ANNUAL REPORT 1999

PROJECT SB-02

ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

CIAT
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October, 1999

PROJECT SB-2: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

PROJECT OVERVIEW

The Challenge: The project's approach to the challenges involved in increasing agricultural productivity, with probably less water and soil, and agricultural competitiveness in tropical developing countries, is based in the application of modern biotechnology to enhance our ability to develop improved strategies for the characterization and sustainable utilization of genetic diversity in crop improvement and conservation. Common bean, cassava and rice are vital to food security and human welfare and along with tropical forrages, are grown in developing countries. Our research on the mandated crops expands to other crops of current or potential economic importance in Latin America.

Objective: To apply modern biotechnology to identify and utilize genetic diversity for broadening the genetic base and increasing the productivity of mandated and non-mandated crops.

Outputs: Improved characterization of genetic diversity of wild and cultivated species and associated organisms. Genes and gene combinations accessed and utilized. Collaboration with public and private sector partners enhanced.

Initiation: 1997
Duration: Five years

Milestones:

1998. Molecular linkage maps and DNA-based markers available for assessing diversity and tagging useful traits of *Phaseolus, Manihot*, and associated organisms. Transgenic resistant plants generated with rice; populations generated with useful traits from wild *Oryza* spp. Collaborative activities with CIAT partners implemented.

1999.DNA-based markers available for other species in CIAT mandated agroecosystems. Modern methods developed for the rapid propagation of cassava and other species in CIAT mandated agroecosystems. Trangenic resistance to insect pests available in cassava. Cryopreservation technique for cassava.

2000.QTL, from wild germplasm identified and mapped involved in yield and quality of rice and beans. Collaborative activities with CIAT partners, including privated sector implemented.

2001. Gene transfer utilized for broadening the genetic base and germplasm enhancement of rice, cassava, beans, and *Brachiaria*. Collaborative activities with CIAT partners implemented.

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

Collaborators: IARCs (IPGRI: systemwide program, CIP and IITA: root-tuber crops research; ISNAR: training, policies. NARS (CORPOICA, ICA, EMBRAPA, INIAs,). AROS of DCs and LDCs. Biodiversity institutions (A. von Humboldt, INBIO, SINCHI, Smithsonian). Corporations and private organizations (Corp. BIOTEC, BRL, Novartis).

CGIAR system linkages: Saving Biodiversity (30%); Enhancement and Breeding (60%); Training (10%).

CIAT project linkages: Inputs to SB-2: Germplasm accessions from gene bank project. Segregant populations from crop productivity projects. Characterized insect and pathogen strains and populations from crop protection projects. GIS services from land use project. Outputs from SB-2: Genetic and molecular information on gene pools, and populations for gene bank, and crop productivity projects. Information and material on identified genes and gene combinations for productivity and crop protection projects. Methods and techniques of propagation and conservation for gene bank and productivity projects. Interspecific hybrids and transgenic stocks for crop productivity and crop protection (IPM) projects.

WORK BREAKDOWN STRUCTURE

PROJECT SB-02: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

PROJECT GOAL

To contribute to increased productivity and to the conservation of agrobiodiversity in tropical countries

PROJECT PURPOSE

To apply modern biotechnology to identify and utilize genetic diversity for broadening the genetic base and increasing the productivity of mandated and non-mandated crops

OUTPUT 1. Genomes of wild and cultivated species and associated organisms characterized.

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

OUTPUT 2. Genes and gene combinations made available for broadening crop genetic bases.

- Utilization of novel genes and gene combinations by means of cellular and molecular genes 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.

PROJECT LOG-FRAME – WORK PLAN 1999

CIAT

AREA: Genetic Resources Research
PROJECT: SB-02: Assessing and utilizing agrobiodiversity through biotechnology
MANAGER: W.Roca

NARRATIVE SUMMARY	MEASURABLE INDICATORS (1999)	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 begining to use information and tools of biotechnology in crop research. • Genetic stocks available to key CIAT partners	CIAT and NARS publications, statistics on agriculture and biodiversity	
PURPOSE Characterized agrobiodiversity, modified crop genetic stocks, and modern molecular and cellular methods/ tools, are used by CIAT and NARS scientists to better understand, utilize and conserve crop genetic diversity	• Information on diversity of wild and cultivated spp • Mapped economic genes, gene complexes • Improved genetic stocks, lines, populations	Publications, Reports, project proposals	Pro-active participation of CIAT and NARS agricultural scientists and biologists.
OUTPUT 1 Genomes characterized: Genomes of wild and cultivated species of mandated and non-mandated crops and associated organisms, characterized	• Molecular information on diversity of mandate/ non mandated crops species, pathogenic/beneficial organisms • Bio-informatic techniques	Publications, Reports, project proposals, germplasm	Availability of up to date genomics equipment, operational funding.
OUTPUT 2 Genomes modified: genes and gene combinations made available for broadening the genetic base of mandated and non-mandated crops	• Transgenic lines of rice and advances with cassava, *Brachiaria* and other crops • Cloned genes and gene constructs • Information on new transformation techniques	Publications, Reports, project proposals, germplasm	Access to genes and gene promoters.
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/workshops, project proposals	Government-industry support to national biotech initiatives
ACTIVITIES **OUTPUT 1: Genomes characterized**	**1999 MILESTONES**		
1.1 Molecular characterization of genetic diversity			
1.1.1 Phylogeny studies in *Phaseolus*	• Parsimony analysis of sequences	Draft article	Material supplied by GRU
1.1.2 Isolation and characterization of SSR in *Brachiaria*	• Sequences of 100 SSRs • Initiate mapping of SSRs	Report	Availability of B. ruzizi x marandu populations
1.1.3 Characterization of cassava genepool structure	• Populations from Guyana • Screened withy SSRs	Report	
1.1.4 Identification and isolation of bean SSRs	• Isolation of clones • Mapping SSRs on Dor 364 x G19833	Maps, Reports	Availability of populations.

