on local inputs and low-cost equipment, which has shown an efficiency of propagation of 1:3-4 in the case of cassava clone M Col–1522. The use of fruit juices and other supplements has helped us maintain this propagation rate.

A simple system was developed to manage and control contamination, and involves periodic checks in LB culture medium. Different points and their coloring in the culture medium are indicators of the level of pollutants.

A total of 6061 plants of six clones (MBra 383, CM 523-7, MCol 1522, HMC1, CM 6740-7 and MPer 183) were produced and planted in the rural greenhouse, with the help of local farmers, for the hardening phase. These materials will be assessed in the field during the second semester of 2002 to gather information on plant development, yield, and total cost of management.

Objective 2

Men and women now have the capacity to work together as a group and can therefore modify the conditions of their community through actions directed to improve the level of education of the population, share experiences with other communities, and produce the seed of other crops using in vitro techniques.

The women participating in the project also participate in activities carried out by governmental entities and other NGOs. They are presented as members of a group and participate actively in the development of new projects.

A criterion of common well being is used to handle all interpersonal problems arising between group members. Actions of solidarity continue to be an important characteristic of most project participants, allowing them to overcome economic problems and issues related to insecurity.

Objective 3

Different cassava clones were collected in several municipalities of Cauca, Colombia, and data were gathered on the clones such as climate, soil conditions, and topography of the collection sites; varietal characteristics; and farmer uses.

A total of 27 clones were identified, using IPGRI's standardized morphological descriptors for cassava.

Two *in situ* seed banks were established with the seed collected in farmer plots, using conservationist tillage practices and low levels of inputs.

One additional set of this material was send to CIAT for termotherapy and molecular analyses.

Other Results

The percentage of plant loss in the farmer-managed greenhouse hardening system is considered low (12.6%) because this was the first time that farmers had handled such a large lot of in vitro produced materials.

Overall management of the in vitro cassava seed production project has allowed participating members to address issues such as education, health care (through food security efforts), community organization, improved agronomic practices, and recovery of home vegetable gardens, among others, thus benefiting the entire community.

Several members of the women's group, who have usually been passive 'information receivers' have now become open questioners about the project. Not only the dedication group members have

showed but also the improved yields obtained with the better quality seed have created expectations among neighbors, and thus encouraged participating women to share results with other groups in the region.

Difficulties with the Work Plan. Project activities have been affected by external factors such as the low prices of cassava roots over the last six months and the legal/illegal importation of products such as cassava starch and starch derivatives.
The multiplication of in vitro produced seed in the field has faced serious difficulties because of severe plant health problems. Pests, for example whiteflies, and viral diseases, such as frog skin, have affected most cassava crops in southwestern Colombia.

In view of the abovementioned situation, the group has shown interest in producing *in vitro* seed of other crops such as banana and pineapple and reducing the work carried out with cassava seed until the market situation of cassava roots and starch improves.

*Communication and Dissemination of Information.*Two local workshops were held on the production of good-quality cassava seed. The first was carried out in Santa Ana with the participation of 20 farmers from neighboring rural areas interested in results obtained by the women's group. The second workshop was held in Santander de Quilichao, with the participation of 19 leaders of farmer organizations from the municipalities of Corinto, Caloto, Santander, Toribio, and Buenos Aires working in a production chain program to produce cassava by contract for the agroindustrial sector, sponsored by the Ministry of Agriculture and the Plant program.

On-going Activities

Evaluation of 6000 cassava plants produced in vitro in farmers' fields to produce high-quality seed and then increase seed production using the rapid propagation system.

Fingerprinting of 26 clones collected using the AFLP molecular marker technique.

Two workshops on fast propagation of seed for entities involved in the commercial planting of cassava in northern Cauca, using the farmer-farmer training technique.

Preparation of a document that summarizes the project's experiences over two years in the use of *in vitro* techniques in farmer-managed cassava seed production.

Ecuador

Introduction

In order to update existing information on the status of cassava in Ecuador, a diagnostic study on the production and utilization of cassava in the Manabi Province of Ecuador was carried out as a pilot study. Manabi is the major cassava producing area of Ecuador. The main objective for this study is to determine the components of the major cassava systems in Manabi and by so doing relate cassava production and use with social and environmental dynamics in the communities. In all, 650 surveys were conducted in Manabi and the data from this already inputted. The analysis is on going.

This study will serve as means for evaluating the status of cassava projects that have already been executed in Ecuador, constitute a guide for the execution of development in the cassava cultivating community of Manabi and also serve as the base line data for planning other development projects.

CBN has in close collaboration with PRGA identified some parameters that could be used to elicit certain urgent information from the data collected so that further work in Ecuador could be commissioned and these are summarized thus:

A number of questions that would give a good indication of preferences that could be analyzed by gender (detectable by the respondent's name), age, wealth (as measured by landholdings and tenure status), principal occupation and agroecological zone (various data from Section A).

Questions that relate to the importance of cassava overall compared to other productive activities or community priorities include:

c17 (preference for cassava vs. other crops),
d2 (importance of cassava for family subsistence)
g1 and g2 (importance of cassava to household well-being), and
h9 (indication of what are considered the most important problems faced by the community).

Questions that indicate preferences, problems and priorities for cassava include:

c1, c2, c4, c5 (input requirements and constraints)
c3 (disease problems)
C7, c10, c11, c13, c14, c19, c20 (varieties known, used, preferred and reasons)
c13, c14, c15, c16 (Yield/production)
c18 (preferred characteristics for cassava plants and reasons) - responses may overlap with previous
c21, c22 (demand for mix of varieties)
c24 (might give a rough idea of the importance of cassava for marketing vs. subsistence)
c25 (importance of being able to delay harvest)
c27 (asks about seed knowledge and supply within the community.
May give an indication of the importance of cassava, as well as the degree of innovation and cohesion present in the community)
d3 (importance of cassava by-products)
e4 (varietal preferences of buyers of cassava or cassava products
e1.1, e2.1, e3.1 (difficulties encountered in marketing fresh cassava, cassava starch and cassava flour)
e10 (difficulties encountered in processing (spec grating))
h4 (productivity and profitability of cassava over time)

Note: Although c26 asks about the knowledge of the respondent about varietal resistance to drought and floods, it does not ask them to indicate if resistance to either drought or flood resistance is a priority.

Questions that give an indication of for whom cassava is important and the degree of voice/asset control that person has are:

c12 (who decides variety selection)
d1 (division of labor for cassava and other household production activities)
e5 (control over income from sales of cassava and cassava products)
g4, g5, g6 (control over household income and expenses)

Other CBN Activities

Familiarization with Projects at Pilot Sites and Monitoring of on-going Activities. The interim CBN coordination has traveled to and has become quite conversant not only with the on-going projects but also with the partners at all the pilot sites at Cauca, Colombia; Cruz das Almas, Brazil and to Quito and Manabi, Ecuador. Plans are underway for initiating the Pilot Site in Cuba.

Small Grants. The criteria for the award of the CBN Small Grants have been developed and the announcements inviting applications have been published. It is hoped that the first set of the grants will be awarded by 01 February 2003.

WebPages . The CBN WebPages will be launched before the end of 2002

Recruitment of CBN Coordination Staff. A search is on for the recruitment of a Regional Coordinator to take over the coordination of the Network in early 2003. Also, in conjunction with PRGA, the TOR for the Social Scientist position has been developed and the search is also on to fill this position.

Fundraising. The Canadian International Development Research Center (IDRC) has generously provided funding in the total sum of has been provided 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 de Jesús (Chusa) Ginés and Ms. Verónica Mera, CBN Coordinator and Social Scientist, respectively, who lost their lives in a tragic airplane accident while on an official trip from their base in Quito, Ecuador, to the headquarters of the International Center for Tropical Agriculture (CIAT), Cali, Colombia. Specifically, the fund aims at achieving the following over the 5-year span:

To provide opportunities and support to female and male master's students
from the developing countries of the world to undertake thesis research addressing key elements of the sustainable use and conservation of agricultural biodiversity, in particular:
a) intellectual property rights and access to agricultural genetic resources
b) molecular characterization of agrobiodiversity
c) community-based conservation of genetic agrobiodiversity.
2. 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.

Information Dissemination and Communication. An international workshop was held at CIAT on *in vitro* cassava dissemination and genetic transformation. This activity was carried out with the collaboration of PBA, CBN, and PRGA as posthumous homage to Chusa Gines and Verónica Mera. The 38 participants included farmers, cassava program technicians of both public and private sectors, and representatives of universities of Colombia, Brazil, Cuba, Ecuador, and Venezuela. This workshop served as a space to share experiences in cassava dissemination of different participating countries, analyze the problems encountered, and discuss potential areas of collaboration.
CBN Coordination participated in the Colombian Second International Seminar on Biotechnology, October 23 – 25, 2002, Cartagena, Colombia.

CBN Coordination also took part in the Workshop titled, "Biosafety of Genetically Modified Organisms of Agricultural Interest: Evaluation and Risk Management", October 28 – 29, 2002, Cartagena, Colombia

3.1.2 Biofortification Challenge program

Staff member played a major role in the development of the Challenge Program Proposal titled "Biofortified Crops for Improved Human Nutrition". The program is aimed at improving the health of the poor by breeding key vitamins and nutrients -- vitamin A, iron, and zinc

into the staple crops eaten by poor people. The term ***'biofortification'*** refers to a strategy of breeding staple food crops, which are relatively high in bioavailable micronutrients as a cost-effective means to reduce micronutrient malnutrition in developing countries. The Biofortification CP involves collaboration between eight CGIAR Centers and a large number of partner institutions in developing and developed countries. It is highly inter-disciplinary requiring expertise in various areas of plant science, human nutrition, and social science. The initial target micronutrients are iron, zinc, and vitamin A.

This new initiative will bring to bear the full potential of agricultural and health science to reduce micronutrient malnutrition in the developing world. This program, if fully funded, will have a significant impact on the health of poor people, particularly children and women in the developing world, more than 3 billion of whom are affected by micronutrient malnutrition.

To prepare the Challenge Program Proposal, CIAT and IFPRI held a number of consultative meetings. .In 2002, two planning meetings were held involving donors and scientists.