NARRATIVE SUMMARY	MEASURABLE INDICATORS (1999)	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
	populations		
1.1.5 Identification of resistance gene analogs (RGAs) in bean	• Sequences of RGAs • Mapping of RGA onto Dor 364 x G19833 RILs	Draft article	
1.1.6 Molecular assessment of Brazil and Nigeria cassava collections using SSRs	• Construction of dendograms	Report	
1.1.7 Characterization of genetic structure of cassava collections in primary and secondary centers of div.			
1.1.8 Molecular evaluation of *P. coccineus* x *P. polyanthus*, core collection			
1.1.9 RAPD analysis of common bean core collection			
1.1.10 Release and distribution of Floramap	• Map released	Report	
1.1.11 Construction of cassava bacterial artificial chromosome (BAC) library			
1.1.12 Genetic variation of CBB pathogen			
1.1.13 Characterization of molecular variability of Colombian *Musa* collection			
1.1.14 Characterization of molecular variability of Colombian Amazonian Collection			
1.1.15 Molecular assesment of selected Colombian biodiversity			
1.2 Identification and mapping of useful genes and gene combinations			
1.2.1 Identification of resistance genes to rice blast	• Linkage analysis on 100F2 from rice blast lineages	Report	Blast screening available
1.2.2 Construction of Brachiaria molecular map	• SSrs mapped of *B. ruziziensis* x *B.decumbens* • Marker available	Report	
1.2.3 tagging whiteflies resistance genes in cassava	• Marker identified • Primers designed	Report	
1.2.4 Identification of low P tolerance QTL in beans			
1.2.5 Yield and antracnose resistance QTL mapping in bean			
1.2.6 Develop *P. coccineus* populations for tagging GBMV and bean maggot resist. Genes.			
1.2.7 Seek molecular markers for high iron content in bean seeds			
1.2.8 Make SCARs for root traits, yield and disease resistance in bean			
1.2.9 Continue saturation of cassava molecular map with PCR based markers (SSRs, ESTs and candidate genes)			

NARRATIVE SUMMARY	MEASURABLE INDICATORS (1999)	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
1.2.10 Mapping of CMD resistance genes			
1.2.11 QTL mapping of earliness, starch content, PDF in cassava			
1.2.12 Mapping CBB resistance genes			
1.2.13 Development of two hybrid system of CBB			
1.2.14 Set up MAS pilot project			
1.2.15 Mapping of apomixis gene in *Brachiaria*			
1.2.16 Analysis of analog resistance gene sin rice and beans			
OUTPUT 2: Genomes modified **2.1 Transfer of novel genes and gene combinations by cellular/molecular techniques**			
2.1.1 Transfer of QTLs from wild rice for improving yield of cultivated rice	• *O. barthii* x Lemont popul screened • Prepare paper of BG90-2 x *O. rufipogon*	Report	Support by breeders
2.1.2 Develop populations of bean x wild bean crossess from Mexico, Argentina, North Peru, Colombia	• Yield data • Populations in field	Report	Support by breeders
2.1.3 Transgenic resistance to RHBV in rice	• F4 and F5 CICA 8 N P-transgenic progeny	Report	--
2.1.4 Testing transgenic RHBV resistance rice	• Data from greenhouse tests	Report	--
2.1.5 *Agrobacterium*-mediated transgenic resistance in rice			
2.1.6 Transgenic resistance to cassava stem borer	• Two Cry A1 (b) gene cassettes prepared and transformation of cv. Venezolana initiated	Report to donor, Ann. Report, publication	Funding from DGIS-Colombia is available
2.1.7 Improving cassava somatic embryogenesis	• Embryogenic cultures of var. Venezolana prepared for transformation	Report to donor, Ann Report	Funding from DGIS-Colombia is available
2.1.8 Bean transformation	• Advances *in Phaseolus* bean *Agrobacterium* mediated transformation	Reports	Continuation (part time) of PhD. scientist
2.1.9 *Brachiaria* genetic transformation	• Methodology patenteed	Reports, Patent	Student availability
2.1.10 Tomato genetic transformation	• Advances in technology		Ph.D. student availability
2.1.11 Cassava micropropagation	• Bioreactor (RITA) technology tested implemented for cv. Venezolana	Reports to donor Ann Report	Funding from DGIS – Colombia available
2.1.12 Development of low input (artesanal) cassava tissue culture scheme	• Basic equipment/facilities and culture conditions designed • Pilot artesanal lab. established in CIAT	Report to donor, Ann Report	Cauca farmers organizations participate
2.1.13 Implementation of cassava relief program in Ecuador	• Ecuador germplasm micropropagated indexed and sent to Ecuador	Report to donor, Ann Report	Ecuador a in organizations, participate
2.1.14 Guanabana micropropagation	• Al least 500 micrografted plants generated	Report, Partner's reports	Partner's (BIOTEC) support continues
2.1.15 Cassava cryopreservation	• Encapsulation/vitrification technique	Publication, Reports	Continued core funding,

NARRATIVE SUMMARY	MEASURABLE INDICATORS (1999)	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
	tested with sub-core collection		system-wide support
2.1.16 Map-based cloning of CBB resistance genes	• Advances in cloning	Report	IRS support
2.1.17 Analysis of analog CBB resistance genes	• Advances in cloning	Report	IRS support
	•		
2.2 Plant – stress interactions	•		
2.2.1. Variability and stability of cassava carotenoids	• Core collection screened • Data on processing effects	Report to donor, Ann Report	Funding from system-wide Program continues
OUTPUT 3: Collaboration enhanced **3.1 Organization of Networks, Workshops, training *courses in biotechnology***			
3.1.1 Workshops and training courses: analysis of genetic diversity, biosafety, molecular breeding, bioinformatics	• At least two training courses organized • At least 10 trainees in service scheme	Reports	Funding available
3.1.2 Attend conferences, workshop			
3.2 Data Bases and Genetic Stocks			
3.2.1 Assembling genetic stocks: *Agrobacterium* strains and gene constructs, plasmid, etc	• Data base for gene constructs, plasmid, and vectors established	Data base, report	Continued core support
3.3 Publications, project concept notes, project proposals, biosafety, IPR			
3.3.1 Preparation of paper manuscripts	• At least 6 papers prepared	Manuscripts, reports	Scientific dedicate time
3.3.2 Preparation of project concept notes/proposals	• At least 6 concept notes/proposals prepared	Concept notes	Scientific dedicate time
3.3.3 Contribution to biosafety and IPR	• *Organize biosafety workshop; attend* IPR course	Regulations updated, implemented	Donor support
3.3.4 Preparation of Ann. Report and EPMR Reports	• Comply deadliness	Reports	Time dedication by team members
3.3.5 Visit partners and donors			

PROJECT SB-2 HIGHLIGHTS 1999

Highlights for the period October 1998 - September 1999 are summarized within each of the project's three outputs:

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

1.1. Combining molecular diversity with GIS and geostatistics, we have initiated the mapping of cassava bacterial blight disease occurrence and pathogen diversity distribution.

1.2. Molecular groups of Middle American bean germplasm were found to correlate with mineral content (iron) and disease resistance (angular leaf spot).

1.3. Collaborative studies with biodiversity institutions were conducted, on the use of molecular markers for genetic resources, characterization, conservation, and gene bank development.

1.4. A marker associated with resistance to the African Cassava Mosaic Disease (ACMD) has been identified, opening the way to introgress ACMD resistance into Latin American germplasm. Similarly, QTLs associated to cassava bacterial blight resistance were identified.

1.5. Resistance gene analogs were linked to resistance to bean golden mosaic virus, and rice blast disease. Likewise, QTLs with large effects on common bean yield were identified.

1.6. A marker assisted selection (MAS) scheme was successfully implemented using a SCAR marker for bean golden mosaic virus resistance. As a consequence, bean breeding effort was reduced in about 60%.

1.7. A GIS -based Flora Map has been prepared for distribution. The system will be used for plant collection, taxonomic and genetic variation studies, and for mapping crop pests and their potential predators.

1.8. Large number of microsatellite markers have been generated for cassava; many have already been mapped in the cassava molecular map.

2. Genes and gene combinations made available for broadening the base of mandated and non-mandated crops.

2.1 Production of transgenic cassava is underway as a component of a management strategy for the control of the stem borer in collaboration with regional organizations. Associated to this work, a bioreactor-based massive multiplication of cassava has been initiated; and on the other hand, a low-cost, artesanal, clonal propagation scheme is being implemented.

2.2 Opening the way to large scale cryopreservation, an encapsulation-dehydration technique was successfully tested with a sub-core collection of cassava germplasm.

2.3 Identification and mapping of QTLs from wild species associated with yield increase of rice continued; a similar approach has shown great promise for common bean.

2.4 Testing, and backcross conversion, of transgenic rice lines resistant to rice hoja blanca virus (RHBV) continued. Simultaneously, a complementary transgenic approach have been tested against RHBV using anti-pathogenic protein gene. On the other hand, *Agrobacterium*-mediated transformation has shown great promise for indica and japonica rices.

2.5 Collaborative work with Colombian organizations continued on micropropagation, and has been initiated in genetic transformation of selected no-tropical fruits.

2.6 A cassava bacterial blight pathogenicity protein gene was cloned, and its interaction with plant's resistance genes studied.

2.7 Work towards *Agrobacterium*-mediated transformation of *Brachiaria* and common bean has prepared progresssed.

3. Collaboration with public and private sector partners enhanced

3.1. Contributing to public awareness in biotechnology and biosafety, SB-2 project members delivered a two-day workshop addressed to the Colombian press.

3.2. An international workshop was organized at CIAT to discuss issues involved in the release of transgenic RHBV resistance rice lines. Latin American countries and US representatives attended the workshop. On the other hand two training courses on biodiversity assessment and molecular plant breeding, were conducted in CIAT.

3.3. A patent on *Brachiaria* plant regeneration and transformation was filed in collaboration with EMBRAPA, Brazil.

3.4. In the period Oct 1998-Sept 1999, SB-2 Project members: (i)published 15 scientifc articles in refereed journals and 17 abstracts and posters; (ii) linkages with a range of private sector organizations were established; (iii) 15 Concept Notes and Proposals have been prepared and/or followed, 10 of these have been funded; (iv) 10 funding organizations contributed to Project's activities in the same period.

CONTENT

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OUTPUT 1 GENOMES OF WILD AND CULTIVATED SPECIES OF MANDATED AND NON-MANDATED CROPS, AND ASSOCIATED ORGANISMS, CHARACTERIZED

ACTIVITY 1.1 Molecular characterization of genetic diversity

MAIN ACHIEVEMENTS

- The distribution of cassava bacterial blight pathogen (*Xam*), and the impact on disease control was assessed. Ongoing work, combining molecular diversity data with GIS and geostatistics will allow to draw maps displaying chances of disease occurrence and pathogen diversity.
- A third genepool in *Phaseolus* diversity, and the definition of Mesoamerican bean races and key useful traits were caracterized. Quantification of spontaneous introgression of Mesoamerican genes in Andean gene pool was carried out, and the respective loci were mapped.
- A molecular group of Middle American bean germplasm highly correlated with mineral content and disease resistance, e.g. a group stood out as an important source of iron content and resistance to bean angular leaf spot.
- Based on correlations between tannin in bean seed coat and diseases, it is implied that seed coat tannin can be reduced without serious effects on plant resistance to diseases.
- Through collaborative work with the Sinchi Institute, the genetic characterization of a collection of *Capsicum* from the Colombian Amazon region was carried using molecular markers. On the other hand, a range of collaborative activities with the A.von Humboldt Institute included the use of molecular techniques for biodiversity and conservation studies of endangered Colombian palms, structure of plant and soil bacteria, genetic variation of pest and river manual diversity, and the initiation of a tissue bank in liquid nitrogen.