In April, scientists gathered to discuss and enhance the technical aspects of the proposal. A meeting of proposed Program collaborators was hosted in Houston, Texas, USA, April, 2002 by the USDA, a member institution of the nutritional genomics team, shortly after notification of selection of biofortification as one of three "fast-track" proposals. Responses to the iSC comments on the December, 2001 version of the proposal were discussed, as well as governance and project management issues. A distinguishing characteristic of this meeting were comments and discussion on the use of biotechnology by participants from the private sector.

In May 2002, more than 30 stakeholders attended a two-day meeting to provide their insights on the proposal A two-day meeting Stakeholder meeting, World Bank, May, 2002 was convened primarily to receive input and suggestions on the proposal and the iSC comments, from a number of institutional perspectives, from those not initially collaborating in the project. A distinguished group was assembled representing NARES, NGOs, the private sector, donors, and scientists from a number of disciplines, in addition to a subgroup of proposed Biofortification Challenge Program collaborators. The tone of the meeting was quite positive and many constructive comments were received. A new draft of the revised proposal was prepared after the stakeholder meeting incorporating these comments and a final version was submitted to the Interim Science Council for their consideration.

The Biofortifcation proposal has now successfully passed the review by the interim Science Council and was strongly recommended by the Executive Science Council for funding. USAID has already funded a CIAT proposal on "Breeding micronutrient-dense, high iron and zinc beans to combat micronutrient deficiency and anemia in Latin America and Africa."

3.1.3 Sequence Databases for Microsatellite Development in Common Bean

MW Blair, M Muñoz
SB-2 Project

Introduction

The development of microsatellite markers is a sequence intensive process as many raw sequences are obtained and processed to come up with a relatively smaller number of positive clones for which a marker can be designed. To facilitate the process of developing microsatellite markers, the management of sequence information needs to be well organized. As described in earlier sections of this report we have been developing a series of microsatellite markers based on small insert and cDNA sequences. The objectives of this work was to create a database using the software Filemaker Pro 5.0 (Claris, Inc.) to house over 2000 sequences of clones that were positive in hybridization assays with simple sequence repeat containign oligonucleotides.

Methodology

The Macintosh version of Filemaker Pro 5.0 was used to create the microsatellite sequence database. Flat file tables were created in MS Excel and related to each other through the database. Two master tables, one for sequences and the other for primers were created for each of the microsatellite discovery projects based on small insert or cDNA clones.

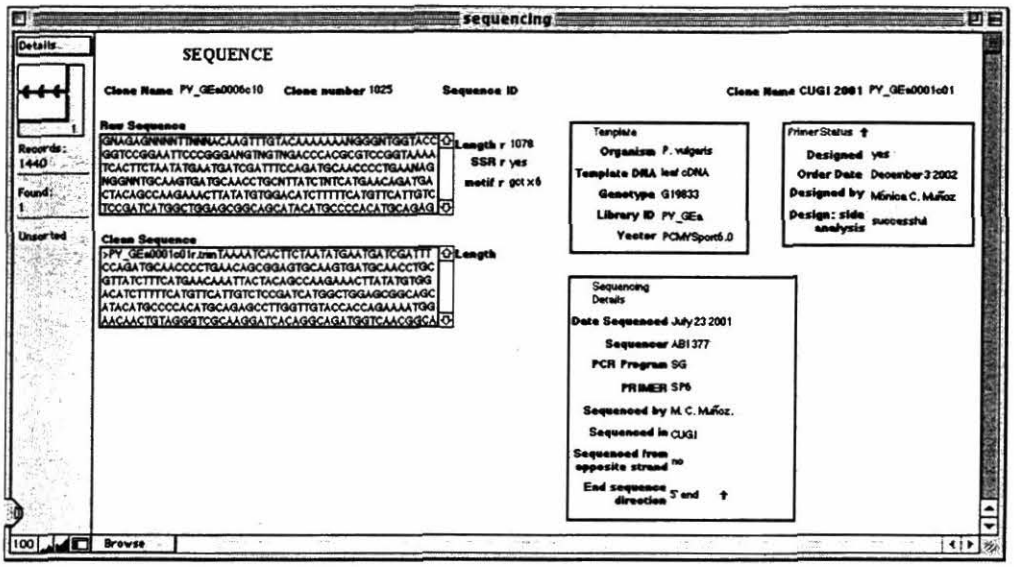
Results and Discussion

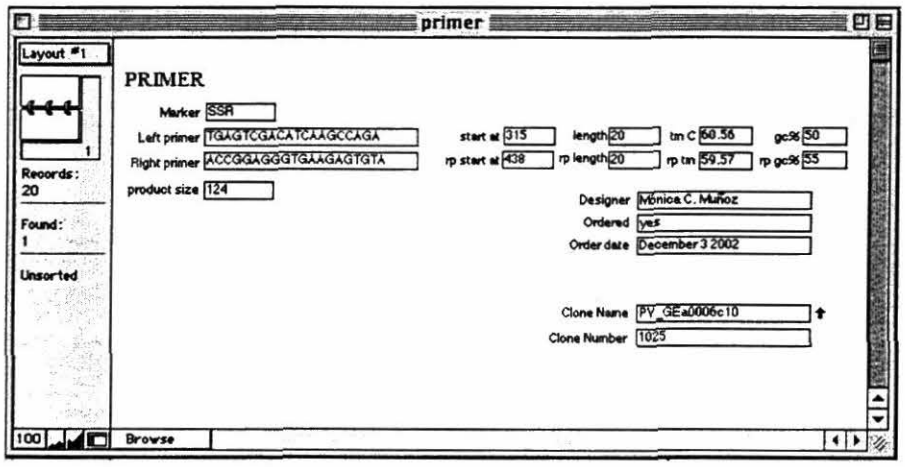
The sequence database contained all the raw 5' EST sequences from the ABI sequencer and for those sequences that were positive for simple sequence repeats, the basic motif was given as well as the number of repeats and the vector/adaptor-trimmed, processed sequence (Figure 1). When simple sequence repeats were not found in the 5' cDNA sequence, the clones 3' sequence was included. A link between 5' and 3' sequences from the same clone is identified via the clone name. The presence of a simple sequence repeat in the 3'cDNA sequence was indicated in the same way as for 5' cDNAs. Details regarding the 'sequencing primer' that was used, and the 'sequenced end' are available next to each clone, and there is a button that links this information with the opposite's end sequence, if it exist. The database also describes whether primers have been designed or not for one particular clone, and if the prior to primer design whether BLAST or ORF searches were conducted. It also links this information to a table where the product size, each primer's sequence, GC%, start and end points, and length, are detailed.

Future Plan

- Add EST sequences for root and leaf cDNA library clones that contain microsatellites to the database

Figure 1. Sequence database for common bean cDNA sequences containing microsatellites: A) sequence and B) primer entries.





3.1.4 A Web-Based Data Base of Simple Sequence Repeat Characterization of Genetic Diversity of Cassava Land Races.

Charles Buitrago, Fernando Rojas, Danny Mauricio Montero, Martin Fregene
CIAT
Funding: IPICs, University of Uppsala, Sweden

Introduction

One of the objectives of the cassava molecular diversity network (MOLCAS) is to make available to cassava researchers every where results of the molecular characterization of cassava genetic diversity. With the completion of the Peruvian, Nigerian and Tanzanian study, a database was

constructed in Oracle to accommodate the results of the above and other studies. Data available for viewing include passport data of the accession, raw SSR gel data, allele sizes, SSR locus information, parameters of genetic diversity and differentiation and principal component analysis (PCA) of genetic distances. The data base will be updated as other country studies become available.

Results

The data-base is organized according to country studies, the Peruvian, Tanzanian and Nigerian country studies are the available ones at the moment. The first page of each country study has links to raw marker data namely, allele sizes per accession and genome location of the marker, where available (Fig 1). Other links are intra-population estimates of genetic diversity, gel images of individual markers and appendixes of additional information from the country study (Fig 1). The URL for the data base is: http://newwebciat/yuca/Molcas/index.htm. Since the inception of the data-base in June, average monthly hits for the first three months averaged 2500 hits per month (Table 2). The high number of visits to the site confirms the importance of the MOLCAS data-base for the cassava community.

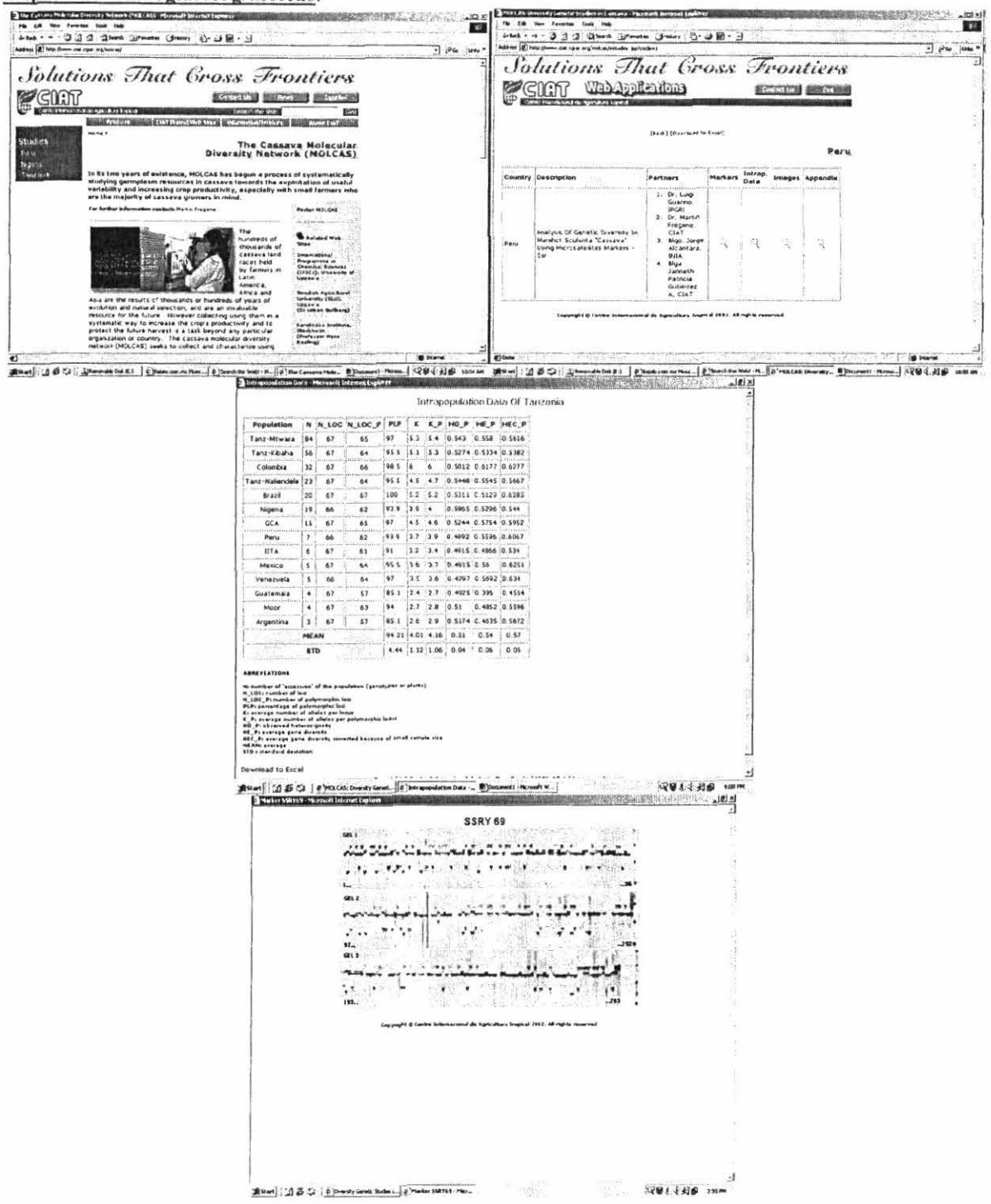
Table 1. Monthly hits at the MOLCAS web site http://www.ciat.cgiar.org/molcas for June, July and August and the total for the 3 months.

Request	Aug 2002	Jul 2002	Jun 2002	Total Hits
/molcas/imagen.jsp	1,033	723	816	2,572
/molcas/locus.jsp	982	743	713	2,438
/molcas/alelosp.jsp	556	84	486	1,131
/molcas/	123	114	94	350
/molcas/markers-det.jsp	110	45	63	220
/molcas/estudios.jsp	96	93	0	196
/molcas/studies.jsp	35	16	67	119
/molcas/intrap_data2.jsp	55	37	22	114
/molcas/pcr_cond.jsp	50	22	20	93
/molcas/imagenbioquim.jsp	50	25	0	75
/molcas/appendix1.jsp	44	19	0	65
/molcas/appendix2.jsp	45	10	0	55
/webapps/molcas/	4	2	46	52
Total	3,183	1933	2327	7,480

Future Perspectives

Include results of other country studies as they become available
Seek for a way to unite data sets from diverse studies.

Figure1. An illustration of the different pages of the SSR diversity data-base on MOLACS web site http://www.ciat.cgiar.org/molcas.



3.1.5 Towards the construction of an EST database and cDNA microarrays for the high-throughput study of cassava physiology and defense

Mauricio Soto Suárez,[1] Silvia Restrepo,[1-2] Chikelu Mba[1]; Valerie Verdier[2] and Joe Tohme[1]
[1]SB-2 Project, CIAT; [2]Institut de Recherche pour le Developpement (IRD)

Introduction

Cassava (*Manihot esculenta* Crantz), a major food crop in the tropics, feeds about 500 million people throughout the world (Restrepo et al., 1999). The application of molecular genetic analysis to the improvement of the cassava crop has been limited as compared to other crops. The construction of EST (Expressed Sequences Tags) databases and DNA microarrays provides powerful strategies for investigating diverse conditions in cassava such as disease resistance and defense responses, starch content and postharvest deterioration. This study refers to the implementation of a database containing functional categories of ESTs and their application in the construction of cDNA microarrays for analyzing plant-pathogen interactions and the study of differences in starch content of different cassava varieties. The plant-pathogen interaction studied corresponded to the cassava–*Xanthomonas axonopodis* pv. *manihotis (Xam)* pathosystem.

The objectives of this study were to:

Evaluate and identify expression patterns involved in the defense response of *M. esculenta* to *Xam* through the construction of an EST database and specific DNA microarrays of cassava.
Evaluate and identify differences in starch content from two cassava varieties through the construction of an EST database and specific DNA microarrays of cassava.
Contribute to the construction of a DNA chip of the Euphorbiaceae family.

Materials and Methods

EST database construction. ESTs of *M. esculenta* were obtained from NCBI (National Center for Biotechnology Information), EMBL (European Molecular Biology Laboratory) and a library constructed at CIAT (Project SB-02: Assessing and utilizing agrobiodiversity through biotechnology) and sequenced at the Université de Perpignan. A putative function was assigned to each sequence based on similarity to sequences of known genes. The BLASTn and BLASTx algorithms were used to query the NCBI. The highest expectation values (E values $<1 \times 10^{-4}$) were retrieved for each sequence.

Sample preparation and cDNA synthesis. Two cv. (M Bra 685 and SG 107-35) were evaluated for differential gene expression over time after inoculation with *Xam* isolate CIO 151. Four-week-old plants of these cultivars were inoculated by leaf and stem puncture, respectively. Stem tissues were collected at 6, 12, 48, and 72 h postinoculation (pi), and 7 days pi. The controls were healthy noninoculated plants and plants inoculated with sterile water. The tissue was preserved in liquid nitrogen, and total RNA was isolated using the Proteinase K method (Hall, 1978). Poly (A) RNA was isolated using Oligotex mRNA Midi kit (QIAGEN, CA). cDNA was synthesized, using a SMART™ PCR cDNA synthesis kit (CLONTECH, CA) from 400-500 ng of mRNA as starting material.

Subtractive library. In order to isolate defense-responding expressed genes to pathogen attack, a method was implemented that can identify the differences between two DNA populations rapidly—Differential Subtraction Chain (DSC) (Luo et al., 1999). A cDNA pool obtained from inoculated plants was used as the "tester," and cDNA pools of healthy noninoculated plants and plants inoculated with sterile water were used as the "driver." The PCR-amplified cDNAs (tester and driver) were purified, digested with *DpnII* and ligated with two separate primer/adaptor sets (Set I: BamIa, BamIb; set II: BamIIa, BamIIb). The ligation products were subjected to PCR amplification under the following conditions: 94^0C for 2 min and 94^0C for 30 sec, 68^0C for 2 min for 30 cycles. For cDNA DSC hybridization, 10 µg of restriction enzyme-digested (37^0C for 2 h) driver was mixed with 100 ng of tester DNA in 32 µl of hybridization buffer (50 mM Hepes, 1 M CTAB). The mixture was heated to 98-100^0C for 5 min, and 8 µl of 5 M NaCl was added. The mixture was then incubated at 67^0C, for 14-16 h. The re-annealed products were purified and re-suspended in 50 µl of 1X mung bean nuclease buffer (New England Biolabs, MA) with 10µl of mung bean nuclease at 30^0C for 25-30 min. An aliquot (5-10%) of the purified DNA product was used for PCR to examine subtraction efficiency, and 2 µl were checked in polyacrylamide gel. The PCR was performed under the following conditions: 94^0C for 2 min and 94^0C for 30 sec, 68^0C for 2 min for 35 cycles. The primer sequence corresponded to BamIa. The remaining product was concentrated and re-suspended in 32 µl of hybridization buffer and reheated to 98^0C for a second round of hybridization.

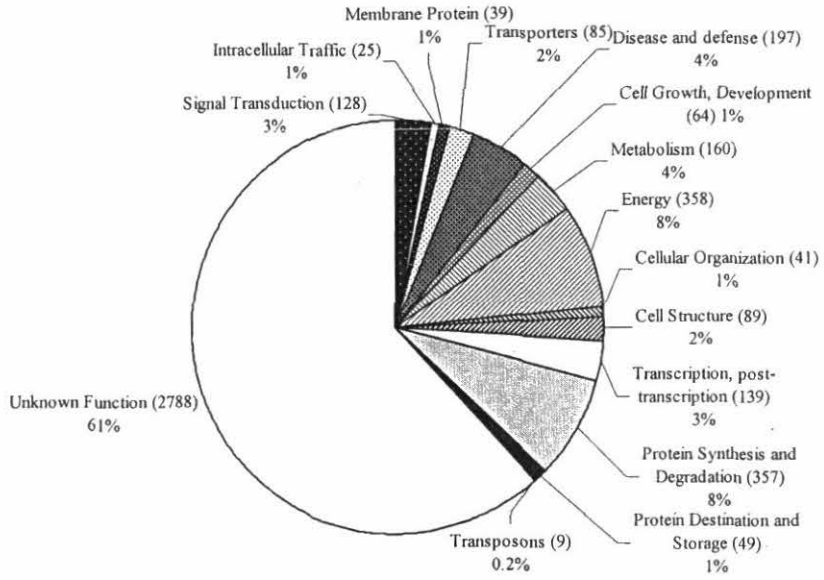
The PCR products of the second and third round hybridizations for SG 107-35 and the third and fourth round hybridizations for M Bra 685 were ligated to pGEM®-Teasy (PROMEGA), and a library was constructed of one 384-well plate for each round.

Sequencing library. At present 48 clones from the third round of hybridization for SG 107-35 and 48 clones from the fourth round of hybridization for M Bra 685 have been sequenced using an automated sequencer (ABI Prism 377). The sequences were edited using Sequencher 4.1 (Gene Codes Corporation) and compared with the GenBank databases using BLASTx and BLASTn algorithms.

Results and Discussion

The data set contains 4800 sequences distributed in 15 functional groups (Figure 1), classified according to a scheme proposed in previous studies (http://www.mcdb.ucla.edu/Research/Goldberg/ESTs/energy.html; Nelson et al., 1997; Somerville and Somerville, 1999). The preponderance (61.5%) of sequences with an unknown function may reflect the lack of molecular biology research on the Euphorbiaceae family.

Among the genes with an assigned function, about 8% were involved in energy utilization, 8% in protein synthesis and degradation, and 4% in disease and defense. The number of ESTs and the percentage of each category are presented in Figure 1.