1.1.1 Assessment of genetic diversity in *Xam* populations from 1996 to 1999

The host population in a plant pathogen and the environmental factors influence the genetic diversity and population structure of pathogens (Adhikari *et al.*, 1994). In

cassava, the development of resistant cultivars has been the primary means of control for bacterial blight. There is concern that the widespread use of resistant genotypes might accelerate the selection of new variants or even races of the pathogen and result in destabilization of crop production (Leach *et al.*, 1992). However, little is known regarding the population dynamics of *Xam*. This study aimed to assess the genetic change in *Xam* populations over time from 1996 to 1999 using RFLP assays. Temporal changes and population dynamics were addressed by comparing samples from four years in different edaphoclimatic zones (ECZs) in Colombia.

Materials and Methods

***Xam* isolates.** All strains were isolated from stem or leaf samples from various cassava plants collected between 1995 and 1999 in different sites. Six sites located throughout four ECZs were visited: Villavicencio and Carimagua (ECZ2), Pivijay (ECZ1), Mondomo and Cajibio (ECZ5), and Santander de Quilichao (ECZ2-5).

RFLP using pthB as probe. Genomic DNA, DNA concentration, digestion, electrophoresis and southern blots were performed as previously described (Restrepo and Verdier, 1997). Membranes were hybridized with a plasmid probe, designated as *pth*B, a 5.6 kb *Eco*RI fragment harboring a pathogenicity gene related to the *avr/pth* gene family . Conditions of hybridization and washes were as previously described (Restrepo and Verdier, 1997).

Statistical analysis. Banding patterns of hybridization on autoradiograms obtained with the RFLP/*pth*B were used to characterize the genetic diversity of *Xam* populations. Each distinct RFLP banding pattern, obtained after hybridization with probe *pth*B, was regarded as a haplotype. The haplotype diversity was estimated using the Nei and Tajima index, (Nei and Tajima, 1981).

$H = [n/(n\text{-}1)](1\text{-}S X_i^2)$, where X_i is the proportion of the ith distinct *pth*B haplotype, and n is the number of strains. The extent of genetic differentiation among groups was estimated by the coefficient of genetic differentiation $G_{ST} = (H_G - H_Y)/H_G$ where H_G is the genetic diversity of each site and H_Y is the average genetic diversity of strains groupings by year of collection. Significance testing for differences in haplotype frequencies over the years in each site or ECZ was performed by a chi-square analysis.

Results

(Figure 1) depicts the change of the genetic composition of the *Xam* population in Mondomo over time. Haplotypes C8 and C9 were the most frequent haplotypes in 1995 and 1996. Recently, the most frequent haplotypes are C36 and C37. These turn over may be influenced by the change in the host population and the introduction of new genotypes.

To examine the effect of environmental factors (host population, climatic conditions among others) on the diversity of the *Xam* population, genetic diversities of strains collected in 4 years were calculated. Genetic diversities calculated for each site were

highest for Carimagua and Villavicencio (ECZ2) and lowest for Cajibio (ECZ5) and Santander de Quilichao (ECZ25). However, genetic diversities were highly variable in each site over time. The coefficient of genetic differentiation based on the groupings of time periods varied from 0.8 (Cajibio) to 0.2 (Villavicencio) (Table 1).

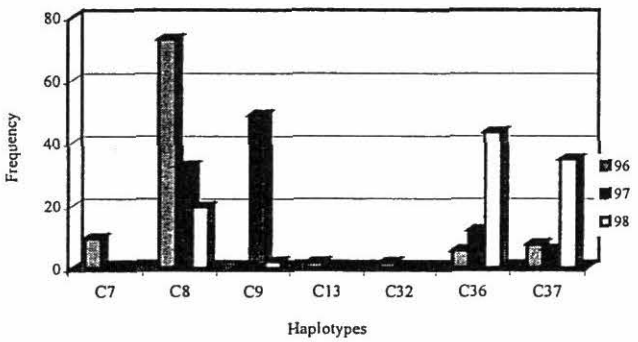

Fig.1. Changes in frequency of RFLP/*pth*B haplotypes during a 3-year period in Mondomo (ECZ5).

Chi-square analysis was used to compare haplotype frequencies over the years of collection. The populations of *Xam* were unstable with regard to haplotype diversities. All of the chi-square values (except for Cajibio) were significant indicating that composition of the population at each site was different over time (Table 1).

Table 1. Haplotypes, Genetic diversities (H), coefficient of genetic differentiation (G) for *Xam* populations collected at six sites in 4 ECZs in Colombia.

ECZ	Site	Year	Haplotypes	X^2	H	G_{SITE}
1	Pivijay	1995	C5 C6 C16 C18		0.41	
		1996	C6 C16 C18		0.33	
		1997	C6 C15 C18 C33		0.41	0.4
		1998	C6 C15 C16 C18 C33 C40 C42 C43C44		0.45	
		1999	C6 C18 C33 C42	118.8 (0.001)[a]	0.33	
2	Carimagua	1995	C1 C2 C4 C12 C13 C19 C21 C22		0.70	
		1996	C2 C4 C12 C13 C14 C21 C26 C32		0.83	0.3
		1997	C4 C12 C13 C17 C27 C32		0.62	
		1999	C12	113.5 (0.001)	0	
	Villavicencio	1995	C2 C3 C4 C12 C13 C19 C23 C24 C25 C26		0.70	
		1996	C2 C4 C12 C13 C23		0.69	0.2
		1997	C4 C12 C17 C27 C28 C29 C34		0.53	
		1998	C4 C12 C17 C19 C27 C29 C45 C46 C47	201.1 (0.001)	0.54	
5	Mondomo	1996	C7 C8 C13 C32 C36 C37		0.37	
		1997	C8 C9 C36 C37		0.48	0.3
		1998	C8 C9 C36 C37 C48	120.1 (0.001)	0.51	
	Cajibio	1995	C7 C8 C9		0.21	0.8
		1996	C7 C8 C13	2.7 (0.446)	0.09	
2-5	Stder Quilichao	1996	C13 C28 C29 C30		0.21	
		1997	C8 C31 C35		0.17	0.7
		1998	C30	66 (0.001)	0	

[a] The chi-square value was significant at the 5% level.

Discussion

Based on information on the temporal distribution of the pathogen's genetic diversity, we can develop a picture of the CBB pathogen's population dynamics in Colombia. The coefficient of genetic differentiation G_{ST} describes the average amount of genetic variation attributed to a particular subdividing factor (e.g. time) relative to the total genetic variation. Most of the sites presented high values of G_{ST}. suggesting a high degree of differentiation between time-periods. In Villavicencio and Carimagua G_{ST} values were low. Genetic diversities of strains grouped according to the time periods were similar and high indicating that the pathogen population has had a consistently high level of diversity over the past 5 years in ECZ2.

Contingency chi-square tests showed that differences in haplotype frequencies over the 4 years were statistically significant, indicating that *Xam* population at the site level were unstable. Genetic drift may have a significant impact on population structure of *Xam* over time. This is probably due to the management and deployment of cassava resistant genotypes in some sites. However, environmental factors may have also a major influence on disease development. Gross environmental differences may also play an important role in *Xam* populations fluctuations and genetic drift of the population.

The fact that plants are exposed to pathogen populations which are genetically diverse and in constant evolution is an important consideration in disease control. Knowledge of pathogen population structure and dynamics is useful in determining the way in which genotypes are deployed in time and space (Leung *et al.*,., 1993). In consequence a continuous monitoring of the pathogen incidence and diversity is necessary. However, monitoring the pathogen population requires extensive and repeated surveys. The development and recent application of the geographical information systems, the geostatistics and the Kriging technique to plant pathology offer an alternative approach. Combining these techniques, a map of the spatial pattern of the disease can be done with the possibility to estimate the variables (incidence, diversity) at unsampled locations (Nelson *et al.*,., 1999; Lecoustre *et al.*,., 1989).

References

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Collaborators

Restrepo, S[1]., Velez, C[1]., Duque, M.C[2], and V. Verdier[1].
[1]IRD-CIAT ; 2. SB-02 Project

1.1.2. Application of geographic Information Systems and Geostatistics in Cassava Bacterial Blight management

Introduction

The characterization of the spatial patterns of disease level can provide important clues to the epidemiology of disease. Patterns must be considered in designing sampling methods and appropriate control measures (Lecoustre *et al.*, 1989). In consequence, efficient methods of analysis and interpretation of spatial patterns are needed to provide the greatest possible information in relation to the time and effort involved. Geostatistics and GIS are very useful tools that can be used to improve our understanding of the temporal and spatial aspects of disease processes (Nelson *et al.*, 1999). In addition, the development of molecular markers for the assessment of the pathogen diversity has created the opportunity to obtain enough data to characterize the pathogen spatially and incorporate this information into management programs. All these tools were used to analyze the spatial patterns of genotypes of *Phytophthora infestans* (Nelson *et al.*, 1999). The *Xam* diversity and population structure in Colombia were previously characterized (Restrepo and Verdier, 1997; Restrepo *et al.*, 1999). The objective of this study are to examine the potential for the use of geographic information systems in evaluating spatial patterns of incidence and diversity of the CBB pathogen, *Xanthomonas axonopodis* pv. *manihotis* (*Xam*).

Materials and Methods

During 1995-1998, cassava-grown fields were visited in 4 edaphoclimatic zones (ECZs) in Colombia. Each field was evaluated for disease incidence and the pathogen diversity for each field was estimated based on the haplotypes collected and their frequency. The number of genotypes cultivated was recorded for each field. Environmental variables such as altitude, precipitation, maximum and minimum temperature were measured each month from january-1995 to december 1998

A correlation analysis was performed to identify the risk variables. Risk variables are those that influence or are highly correlated to the disease incidence or the pathogen diversity.

Results and discussion

The principal result of the analysis was the identification of the variables that influence the incidence of the disease and/or diversity of the pathogen. The higher correlation was found between the pathogen diversity and the number of cultivars grown in the field ($r = 0.70$), confirming the influence of the host on the *Xam* population structure. There was also a high correlation between diversity and altitude suggesting that in fields located at high altitude the diversity is lower ($r = -0.63$). Diversity is also influenced by minimum temperature and temperature at night.

The information suggests the existence of "conducive" environments. However, a conducive environment does not imply that all fields in an environment are at uniform high risk of CBB incidence. In a conducive environment fields have a greater probability of serious CBB incidence but the final outcome will be influenced by a combination of the risk variables (Nelson *et al.*, 1994).

Some of the features of CBB incidence and *Xam* diversity are apparent from direct observation. Geostatistics and GIS will help to confirm, refine and quantify the analysis. In addition, some features of spatial heterogeneity, such as gradients, aggregation patterns, and punctual anomalies, cannot be detected by direct observation. Geostatistics can detect such patterns and also can help to relate various biological processes that lead to the spatial pattern of the disease (Lecoustre *et al.*, 1989; Chellemi *et al.*, 1988).