- **Figure 1. Functional groups of ESTs. The number of ESTs in each category and their percentage are indicated.**

A total of 96 clones have been sequenced and compared with GenBank databases. Significant homologies with known or putative genes have been found for 23 sequences: 14 for M Bra 685 and 9 for SG 107-35 (Table 1).

For M Bra 685, five sequences showed homology with plant resistance or defense-related proteins and three for SG 107-35 (Table 1). Nevertheless, proteins classified in other functional groups such as intracellular traffic and signal transduction can be involved in disease resistance.

Among the homologies showed by the sequenced clones we found:

Catalase CAT1 is reported as involved in oxidative processes of deterioration response from cassava (Reilly et al., 2001).

Translation initiation factor 2B plays an important role in regulating the initiation of protein synthesis (Moon et al., 2001).

Calmodulin is reported as being involved in wounding and pathogen responses (Cheong et al., 2002).

Table 1. M Bra 685 and SG107-35 clones with respective BLASTx E value

Functional category and homology	M Bra 685	SG107-35
Disease & defense	**E value**	**E value**
Catalase CAT1	3E-62	
Glutaredoxin	3E-38	3E-22
Translation initiation factor 2B	8 E-12	
DNA J protein		5E-24
Putative esterase	1 E-7	
UDP – glucose dehydrogenase		3E-36
Calmodulin	5E-43	
Intracellular traffic		
Nonclathrin coat protein	3E-23	
NTGP1		1 E-5
Signal transduction		
Specific GTPase activating protein (RhoGAP)	1 E-13	
Protein synthesis & degradation		
Ribosomal protein	3E-23	
Protein destination & storage		
Ubiquitin precursor	3E-36	
Cell structure		
Ankyrin–like protein		1E-15
Others		
SecA-type chloroplast protein transport factor	2 E-15	
Phospho-2-dehydro-3-deoxyheptonate aldolase 1	4E-20	
Unknown	3E-26	
F14D16.29	2E-44	
Putative protein	2 E-18	
Unknown protein		1E-70
Hypothetical protein		1 E-5
Hypothetical protein TC0129		6 E-15
Expressed protein		2 E-14

Glutaredoxin prevents oxidative damage in sieve tubes from *Ricinus communis* (Szederkenyi et al., 1997).

UDP-glucose dehydrogenase is important in *Xanthomonas campestris* pv. *campestris* infection in crucifers (Chang et al., 2001).

Future Activities

- Additional Development of an EST database containing approximately 10,000 sequences organized in functional groups and implemented in FileMarker Pro 5.0
- Sequencing of subtractive libraries
- Microarray construction with clones from the disease and defense functional group of the EST database and clones from the subtractive library
- Hybridization of the microarrays with mRNA-derived probes from plants inoculated and collected at 12, 72 h and 7 days pi.
- Microarray construction with ESTs related to starch content and hybridization with mRNA-

derived probes from tissue collected from cassava cv. M Per 183 and CM 527-7

- Microarray construction with cassava clones from all the different functional groups and with clones from other Euphorbiaceae species.

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3.1.6 Development of a Biotechnology Program for Public Schools

R.H. Escobar[1], F. Escobar[1], G. Gallego[1], D.F.Cortez[1], A.L. Chavez[1], A. Bohorquez[1], A.L. Caicedo[2], A.S. Narvaez[3], E. Lozano[3] , J. Lugo [3]and J. Tohme[1]
[1] SB2, [2] Washington University- San Louis, [3] Centro Auxiliar de Servicios Docentes, Sede Cali.

Introduction

Technology may be considered part of the development motor of society. It is therefore necessary that the general public be assisted to keep up with the pace of technological changes. CIAT has been considered as a promoter research site in the region. Taking care of that, many activities have been developed to motivate knowledge diffusion, such as "Biotechnology for not Biotechnologist" and "Open-House" where people don't were afraid to ask everything than they want to know about genetic transformation. Other activities have been developed to attend journalist, police makers, business managers, universities, public and private schools, ONG and private industry among others, allow us demystify biotechnology. Styles (2002), consider that if biotechnology has a potential role in our society, scientist must be proactive in educating the general public.

We consider that if people had good knowledge about how biotechnology is, they could take decisions and adopt more easily the changes. This will also involve educating the educators.

Materials and Methods

A multidisciplinary working group was establishing to perceive how many biology teachers know about new technologies, biotechnology and his art-status.
Some biological-sciences teachers were invited to attend a meeting at CIAT. They receive one day of basic biotech theory.
Some themes were selected by research and teacher to include in practical section.
A basic manual was written to use as guidebook.
Teacher reviews it and make suggestion about pedagogic form and how were the best form to present very pleased the information.
A practical guide for laboratory experimentation was developed.
Science-biology teacher was invite to attend a practical 2-day section. In the round-table they judge the activities.
Conclusions and suggestion were take into consideration to adjust both manuals.
Science teachers are implementing biotechnology lab activities and they are thinking to involve in normal curriculum agenda.

Results and Discussion

Teachers from public school had difficulties with its up dating, reading technical paper in English and Internet access. Biological sciences are very challenging to teach in school, for that reason a relevant issue is that they could maintain access to technical support.
In some cases teachers consider that biotechnology is expensive technique. They do not consider that it's allowed doing in his/her lab.
Research has the information but teachers have the manner to facilitate this sort of learning by their students.

A guidebook was under checking taking care of teacher's suggestion. A guidebook including cell and molecular biology themes such as cell division, tissue culture, DNA extraction, PCR and other molecular techniques and evolution among other topics were wrote.

Manuals involve how could they build low cost equipment to implement the biotechnology activities suggested in its curriculum.

A B C

Figure 1: (A) Science-biology teacher laboratory section. (B-C) "Knowing DNA"

Conclusions

A guidebook including theory and biotechnology lab experiments has been developed to use in normal science courses.
Educating our consumers will be invested for the future and they will be ready to accept challenges that technological progress present. Improve public relations is only one element of a strategic plant for the future, and education is one strand of the plan.

Future activities

Diffusion on biotech module will be with teacher-teacher interaction through CASD training implementation.
When this module was implemented as normal course, a feedback meting will be plane to know how was the adoption.

References

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Balkwill F. and Rolh M. 2002. Have a nice DNA. Cold spring Harvor Laboratory Press. 33pp

Rainis K and Nassis G. 1998. Biotechnology projects for young scientists. Grolier publishing. 158 pp

3.1.7 Cassava BAC library- Collaboration with Clemson University

Clemson University constructed as part of a collaborative project funded by USAID, a BAC library for cassava using the clone MECU72. This clone carries resistance to the whitefly pest *Aleurothachellus socialis*. The library contains 73,728 clones stored in 192 384-well microtiter plates. A random sampling of BACs indicated an average insert size of 93 kb. Based on a genome size of 760 Mb, library coverage is approximately 10 haploid genome equivalents. Both whitefly resistance and ACMV will be targets for map-based cloning using the BAC libraries as tools.

3.1.8 Gene Libraries/construct

cDNA

- From roots of cassava cv. CM523-7, for analysis of Post Harvest Deterioration (PHD), Samples taken at 0,3,4,12,24,48 and 72 hours post-harvesting
- From mature roots of high –carotene-content cassava cv Mper297
- From roots of cassavacv. CM523-7 and Mper 183, for starch quality
- Full-length cDNA from total inflorescence of *Brachiaria* (*B. ruziziencis* and *B. decumbens*), to analyize genes involved in Apomixis
- Bulked, full-length cDNA from embryo sacs of sexual and apomictic Brachiaria individuals
- Forward and reverse substractive cDNA libraries of *B.ruziziencis* and *B.decumbens*
- Forward and reverse substractive cDNA libraries from embryo sacs of sexual nd apomictic *Brachiaria* individuals.
- From ten day old *Brachiaria* seedlings
- From rice cv. IRAT13 infected with Pyricularia isolate CICA 9-31-4

Genomics

- From cassava leaves for PHD
- From bean cv. G19833 for isolation of TY1 copy retroelements
- For microsatellite isolation in beans, *Brachiaria* and palms
- For Diversity Array Technology (DarT) in bean, cassava, *Brachiaria* and rice.

3.1.9 Visiting collaboration national and international

Myriam Cristina Duque. Conferencia sobre Métodos Estadísticos Aplicables a la Biología Molecular. DANAC. Venezuela, Nov. 2002

Chikelu Mba. Perpignan University. Nov, 2001

Joe Tohme, Paul Chavarriaga, Roosevelt Escobar, Chikelu.Mba, Diego Cortes. 2 International Seminar on Agriculture Biotechnology. Cartagena. Oct, 2002

Joe Tohme. Bioinformatics, Dinamarca. Oct. 2002

Daniel Debouck. FAO – Rome. Oct, 2002

Z. Lentini. Braunnsweig, Germany. October, 2002
Visit the Federal Biological Research Center for Agriculture and Forestry (BBA). Contact: Joachim Schiemman
Follow up collaboration Gene Flow Project funded by BMZ
Get acquainted with current technology for precision genetic engineering including elimination of marker genes in transgenic plants, site specific gene precision insertion, bioprospection of specific promoters
Discussion of potential future project
Give a seminar

Z. Lentini. Beijing, China. October, 2002
Attend the The 7th International Symposium on the Biosafety of Genetically Modified Organisms
Present two posters
Contact with donors for potential future projects

Z. Lentini. Cartagena, Colombia. October, 2002
Attend the International Workshop on the Biosafety of GMOs organized by OEA and Cambiotec, Canada. Give a talk as Invited Speaker.

Z. Lentini. Hannover – Germany. October 2002
Visit the University of Hannover. Contact: Hans-Jorg Jacobsen
Follow up collaboration Gene Flow Project funded by BMZ
Give a seminar

Z. Lentini. Bogotá – Colombia. February, June, and October, 2002
Colciencias, Biotechnology Council. Contact: Myriam de Peña, Director
Participation as a Council Member on regular meetings for project revision and approvals, and decision making on National Biotechnology Programs

Joe Tohme. Cassava Global Plan. Bellagio, Italy. Sept, 2002

Eliana Gaitán. Manejo Luminex – Austin, USA. Sept., 2002

J. Tohme, A. Bellotti, H. Ceballos. Meeting Cassava Global Plan – Bellagio, Italy. Sept, 2002

Beebe. Brazil, September, 2002. Attendance at national been meeting. Vicosa, M.G.