The final objective is the preparation of maps that display the probabilities of occurrence of disease severity, pathogen diversity and even of pathogen haplotypes. The probability of occurrence of haplotypes can be estimated by indicator Kriging (Nelson *et al.*, 1999). Indicator variables can be used for nonnumerical data, such as the presence or absence of a particular haplotype.

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Collaborators

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1.1.3 Role of the host in causing differentiations among *Xanthomonas axonopodis* pv. *manihotis* strains.

Introduction

Cassava Bacterial Blight (CBB), is one of the most important diseases that affects cassava. The causal agent is the bacteria *Xanthomonas axonopodis* pv.*manihotis* (*Xam*). The use and development of resistant cultivars is an important component for controlling the disease, but resistance can be overcome by changes that occur in the pathogen population structure. The genetic diversity in pathogen populations is an important consideration in choosing pathogen management tactics and there is a need for increased understanding of how the host affect the genetic structure of pathogen populations. A study carried out by Restrepo and Verdier (1997) in Colombia, led to the characterization of the genetic diversity in the pathogen population. RFLP and AFLP were used to assess genetic diversity among *Xam* strains. The molecular analysis is linked to pathogenicity assays in order to obtain a general picture of *Xam* population structure in Colombia. We also test the hypothesis that the host can exert a selection pressure on *Xam* population structure.

Materials and Methods

Bacterial strains. *Xam* isolates were collected in different fields located in four ecological zones in 1997. Collections were made from different improved or traditional cultivars adapted to each ecozone.

Molecular characterization. RFLP and AFLP markers were performed as previously described (Restrepo *et al.,* 1999). Two primer combinations were used for the AFLP technique, *Eco*RI+T/*Mse*I+T and *Eco*RI+T/*Mse*I+A. Each banding pattern was regarded as a haplotype.

Statistical analysis. We used NTSYS-PC to calculate a similarity matrix using Jaccard's coefficient of similarity. Cluster analysis was made with the UPGMA method. The diversity of strains from each field, ECZ and the entire country were estimated using the Nei and Tajima's diversity index (H_T) (Nei and Tajima, 1981). A correlation coefficient was calculated between the diversity index and the number of cultivars planted in each field. Analysis of molecular variance (AMOVA) (Excoffier *et al.,* 1992) was used to estimate variance components for RFLP haplotypes, partitioning the variation among

individuals within and among sites and ECZs. Genetic distance calculated as a Euclidean metric variance was computed between all pairs of isolates. Significance levels for variance component estimates were computed by nonparametric permutation procedures.

Pathogenicity tests. Strains corresponding to the RFLP/pthB haplotypes detected were stem inoculated on a set of fourteen differential cultivars. The area under disease progress curve (AUDPC) was calculated for each strain x cultivar interaction.

Results

We found 19 distinct *pth*B haplotypes among the 272 Colombian isolates. Nine of the haplotypes (C4, C6, C8, C9, C12, C13, C15, C17, and C18) found in 1995-1996 were recovered in 1997, and 10 haplotypes (C27, C28, C29, C30, C31, C33, C34, C35, C36, and C37) were new. Pathogen diversity was high within the fields with several improved cultivars. In contrast, diversity index was 0 for sites A and F where only one or two cultivars were cultivated (Table 1). A large part of the total variation was among ECZ-populations. Variance among field populations within ECZs was very low (Table 2). With only one exception, all the strains sharing the same AFLP haplotype were collected in the same field. The percentage of polymorphic bands was 90% for both combinations, showing a high level of polymorphism detected by AFLP data.

The correlation between the geographical origin of the strains at field level and DNA polymorphism is higher for AFLP than for RFLP data. For example, using RFLP, strains collected from fields C, D, and E located in ECZ 2 were frequently grouped together. According to AFLP data, these strains were grouped in three distinct clusters. However, the finest correlation between geographical origin and DNA polymorphism was obtained by combining the RFLP and AFLP data. Bootstrap values are higher for the AFLP and AFLP/RFLP dendrograms, showing that clusters obtained by AFLP are more robust and more reliable for phylogenetic studies.

Different pathotypes were defined using a differential set of cultivars. Four pathotypes were characterized, three previously detected (pathotypes 1-2, 2-3, and 5-1) and one new (pathotype 2-9). AUDPC values were significantly different according to the strains ECZ of origin. AUDPC values were higher for strains collected in ECZs 2/5 and 5.

Conclusions

The *Xam* population has shown a high degree of genetic diversity and the role of host diversity in structuring pathogen population was assessed. Results emphasize the host-induced selection on *Xam* populations and the potential of molecular markers as tools for identifying and monitoring *Xam* genetic variability. Diversity at loci involved in host pathogen interactions seems to be affected by differences in host population structure and this diversity is detected by RFLP/pthB.

Depending on the purposes of the study, RFLP and AFLP or the combination of both may be suitable to use. The RFLP / pthB is useful to estimate genetic diversity since the distribution of the haplotypes reflects the adaptation of the host and the pathogen to the ecosystem. We recommend to consider the AFLP data or the AFLP and RFLP combined to make phenetic inferences on the *Xam* population. The pathogen were highly differentiated for virulence factors in the four ECZs and different pathotypes were defined in each ECZ.

Ongoing Work

To develop a spatial simulation model to know the influence of various ecological and genetic factors on the distribution and potential change of *Xam* population structure.
To study the *Xam* population behavior during one crop production cycle.

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1.1.4 Evidence of a third gene pool in cultivated common bean

Background

It is widely recognized and documented that two major gene pools exist in common bean (*P. vulgaris*), based on evidence of plant morphology, seed proteins, isoenzymes and DNA markers. However, it is also known that wild *P. vulgaris* displays genetic variability that is not represented in cultivated bean, especially in Colombia, Ecuador and N. Peru. There is also some evidence that some incipient domestication took place in Colombia. The analysis that we are reporting was based on data taken on the bean core collection, and was focussed on defining the gene pool structure of cultivated bean.

Materials and Methods

The core collection was evaluated for plant morphological traits, for seed proteins (phaseolin, lectins and alpha-amylase inhibitors-αAI) and for quantitative traits of protein content, % of each major protein fraction in the seed, and seed and pod size. Data were analyzed in a step-wise fashion, incorporating different types of data in different combinations. This implied analyzing categorical and quantitative data separately and together, using methods reported for this purpose. Subsequently, the structure that was formed was compared with phenotypic data of various types: disease reaction and seed mineral content, among others.

Results

The simplest analysis undertaken was based on categorical data of the three seed protein types. This analysis demonstrated that most accessions fell into two major groups representing the two principal gene pools. However, accessions with 'CH' phaseolin type grouped apart from others, reflecting its association with unusual lectin and αAI types. 'C' phaseolin type fell into an intermediate position between the Middle American and the Andean gene pools. When categorical data of plant morphology were added to the analysis, the structure was maintained, and the difference of the 'C' types was accentuated.

However, when quantitative data such as seed and pod size were added to the analysis, the results returned to the well-known structure of two gene pools. That is, the 'CH' types grouped with the Middle American gene pool, and the 'C' types grouped with the Andean gene pool. Apparently the traits that reflect human intervention and selection influenced this analysis heavily and obscured the effects of the protein types.

When the putative N. Andean gene pool containing 'CH' phaseolin was compared with the two major gene pools based on numerous traits, it was found to distinguish itself in better resistance to the Middle American races of *Phaeoisariopsis griseola* (the angular leaf spot pathogen) and in higher iron levels. For other pathogens that present specialization to gene pools (Andean races of ALS; Andean and Middle American races of the anthracnose pathogen), and for Common Bacterial Blight, the N. Andean pool gave a reaction more similar to the Andean pool, in spite of being morphologically similar to the Middle American pool. This suggests that this pool evolved *in situ* in the Northern Andes, in association with the local pathotypes.

The significance of the 'C' types is yet to be clarified. While the possibility of a gene pool centered on 'CH' type is substantiated by data derived from the structure of wild bean, no such auxiliary data exists for the 'C' type accessions.

Conclusions

Variability exists in cultivated bean in the N. Andes that does not pertain to either major gene pool, and which is probably derived from incipient domestication events and/or introgression through spontaneous cross-pollenation. Further study may justify considering this to be an independent gene pool. However, this gene pool is probably heavily introgressed with the two major gene pools and has probably lost much of its integrity. It should be the object of special efforts at conservation, especially considering that its small seeded types are in no way commercial in the region and are subject to rapid genetic erosion.

Collaborators

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1.1.5 Relationship between seed coat polyphenolics and disease resistance in common bean

Background

Common beans contain a number of antinutritional factors as do most grain legumes. Proanthocyanidins (condensed tannins) are one of several antinutritional factors present in the dry bean and are located mainly in the seed coat or testa. Tannins alter the nutritional quality of plant products in several ways. They react with minerals, especially with iron, and with the proteins of the cotyledons of common bean, forming an indigestible complex. One possibility to improve the nutritional value of bean is to reduce tannin content, but it is not known if this would have a negative effect on resistance to plant disease. The objective of this study was to examine the relationship between seed coat polyphenolic content and resistance to three diseases and one insect pest in common bean, to determine the possible effects of altering seed coat tannin content.

Materials and Methods

Polyphenolics were extracted from a total of 1059 accessions in the bean core collection, representing all seed colors except white and non-commercial types. White beans have minimal tannin content and therefore were excluded from the analysis. Tannins are thought to be associated with seed pigments; therefore the non-commercial colors were also excluded as these colors are not of practical utility. The 797 accessions from primary centers were investigated in the present study. These accessions were previously subjected to a multivariate analysis to classify them into three gene pools and two intermediate groups. This classification was used to study the effects of tannins among and within gene pools. Only the two major gene pools presented sufficient numbers of accessions and were included in the analysis. Tannins were extracted from the seed coat,

dried and weighed. Disease data had been obtained previously, and were related to tannin content through linear correlations.

Results

The Middle American gene pool was found to have significantly higher tannin content than the Andean gene pool, therefore the two gene pools were considered separately. In the Mesoamerican pool, red and black beans have significantly higher tannin than those of cream, yellow and pink classes but don't differ significantly from purple (Table 1). Tannins of cream colored beans are only higher than those of yellow beans, which are the lowest in tannin content.

When correlations were calculated within the Mesoamerican gene pool, a complex picture emerged. Significant positive correlations were observed with tannins in the cream color class with Middle American races of ALS and Empoasca; in yellow beans with Empoasca; and in black beans with Andean races of ANT. However, red and purple beans present negative correlations with Andean races of ANT. Tannins in black beans also correlate negatively with Empoasca. Therefore, the tannin content and the correlation pattern varies for different seed colors, even in a particular gene pool.

In the Andean gene pool, red beans have the highest amount of tannin (0.0278 g/.25g testa) while black beans contain the lowest (0.0226 g/.25g testa). Surprisingly, tannins in black bean are significantly lower than those of cream, yellow and red but don't differ significantly from purple beans. Tannins in all colors have positive correlations with CBBleaf and CBBpod, some of these being highly significant. Significant negative correlations were observed within the Andean pool with tannins in the cream class with Middle American races of ALS and Andean races of ALS, in the pink beans with Andean races of ANT, and in black beans with Andean races of ALS .

Table 1. The correlation matrix of tannins in different seed colors with different diseases for Mesoamerican gene pools.