Beebe. México. September 2002. Attendance in BNF Project coordination meeting. Cuernavaca, Morales.

Joe Tohme. CORPOICA – CEGA. August, 2002

Z. Lentini. Villa de Leiva – Colombia. July 2002
Institute von Humboldt. Contact: Rodrigo Artunduaga, and María Teresa Palacios
Write up proposal for the Implementation of Cartagena Protocol on Biosafety of GMOs in Colombia, Sponsor: GEF/ World Bank

E. Gaitán. Laboratorio de Vanderville Microarrays. Nasville. July, 2002

C. Mba, R. Escobar. CBN- Ecuador, July, 2002

Myriam Cristina Duque. Congreso Internacional de Fitopatología. Sociedad Mexicana de Fitopatología. México – Monterrey. July, 2002

Z. Lentini. Bogotá – Colombia. July 2002
World Bank. Contact: Mike McHannon
Final writing of proposal for the Implementation of Cartagena Protocol on Biosafety in Colombia for the GEF/ World Bank
Visit ETH. Contacts: Christof Sautter and Whilem Gruissen.
Discuss collaboration in rice and cassava biotechnology
Give a seminar

Z. Lentini. Basel –Germany. June 2002.
Visit Syngenta. Contacts: Willy De Grief and Bruce Lee
Discuss potential collaboration on assistance for biosafety work at CIAT and access to proprietary technology for CIAT use

Z. Lentini. Zurich - Switzerland. June 2002
Follow up collaboration of Rockefeller Project on Deployment of RHBV Resistant Transgenic Plants to Farmers and Advisory Support on RHBV resistance field evaluations and biosafety.
Coordination of the 2nd BMZ Project Meeting on Gene Flow Analysis

Z. Lentini. Torino – Italy. June 2002.
Attend RicEU Conference on rice Biotechnology
Give two talks as Invited Speaker. Contacts: John Snape (John Inness Institute - England; Emmanuel Guiderdoni, CIRAD – France)

S. Beebe. El Salvador and Costa Rica, Nicaragua and Honduras in 2002, to review field trials including drought tolerance studies.

J. Tohme, Biofortification Project. Washington. June, 2002

Joe Tohme. Alianza Andina - Instituto von Humboldt. May , 2002

Chikelu MBA visiting CNPMF/EMBRAPA. May, 2002

Z. Lentini. San José de Costa Rica – Costa Rica. May 2002

University of Costa Rica, San José and Alajuela Experimental Station. Contacts: Ana Mercedes Espinoza, Ana Sittenfeld, and Rodolfo Araya.

Microsatellites de musa en Guadalupe – Venezuela. May, 2002

Roosevelt Escobar, Gerardo Gallego. Workshop on Bioprospección. Universidad Nacional, Bogotá. April, 2002

S.Beebe. Cuba 2002 To review field trials.

Roosevelt Escobar, CBN- Brasil, Proyecto Bajo Costo. April, 2002

Beebe. PCCMCA. Dominican Republic, April 13-20, 2002.

Z. Lentini. Bogotá – Colombia. April 2002.
Attend meeting at the Ministry of Environment
Discussion on development for the Colombian National Biosafety Law

Joe Tohme Antioquia University. March, 2002

Fernando Rojas, Workshop on Bioinformatics. National Center for Genome Resources (NCGR). Santafé Estado New México, March 11-15, 2002

Joe Tohme. USAID Biofortification Project. Feb. 2002

Joe Tohme IRRI. Philippines. CIAT – JIRCA. Feb, 2002

Myriam Cristina Duque. Detección de QTLs. Cornell University. S.McCouch. Feb, 2002

Joe Tohme. Instituto von Humboldt – Dr. Juan Lucas Restrepo, Jan., 2002

450 students from more than 20 national universities and institutions visited SB2 during Sept/01 – Oct, 2002

3.1.10 Training, Workshops, Conferences: International, National and for CIAT Personnel

Tania Quesada, M.Sc. University of Costa Rica. Training on microsatellite analysis of rice and their use for gene flow analysis into wild/ weedy relatives. March 2002

Griselda Arrieta, M.Sc.. University of Costa Rica. Training on rice crossing, transgenic field testing, biosafety, and RHBV evaluation. March 2002

Fabiana Malcarne, Ph.D.Universidad Central de Venezuela, sede Maracay, genetic transformation, field testing, biosafety, gene flow. April – May, 2002

Erika Arnao, M.Sc. Universidad Central de Venezuela, sede Maracay, microsatellite for rice characterization.

Juan Manuel Beltrán. Universidad de Sucre, Colombia

Javier Beltrán, Universidad de Sucre, Colombia. March 4 – 8, 2002

Davi Junghands, EMBRAPA, Brasil. March 4 –15, 2002

Arrieta Griselda, Universidad de Costa Rica. March 10-23, 2002

Malacarne Fabiana, Centro de Investigaciones en Biotecnología Agrícola, Argentina. April 15-May 15, 2002

Quesada Vargas, Tania, Universidad de Costa Rica, Marzo 10 – 23, 2002

J. Tohme. Biointegra. Conference. Bogotá, July, 2002

Eliana Gaitan received training in Luminex-100 Instrument. University of Florida, Miami. Dec, 2001 and N. York, April, 2002.

E Gaitan received training in L-2/L-2/L-4 Liquid Handling for Genesis with disposable tip option and low volume option. TECAN. Switzerland, March 2002.

Iván Acosta. Training Yale University at Steve Dellaporta's laboratory. May, 2002

E. Gaitán. One week visiting Shawn Levy's Lab. In Vanderbildt University: Spotting conditions of SPBIO I, July 2002.

G. Mosquera and S. Restrepo, Universidad Nacional de Colombia. Genomics of a plant-pathogen Interaction. July 23, 2002

E. Gaitán. Instrument installation and Genesis/ RSP-150 instrument training. Two days training in CIAT, April 2002 .

CIAT and FIDAR offered the workshop on "Cassava Propagation and Integrated Pest Management" for twenty small-scale farmers from Santa Ana (Cauca, Colombia). Roosevelt Escobar, Carlos Julio Herrera and Javier López were the instructors. July 9/2002.

SB2 Staff offered the workshop " Herramientas de la Biotecnología Moderna para el Mejoramiento de Plantas", on June 25/2002, at the Universidad Nacional, Bogotá, Colombia. The objective was to inform and update students, professors and the general public, on the latest advances on plant transformation, biosafety of transgenic plants and genomics. Over 100 people attended.

Adriana Almeida was a speaker in the "Taller sobre Bioseguridad de Plantas Transgénicas". Extracción y análisis de ADN. Sept. 20, 2002

Paul Chavarriaga was a speaker in the "International Workshop on Genetic Engineering for Colombian Agriculture", September 12-13/2002, Universidad Nacional, Bogotá, Colombia.

SB2 Staff organized the workshop "Biotechnology and the Media" September 29-30, 2001, CIAT, Cali.

SB2 Staff organized the workshop "Biosafety of Transgenic Plants", Ministries of Agriculture and Environment of Colombia. CIAT, Cali, Colombia, September 20 – 21, 2002

Astudillo, C, Blair MW "Identificacion de genes para contenido de hierro y zinc en dos poblaciones de frijol comun (*Phaseolus vulgaris*) y mapeo de genes candidatos de ferriting". Primer Coloquio Internacional y Segundo Nacional Sobre Nutricion en la Universidad de Antioquia Medellin. August 8 – 9, 2002.

Blair MW, Beebe S, Astudillo C, Rengifo J, Giraldo MC, Pedraza F, Tohme J, Graham R "Progress and potential for improving micronutrient content in common beans" Invited Speaker – Legume section. Plant and Animal Genome, San Diego, California, USA, January 12-16, 2002. Proceedings

Blair MW, "Bioligical Nitrogen Fixation and bean genomics" ENSA, Montpellier, France. March 6, 2002

M. Fregene visit to the Iwate Biotechnology Research Center (IBRC), Kitakami, Iwate, Japan. Transient assay of a CMD candidate gene for resistance to the African Cassava Mosaic virus. March 23-April 19, 2002

M.Fregene, visit to Uganda. Set-up of a SSR marker lab in Kampala (medical biotech labs) and visit to NARS partners. May 15-May 228 2002

M.Fregene, visit to India. Visit to CTCRI on the MoU between CIAT and CTCRI on germplasm transfer and training July 20 to July 30, 2002.

M.Fregene,attend the Meeting on Biotechnology, Breeding and Seed System s for African Crops in Entebbe Nov 3-7.

Blair MW, Buendia HF, Hoyos A, Mahuku G, Cardona C "Mejoramiento de Frijol Rojo Moteado Caribeño en el CIAT" XLVI IAnnual Meeting PCCMCA. Santo Domingo, Dominican Republic. April 2-6, 2001. Proceedings.

Blair MW "Micronutrient improvement in common beans" Phaseomics meetings, Geneva, Switzerland May 15, 2002

Blair MW, Beebe S, Astudillo C, Rengifo J, Giraldo MC, Tohme J, Graham R"Role of Nutritional Genomics in Improving Micronutrient Content in Common Beans" Invited speaker. International Legume Genetics and Genomics Conference, Minneapolis, Minnesota, USA. June 2-6, 2002

Blair MW, "Tecnicas modernas de fitomejoramiento aplicado al frijol" Invited Speaker. VII Reunion Boliviana de Leguminosas y Rhizobiologia, Santa Cruz, Bolivia, Sept 17-20, 2002

Blair MW, "Uso de marcadores moleculares en estudios genéticos de frijol" CIP, Sept 23, 2002

Blair MW, "Aplicacion de la biotechnologia al mejoramiento del frijol comun" Universidad Nacional Agraria La Molina, Lima, Peru, Sept 27, 2002

Iriarte, GA, Blair MW "Identificacion de marcadores asociados a QTL's para caracteristicas agronimicas en una retrocruza avanzada entre una accesion silvestre y una variedad andina

mejorada de frijol comun". XXXVIII Congreso Colombiano de Ciencias Biologicas en San Juan de Pasto. October 1 – 4, 2002.

Muñoz, L.C., Blair MW " Analisis de introgresion en hibridos interespecificos en frijol". Congreso de Biotecnologia en la Universidad del Cauca – Popayan February 15, 2002.

Muñoz C, Blair MW, Debouck D "Diversidad en frijol tepary" XLVI Annual Meeting PCCMCA. San Jose, Costa Rica. April 2-6, 2002. Proceedings.

Muñoz C, Blair MW, Debouck D "Diversidad Genetica en Frijol Tepari (*Phaseolus acutifolius*)" VIII Congreso Latinoamericano de Botánica, Cartagena, Colombia. October 13-18, 2002.

Muñoz C "Aplicacion de las tecnicas de cultivo de tejidos en frijol" Congreso de Biotecnologia en la Universidad del Cauca - Popayan.

Santana GE, Blair MW, García O "cruzas anchas para el mejoramiento de fríjoles volubles andinos: cargamantos y nuñas" XLVI Annual Meeting PCCMCA. San Jose, Costa Rica. April 2-6, 2002. Proceedings.

Carlos Cesar Caula - Universidad Nacional – Palmira, Colombia (Feb 2002 onward) – training in microsatellite mapping and gene tagging.

Monica Muñoz – COLCIENCIAS - Young Investigator trainee – (May 2001 – May 2002) report: "Analisis de una genoteca y una biblioteca de genes de fríjol común (*Phaseolus vulgaris* L.) para identificar marcadores moleculares útiles en la selección de variedades rendidoras".

Steve Beebe. México. September 2002. To attend a workshop on biotechnology and human nutrition.

Gloria Esperanza Santana – CORPOICA – Rionegro, Antioquia, Colombia (Aug – Sept 2002) training in molecular marker techniques and indirect selection for BCMV resistance.

Matthew Blair – INRA-ENSA, Montpellier, France – March, 2002. cDNA library construction and macroarray hybridization

Carmenza Muñoz – cDNA library construction for common bean nodular cortex and physiological studies on nitrogen fixation potential of tepary beans under phosphorous deficiency. INRA-ENSA, Montpellier, France – May-July, 2002.

Monica Muñoz – Development and sequencing of small insert and cDNA libraries and clones, Clemson University, South Carolina, USA, July-December 2001; July 2002

53 trainees from national an international institutions received training on diverse aspects of biotechnology during 2002

3.1.11 Scientific Meetings National and International

Curso Intensivo Latinoamericano en Biotecnología. BIOLAC. Instituto IDEA. Caracas – Venezuela. March, 2002 SB-2

Genoma Conference. Chile University. March, 2002

II International Congress on Human Genetics. Universidad Nacional, Bogotá, Colombia, April, 2002

Symposium on Model Food Legumes, Geneve, Switzerland, May, 2002

Phaseomics: beans (*Phaseolus* spp.) - model food legumes. Geneve, Switzerland, May 16-18, 2002.

First International Symposium on Liquid Systems in Noruega (May-June, 2002)

First International Symposium on Tissue Culture in Liquid Media for *in vitro* Mass Propagation of Plants, Oslo, Norway, May 30 - June 1, 2002.

Workshop on Métodos Estadisticos aplicados en Biología Molecular. DANAC. Venezuela. Nov, 2002

XXXIVVV Congress of the ACCB, Pasto, Colombia, Sept. 2002.

II International Seminar on Agricultural Biotechnology, organized by PBA in Cartagena, Colombia, October 23-25, 2002

3 erd. International Rice Blast Conference. Tsukuba, Japon. 11-14 September 2002.

International Rice Conference , Beijing,China 16-20 Septiembre 2002.

Workshop on biotechnology and human nutrition. Mexico, March 2-7, 2001.

Planning workshop. Haiti, February, 2002.

PCCMCA. Dominican Republic, April 13-20, 2002.

San Diego, California, USA Jan 11-17, 2002, to attend Plant Animal Genome as invited speaker for Legume Genetics session

Ardmore, Oklahoma, USA Jan 21-23, to visit the Noble Foundation

Quito and Guaranda, Ecuador, Feb 12-18, to visit field experiments, farm sites and plan for collaborative activities with INIAP and CRSP-MSU partners.

Montpellier, France, Mar 1-31, 2002. Laboratory exchange with ENRA-INSA collaborators on an Agropolis funded project

Houston, Texas, USA, Apr 15-19, 2002, to attend technical workshop for the USAID Biofortification project

Geneva and Zurich, Switzerland, May 15-24, 2002, to attend a workshop on Phaseolus genomics and meet with co-advisor at ETH.

Minneapolis, Minnesota, USA, June 1-9, 2002, to attend International Conference on Legume Genetics and Genomics as invited speaker in World Agriculture session

Lansing and Detroit, Michigan, USA, June 11-15, 2002, to visit collaborators at Michigan State University and attend CRSP technical committee meeting

Ibague, Colombia, August 9, 2002 to participate in thesis defense at Univ. of Tolima

Prosser, Washington, USA, August 28-31, 2002, to visit collaborator at USDA-ARS

Santa Cruz and Cochabamba, Bolivia, Sept 17-22, 2002, to attend Legume conference and plan for collaborative activities with UAGRM partners.

Lima and Chiclayo, Peru, Sept 23-28, 2002, to plan for collaborative activities with PROMPEX and INIA partners.

Myriam Cristina Duque. Primer curso internacional sobre mejoramiento de frijol asistido por el uso de marcadores moleculares. Octubre 21 - Noviembre 15, 2002

Activity 3.2 Publications

3.2.1 Refereed Journals, Books

Fregene, M,; Tohme, J.; Roca, W.; Chavarriaga, P.; Escobar, R.; Ceballos, H. 2002 Biotecnología para la yuca. *In*: La yuca en el tercer milenio: Sistemas modernos de producción, procesamiento y utilización. Ceballos H y Ospina B (eds). Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, p. 377-405

Fregene, M. Okogbening, E.; Mba, C.; Angel, F.; Suarez, MC; Gutierrez, J.; Chavarriaga, P.; Roca, W. Bonierbale, M. and Tohme, J. 2002Genome mapping in cassava improvement: challengues, achievement and opportunities

Fregene, M.;and Pounti-Kaerlas, J. 2002. Cassava Biotechnology

Verdier, V.; Ojeda, S. and Mosquera, G. 2002. Methods for detecting the cassava bacterial blight pathogen: a practical approach for managing the disease.

Akano, A.; Barrera, E.; Dixon; A.G.O.; Fregene, M. 2002. Molecular genetic mapping of resistance to the African Cassava Mosaic Disease. Theor. And Appl. Genet. 105:521-525

Islam, F.M.A.; Basford K.E.; Redden, R.J.; Gonzalez, A.V.; Kroonenberg, P.M.; and Beebe, S. 2002. Genetic variability in cultivated common bean beyond the two major gene pools.

Torres-Gonzalez, A.M.; Toro, O. and Debouck, D. 2002. *Phaseolus talamancensis*, a new wild bean species (leguninosae, phaseoline) from montane forest of Eastern Costa Rica.

Bellotti A.;Roca, W.; Tohme, J.; Chavarriaga, P.; Escobar, R.; Herrera, C. 2002. Biotecnología para el manejo de plagas en la producción de semilla limpia. *In*: La yuca en el tercer milenio: Sistemas modernos de producción, procesamiento y utilización. Ceballos H y Ospina B (eds). Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, p. 255-261

Nguyen, VT,; Nguyen, Bay D.; Sarkarung, S.; Martinez, C.; Paterson, AH.; Henry, T.Nguyen. 2002. Maping genes controlling Al tolerance in rice: Comparing different genetic backgrounds. 2002. Molecular Genetics and Genomics. Published on line June 07/02.

Mahuku, G.M.; Jara, C.;Cajiao, C.;Beebe, S. Sources of resistance to angular leaf spot (*Phaeoisariopsis griseola*) in common bean core collection, wild *Phaseolus vulgaris* and secondary gene pool. Euphytica. (Accepted)

Islam, F.M.A.; Rengifo, J.; Redden, RJ.; Basford, KE.; Beebe, S. Association between seed coat polyphenolics (tannins) and disease resistance in common bean. Plant Foods for Human Nutrition. (In press)

Gonzalez, C.; Restrepo, S.; Tohme, J. and Verdier, V. 2002. Characterization of pathogenic and non pathogenic strains of Xanthomonas axonopodis pv manihotis by PCR based fingerprinting techniques, FEMS Microbiology (in press)

House, W.A.; Welch, RM.; Beebe, S.; Z. Cheng. Potential for increasing the amounts of bio-available zinc in dry beans (*Phaseolus vulgaris* L.) through plant breeding. Journal of Agricultural and Food Science (Accepted).

Gaitán-Solís E., M.C. Duque, K.J. Edwards, and J. Tohme. Microsatellite repeats in common bean (*Phaseolus vulgaris*): isolation, characterization, and cross-species amplification in *Phaseolus sp*. Accepted for publication in Crop Science, 2002.

López CE., I. Acosta, C. Jara, F. Pedraza, E. Gaitan-Solis, G. Gallego, S. Beebe and J. Tohme. Identifying resistance genes analogs associated with resistances to different pathogens in common bean. Accepted for publication in Phytopathology

Banguera-Hinestroza E., H. Cárdenas, M. Ruiz-García, M. Marmontel, E. Gaitán, R. Vázquez and F. García-Vallejo Molecular Identification of Evolutionary Significant Units in the Amazon River Dolphin *Inia* Sp. (Cetacea: Iniidae) . Accepted for publication in Journal of Heredity

Rosero-Galindo C., E. Gaitan-Solis, H. Cárdenas-Henao, J. Tohme and N. Toro-Perea. Polymorphic microsatellites in a mangrove species, *Rhizophora mangle* L. (Rhizophoraceae) Molecular Ecology Notes. (vol 2) Issue 3 Page 281 - September 2002

Martinez AK, E. Gaitan-Solis, MC Duque, R. Bernal and J. Tohme Microsatellite. loci in Bactris gasipaes (Aracaceae): their isolation and characterization. Accepted 9 may 2002. Molecular Ecology Notes (2002)

E. Gaitán-Solís, M.C.Duque, K.J. Edwards, and J. Tohme. 2002. Microsatellite repeats in common bean (*Phaseolus vulgaris*): isolation, characterization, and cross-species amplification in *Phaseolus* sp.. Accepted for publication in Crop Science.

C.E.López.; I. Acosta.; C. Jara.; F. Pedraza.; Gaitán-Solís, E..; Gallego, G.; S.Beebe and J. Tohme. 2002. Identifying resistance genes analogs associated with resistance to different pathogens in common bean. Accepted for publication in Phytopathology.

Lentini Z., Lozano I, Tabares E., Fory L., Domínguez J., Cuervo M., Calvert L. 2002. Expression and inheritance of hypersensitive resistance to rice hoja blanca virus mediated by the viral nucleocapsid protein gene in transgenic rice. Theoretical and Applied Genetics (*B1415, In Press*)

Lentini, Z. 2002. Unique Challenges and Opportunities for Environmental Assessment of GMOs in the Tropics. *In*: GMOs and the Environment. OECD. Raleigh-Durham, the United States *(In Press)*

Lentini, Z. 2002. Gene Flow Analysis for Assessing the Safety of GMOs in the Neo-Tropics: The case of beans and rice. *In:* GMOs: Real Risks or Chimeras? UNIDO, Austria *(In Press)*.

Okogbenin, E.; and Fregene, M. 2001. Genetic Analysis and QTL Mapping of Early Bulking in an F1 Segregating Population from Non-inbred Parents in Cassava (*Manihot esculenta* Crantz) (Theor and Appl Genet published online September 10)

3.2.2 Proceedings, Abstracts and Others

Correa-Victoria, F.; Tarreau,D.; Martinez,C.; Escobar,F.; Prado, G.; Aricapa, G. 2002. Studies on the rice blast pathogen, resistance genes, and implications for breeding for durable blast resistance in Colombia. Abstracts.3rd International Rice Blast Conference. 11-14 Septiembre 2002.Tsukuba, Japan p64.

C.P.Martinez, P.Moncada, J.Lopez, A.Almeida, G.Gallego, J.Borrero, M.C.Duque, F. Correa, C.Bruzzone, J.Tohme, and Z.Lentini. 2002. Gene Technology: Expanding Genetic Diversity and Adding Value to rice. Invited Key Note Speaker. RicEU Conference. Torino, Italy, June 6-8, 2002.

Martinez, CP.; Moncada, P.; Lopez, J.; Almeida, A.; Gallego, G.; Borrero, J.; Duque,MC.; Correa, F.; Bruzzone, C.; Tohme, J. 2002. Utilization of new alleles from wild rice species to improve cultivated rice in Latin America. Abstracts International Rice Conference. 16-20 September, 2002, Beijing, China, p 271.

P. Ruíz, J.J. Vasquez, E. Corredor, E.Gonzalez, L.F. Fory, A. Mora, J. Silva, M.C. Duque, and Z.Lentini. 2002. Poster No. 14, page. 287. *In:* The 7th International Symposium on the Biosafety of Genetically Modified Organisms. Beijing, China. October 10-16, 2002.

E. Gonzalez, L.F. Fory, J.J. Vasquez, P. Ruíz, E. Corredor, A. Mora, J. Silva, M.C. Duque, and Z.Lentini. 2002. Poster 16, page. 288. *In:* The 7th International Symposium on the Biosafety of Genetically Modified Organisms. Beijing, China. October 10-16, 2002.

Z. Lentini, E. Tabares, L. Fory, A. Mora, E. Gonzalez, J.J. Vasquez, P. Ruíz. 2002. Expression and Inheritance of RHBV in Transgenic Rice Breeding and Biosafety in the Neo-Tropics. Invited Key Note Speaker. *RicEU Conference*. Torino, Italy, June 6-8, 2002.

C.P. Flórez, M. Emmerling, G. Spangenberg y Z. Lentini. 2002. Aislamiento y Caracterización de Genes de la Biosíntesis de Ligninas de *Brachiaria decumbens* Stapf. Poster. First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

E. González, J. Vásquez, P. Ruíz, E. Corredor; L. Fory, A. Mora, J. Silva, M. Duque y Z. Lentini. 2002. Caracterización Molecular de Arroz Rojo Colectado en Huila y Centro Internacional de Agricultura Tropical (CIAT). Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

E. Tabares, L. Fory, L. Duque, F. Angel, G. Delgado, and Z. Lentini. 2002. *Análisis comparativo de* la eficiencia en la transformación genetica de arroz utilizando el bombardeo de particulas y *Agrobacterium tumefaciens* . Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

L. Fory , E. Tabares, I. Lozano, A. Mora., G. Delgado, T. Agrono, C. Ordoñez, M.C. Duque, L. Calvert, Z. Lentini. Arroz Transgénico con Resistencia Al Virus de la Hoja Blanca del Arroz (RHBV) en Campo. Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia

Ladino, YJ.; Mancilla, LI.; López, D.; Echeverry, M.; Chavarriaga, P.; Tohme, J.; Roca, W. 2002 "Transformación genética de yuca (*Manihot esculenta* Crantz) con un gen *cry*1Ab para resistencia a insectos" *In*: First Colombian Congress of Biotechnology. Poster and Proceedings. JAVEGRAF, Bogotá D.C., pp 70.

López, D.; Montoya, JE.; Escobar, RH.; Chavarriaga, P.; Roca, W.; Tohme, J. 2002 "Inducción de callo embriogénico friable (CEF) en clones de yuca (*Manihot esculenta* Crantz) y mejoramiento de la regeneración de plantas utilizando el sistema de inmersión temporal. *In*: First Colombian Congress of Biotechnology. Poster and Proceedings. JAVEGRAF, Bogotá D.C., pp 69.

Escobar, RH.; Hernández, CM.; Restrepo, JM.; Ospina, GI.;Tohme, J.; Roca, W. 2002 Desarrollo participativo de un sistema *in vitro* de bajo costo para yuca. Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

Escobar,RH.; Manrique, NC.; Gil, F.; Santos, LG.; Tohme, T.; Debouck, D.; Roca, W. 2002. La crioconservación: una estratégia complementaria para mantener la yuca como recurso genético. Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

Galindo, LM.; E.Gaitán-Solis and J. Tohme. 2002 Aislamiento y caracterización de las secciones Ribonucleasa LTR de retrotransposones del grupo TyI-copia en fríjol común *Phaseolus vulgaris* para estudios de marcadores moleculares. Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

L.M. Galindo, A.Mejía, W.Roca y J. Tohme. 2002 transformacion genetica de frijol (*phaseolus sp*) mediante el uso de *agrobacterium tumefaciens.* Poster, First Colombian Congress of Biotechnology, June 26-28, Bogotá, Colombia.

Escobar, RH.; Muñoz, L.; Montoya, JE.; Tohme, J.; Roca, WM. 2002 Implementación del sistema RITA® en la propagación a gran escala y en la embriogénesis somática de yuca.

Ramirez, C.; Herrera, CJ.; Chavarriaga, P.; Tohme, J. Bellotti, A. 2001 Contributions towards the knowledge on the biology, behavior and distribution of the stem borer (*Chilomima clarkei*; Lepidoptera: Pyralidae) in Tolima, Colombia. Abstracts Fifth International Sientific Meeting of the Cassava Biotechnology Network. November 4-9, 2001, St Louis, MO, USA. S8-17

Ladino, YJ.; Mancilla, LI.; Chavarriaga, P.; Tohme, J.; Roca, WM. 2001 Transformation of cassava cv. TMS60444 (Mnig11) with A. tumefaciens carrying a cry1Ab gene for insect resistance. Abstracts Fifth International Sientific Meeting of the Cassava Biotechnology Network. November 4-9, 2001, St Louis, MO, USA. S7-16

López, D.; Chavarriaga, P.; Tohme, J.; Roca, WM. 2001 Induction of friable embryogenic callus and plant regeneration in Colombian commercial cultivars. Abstracts Fifth International Sientific Meeting of the Cassava Biotechnology Network. November 4-9, 2001, St Louis, MO, USA. S7-17

Montoya, JE.; Escobar, RH.; Chavarriaga, P.; Tohme, J.; Roca, WM. 2001 Optimizing plant regeneration from friable embryogenic callus using temporary immersion systems. Abstracts Fifth International Sientific Meeting of the Cassava Biotechnology Network. November 4-9, 2001, St Louis, MO, USA. S8-19

Escobar, RH.; Hernandez, C.; Restrepo, J.; Ospina, G.; Tohme, J.; Roca, WM. 2001 Innvolvement of farmers in the development of cassava low-cost input, *in vitro* propagation methods. Abstracts Fifth International Sientific Meeting of the Cassava Biotechnology Network. November 4-9, 2001, St Louis, MO, USA. S1-08.

Escobar, RH.; Manrique, NC.;Gil, F.; Tohme, J.; Debuck, DG. 2001. Preliminary studies on the cryopreservation of meristems and seeds of wild *Manihot* species. Abstracts Fifth International Sientific Meeting of the Cassava Biotechnology Network. November 4-9, 2001, St Louis, MO, USA. S7-08.

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Beebe, S.; Teran, H.; Rao, I. 2002. Evaluación de poblaciones para combinar tolerancia a sequía con resistencia a BGMV en frijol de grano rojo y negro en CIAT, Cali, Colombia.Paper presented at the annual meeting of the PCCMCA, Santo Domingo, Dominican Republic, April, 2002.

Beebe, S.; Blair, M.; Tohme, J. 2002. Oportunidades del Uso de la Biotecnología para Modificar el Valor Nutricional de los Alimentos: el Caso del Fríjol.

Beebe, S. ; Miklas, P.; Quintero, C.; Pedraza, F.; Terán, H.; Tohme, J. 2002. Implementación de un Sistema de Alta Capacidad para Selección Asistida por marcadores en Fríjol Común. A poster presented at the Congresso Nacional de Feijao. Vicosa, Minas Gerais, Brazil. September,8-12, 2002.

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Blair MW, Pantoja W, Pedraza F, Cregan P, Fatokun C (2002) Legume microsatellites in common bean. Annual Report of the Bean Improvement Cooperative. 45: 242-44

Broughton WJ, Hernandez G, Blair MW, Beebe SE, Gepts P, Vanderleyden J (2002) Beans (*Phaseolus* spp.) – Model Food Legumes. Plant and Soil (accepted)

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Jones, P. G. ; Beebe, S. 2001. Predicting the impact of Climate Change on the distribution of plant genetic resources in wild bean *Phaseolus vulgaris L.* in Central America. Paper presented at the Third International Conference on Geospatial Information in Agriculture and Forestry. 5-7 November, Denver, Colorado

Thomson,MJ.; Septiningsih, EM.; Trijatmiko, KR.; Alcala, J.;Martinez, C.; McClung,A.; Moelpojawiro,S.; McCouch, SR..2002. Targeting functional nucleotide polymorphisms (FNPs) underlying QTL introgressed from O.*rufipogon*.

Mahuku, G.; Jara, C.; Teran, H.; Beebe, S. 2002. Resistencia a mancha angular en G10474, un frijol voluble de Guatemala. Paper presented at the annual meeting of the PCCMCA, Santo Domingo, Dominican Republic, April, 2002.

Mahuku, G., Jara, C.; Teran, H.; Beebe, S. 2002. Inheritance and Characterization of Angular Leaf Spot Resistance in G10474, a climber from the highlands of Guatemala.

3.2.3 Thesis

"Identificacion de QTLs para rendimiento y sus componentes en una población de DHs derivada del cruce Caiapo/*O.glaberrima* " Carolina Castaño Rodriguez. Universidad de los Andes.

"Evaluación de nuevas metodologías en la regeneración de plantas de yuca (*Manihot esculenta*, Crantz) a partir de callo embriogénico friable utilizando RITA®", Juan Esteban Montoya, Universidad Nacional de Colombia-Medellín.

"Estudio exploratorio para desarrollar una metodología de crioconservación de callo embriogénico friable (CEF) de yuca (*Manihot esculenta* Crantz) variedades MCol 2215 y MNig 11", Luis Guillermo Santos, Universidad Nacional de Colombia-Palmira.

"Evaluación preliminar de la expresión del gen *bar* en plantas transgénicas de yuca mantenidas en reproducción vegetativa por cerca de diez años", Morgan Echeverry, Universidad del Valle, Cali, Colombia.

"Aportes al estudio de la biología, comportamiento y distribución del barrenador del tallo de la yuca *Chilomima clarkei* (Lepidoptera : Pyralidae), en el departamento del Tolima", Carolina Ramírez, Universidad del Tolima, Ibagué, Colombia

"Saturación del mapa genético molecular de yuca (*M. esculenta*, Crantz) usando marcadores basados en PCR", Tania García, Universidad del Valle, Cali, Colombia.

"Caracterización Morfológica, fenológica, y genética de Biotipos de Arroz Rojo (Oryza sativa f. expontánea) colectados en el Municipio de Saldaña (Tolima.) Juan José Vásquez, BSc. Universidad de Los Andes, Bogotá

Activity 3.3 New Projects and Donor

3.3.1 Projects approved or on going

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 . The Rockefeller Foundation. Approved January 2001-2004 (Partnertship: CIAT and Univ. of Costa Rica).

"Candidate Genes for Tolerance of Symbiotic Nitrogen Fixation (SNF) to Phosphorus Deficiency in Common Bean (*Phaseolus vulgaris L.*)". Approved by *Plate-forme de recherches avancées Agropolis – 2ème appel d'offre (2001 – 2003).* (partnership INRA, CIAT and INIFAP, Mexico)

Gene Flow Analysis for Assessing the Safety of Bio-Engineered Crops in the Tropics. BMZ. 2000-2003. (Partnership: CIAT, Univ. of Costa Rica, Hannover University, and Federal Biology Institute-Germany).

Genoplante – Evaluation & Multiplication of 5000 TDNA – mutant rice lines. 2002 – 2003

Biotechnology assisted development, deployment and nutritional efficacy testing of high mineral beans to combat iron deficiency anemia in East Africa. Approved by USAID.

Rice functional genomics consortium. Yale University (2001- 2003)

"Candidate Genes for Tolerance of Symbiotic Nitrogen Fixation (SNF) to Phosphorus Deficiency in Common Bean (*Phaseolus vulgaris L.*)". Approved by *Plate-forme de recherches avancées Agropolis – 2ème appel d'offre.* (partnership INRA, CIAT and INIFAP, Mexico) (2001 – 2003)

"Breeding staple-food crops with high micronutrient density for better human nutrition (Sub-project: Improvement of Common Bean) project funded by DANIDA and coordinated at IFPRI (1998-2002)

"Breeding staple crops for improved micronutrient value", a proposal approved by USAID for biofortification research (2002-2004)

"Frijol voluble para la zona Andina" Approved by Fontagro/BID in agreement with IICA (2002-2005).

"Mejoramiento de frejol para el programa nacional de leguminosas de Bolivia" bilateral project approved by COSUDE – La Paz (2002-2004)

"Mejoramiento de frijol para Profriza II – Peru" bilateral project approved by COSUDE - Lima (2002-2004)

"Estudio de la factibilidad de la selección asistida por marcadores para obtener cultivares de frijol con resistencia simultanea al virus del mosaico común y la antracnosis" Approved by Ministerio Agricultura – Colombia (submitted by CORPOICA - Rio Negro, with activities at CIAT-2002.

3.3.2 Projects submitted, in preparation and concept notes

Bean Genomics for Improved Drought Tolerance in Central America. Submitted to BMZ.

Plant Research for Food of the Future: Biofortification in bean. Submitted to DANIDA.

Research, Monitoring and Implementation of Cartagena Protocol on Biosafety of GMOs in Colombia . Submitted to GEF/ World Bank (US$ 1 million), August 2002. ICA, INVIMA, Institute von Humboldt, Ministries of Agriculture, Health, Environment, Planning and Foreign Commerce, Colciencias, and CIAT.

Genes, Environment and Biological Response: Complex Systems that Defy Understanding. Submitted to :The James S. McDonnell Foundation

Nutrirional genomics, nutritional diversity: importance of phaseolus species for nutritional quality in East Africa. Universitet GENT

Plant Research for Food of the Future. Arhus Universitet.

Bean genomics for improved drought tolerance in Central America. Submitted to: Bundesministerium fur Wirtschaftliche – Zusammernbarbeit und Entwicklung (BMZ)

"Bean genomics for improved drought tolerance in Africa and Latin America", concept note prepared for BMZ-Germany.

"Breeding staple crops for improved micronutrient value", a proposal submitted to a consortium convoked by the Gates Foundation, to improve the nutritional status of bean

"Bioavailability and clinical response to the consumption of high mineral beans and quality protein maize" proposal presented to the Micronutrient Initiative for funding of nutrition research in Colombia (submitted by Universidad del Valle with CIAT).

"Comparative genomics and genetics in legumes" a collaborative research project between CIAT and University of Aarhus, concept note prepared for DANIDA

"Expressed sequence tags for common bean" a pre-proposal submitted to Brazilian (Sao Paulo) funding agency FAPESP by ESALQ, Univ. de Sao Paulo with participation by CIAT

"Nutritional efficacy testing of a biofortified diet of high mineral beans and vitamin A rich sweet potatoes to combat iron deficiency anemia in East Africa", concept note prepared for Micronutrient Initiative.

"Nitrogen fixation capacity of tepary bean". South Initiative-Belgium. (submitted by Univ. of Lueven with CIAT collaboration)

"Phaseomics" wRUIG-GIAN. (submitted by Univ. of Geneva with CIAT collaboration)

"Manejo de germoplasma local y aumento de la agrobiodiversidad de frijol y maiz con variedades biofortificadas para mejorar la nutricion en comunidades rurales y urbanas de Nariño" Cuenta de las Americas. (submitted by FIDAR with CIAT).

"Mejoramiento de la nutrición humana en comunidades pobres de America Latina utilizando maiz (QPM) y frijol común biofortificado con micronutrientes" proposal presented to Fontagro (IDB), to improve nutrition in rural and urban communitities in Colombia and Guatemala with NGO partners (submitted by FIDAR with CIAT).

Desarrollo de germoplasma mejorado de arroz con resistencia al añublo de la vaina causado por Rhizoctonia.

Alternativas para introducir mayor valor agregado a la producción de arroz en Colombia – MADR – Colombia .

3.3.3 Projects funded and their Donors (Oct, 2001 – Sept. 2002)

Canada

- **International Development Research Centre. (IDRC)**

  Strategies for integrating small-scale end-users in cassava biotechnology research (Latin America)

- **Natural Resources International . (NRI)**

  Identifying target points for the control of post-harvest physiological deterioration in cassava

Colombia

- **Fundación para la Investigación y el Desarrollo Agrícola. (FIDAR)**

  Rice Functional Genomics Consortium

- **Ministry of Agriculture and Rural Development. (MADR)**

  Regeneration capacity and genetic transformation potential of commercial cassava varieties in Colombia

  Propagation and certification of FSD-free cassava

  Biotech Fruits

- **Corporación BIOTEC**

  Molecular and agromorphological characterization of native genetic variability of soursop and related Annonaceae species

- **Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología. (COLCIENCIAS)**

  Characterization of cassava resistance to vascular bacteriosis and its use in breeding

- **Centro de Estudios Ganaderos y Agrícolas. (CEGA)**

  Control of the cassava stem borer on Colombia's North Coast

  Production and management of high quality seed for the socioeconomic development of small and intermediate cassava producers on Colombia's Atlantic Coast

  Ensuring Stable and Durable Resistance of Rice to Pathogens and Pests: Rice Hoja Blanca Virus, Rhizoctonia Solani and Sogata

- **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*

Cote D'ivoire

- **West Africa Rice Development Association. (WARDA)**

Interspecific hybridization proyect

France

- **Advanced Research Platform. (AGROPOLIS)**
- **Institut de recherche pour le developpement. (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

Rome

- **Food and Agriculture Organization. (FAO)**

Meeting to measure baselines of genetic diversity

The Netherlands

- **Ministry of Foreign Affairs and Trade. (MFA)**
- **Directorate General International Cooperation. (DGIS)**

Cassava Biotechnology Network III – CBN