Seed color	Sample	Mean	Mean differ	Vol	ALS And	ANT and	ALS mes	ANT mes	CBB leaf	CBB pod	Empoasca
Cream	81	0.0310	Y**,R**, B**	.033	-.036	-.028	.288**	-.018	.032	-.189	.267*
Yellow	78	0.0285	R**,B**,P*	.201	-.005	-.202	-.076	-.003	.093	.090	.303**
Pink	14	0.0307	R**, B**	-.098	.187	.305	-.408	.198	.067	-.157	.141
Red	53	0.0342		-.162	-.181	-.270*	.232	.104	.052	.048	.034
Purple	38	0.0316		.234	0.0	-.364*	.277	.115	.136	.207	-.017
Black	167	0.0340		.207**	-.069	.296**	-.093	.355**	.127	-.080	-.308**

** and * are respectively for P≤0.01 and P≤0.05; Y=yellow, R=red, and B=black

Table 2. The correlation matrix of tannins in different seed colors with different diseases for Andean gene pools.

Seed color	Sample	Mean	Mean difference	Vol	ALS And	ANT and	ALS mes	ANT mes	CBB leaf	CBB pod	Empoasca
Cream	86	0.0265	B*	.374**	-.222*	-.118	-.219*	.118	.373**	.398**	.122
Yellow	50	0.0278	B**	.433**	-.007	-.033	-.093	-.120	.259	.167	.261
Pink	19	0.0268		.395	.194	-.533*	.005	.263	.360	.419	-.108
Red	40	0.0278	B**	.172	.104	.091	-.282	-.072	.152	.329*	-.141
Purple	58	0.0255		.382**	-.140	.210	-.098	.137	.326*	.149	-.210
Black	23	0.0226		-.039	-.442*	-.353	.204	.341	.290	.512*	-.122

** and * are respectively for P≤0.01 and P≤0.05; B=black

Conclusions

The relationship of seed coat tannin and disease resistance was complex and depended on the particular gene pool, color class, disease and races of the pathogen. Although black beans of the Middle American gene pool registered among the highest values of total tannin, in the Andean pool the black beans had the lowest levels. Surprisingly, positive correlations (ie, more tannin being associated with more disease) were as common or more frequent than negative correlations. This positive relationship was especially strong with CBB. Apparently seed coat tannin can be reduced without serious effects on plant resistance to diseases.

Collaborators

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1.1.6 Phenotypic, agronomic and nutritional differences among Middle American races of cultivated common bean

Background

Within the Middle American gene pool, three races have been proposed, based on differences in plant and seed morphology and adaptation regimes: race M (Mesoamerica), race D (Durango) and race J (Jalisco). Races were defined in terms of morphology and general agro-ecological adaptation. However, RAPD analysis revealed additional variability in Middle American beans. Races M and D displayed sub-races, and Guatemalan germplasm contained accessions of climbing bean that did not group with any of the previously defined races. Thus, a fourth race G (race Guatemala), was proposed. However, no broad and statistically validated evaluation of the differences among races has been undertaken. Neither has race Guatemala been characterized for its useful traits, nor have the practical implications of subraces been explored. The objective of the study is to determine

what differences exists among Middle American races of common bean as defined by RAPD analysis, for disease resistance, nutritional characters and yield potentials in different environments.

Materials and Methods

A total of 479 landraces of Middle American origin were chosen for RAPD analysis to confirm the races and sub-races defined previously with RAPD. Of these, all were from primary centers of diversity and were included in the CIAT bean core collection. Six races or subraces were confirmed with the analysis of these 479 RAPD: M1, representing small black germplasm including ICA 'Pijao'; M2 representing Central American landraces; D1 including typical race Durango Pinto and other commercial types; D2 composed of habit 3 and 4 small to medium seeded types; and races J and G. The race M1 was represented by 96 accessions; M2 by 78 accessions; D1 by 38; D2 by 92; J by 64; and race G by 105 accessions. The classification so obtained was compared with the data taken previously on the core collection: yield with and without P stress in Darién; yield without fertility stress in Popayán; seed mineral content; and disease reactions.

Results

In terms of yield of bush types in Darién, subrace D2 gave the highest mean yield in bush beans in the P stressed treatment, significantly higher than those of other races except M2 and about 10% more than subrace D1 to which it is related genetically. Race G presented the lowest yield of bush types under the same low P condition. In the high P treatment in Darién, race M1 in bush types had the highest mean yield followed by race J. The lowest yield was of race D1 which yielded significantly less than races M1, M2, D2 and J. In this treatment also, D2 again yielded about 10% more than D1. In the high P treatment in Popayán, race M1 again had the highest mean yield, significantly higher than those of races D1 and D2; and race M2 also yielded significantly more than races D1 and D2. Again, in the high P treatment subrace D1 yielded the least among the several subraces. In climbing beans few significant differences in yield could be discerned, except that races J and G outyielded subraces D2 and M2.

The lowest mean disease score with Andean isolates of angular leaf spot was observed in subraces M1 (1.91) and M2 (1.78); the highest scores were of subraces D1 (3.08) and D2 (2.56). Races D1 and D2 also gave the highest disease scores of ALS with Mesoamerican isolates: 7.58 for D1 and 7.54 for D2, in contrast with 6.28 for M1 and 6.64 for race G. With Mesoamerican isolates of ALS, subrace M2 was significantly more susceptible than subrace M1 and race G. In the case of anthracnose, subraces D1 and D2 had low scores with either Andean or Mesoamerican isolates, while M1 presented the highest scores with either pathogen population, significantly more than M2. The mean scores of common bacterial blight on leaf were lowest for race Durango and especially subrace D2 which presented significantly lower scores than any other group. The two races of climbing bean, J and G, were discriminated by their reaction to Mesoamerican isolates of ALS, for which race G was more resistant.

The highest protein contents were observed in races M1 and M2; the lowest were in races D1 and D2 while J and G were intermediate, thus forming three groups that were all significantly different from each other. Race G contained the highest phaseolin, lectin and tripsin inhibitors but the lowest αAI. Therefore, race G presented an unusual profile of protein fractions. In the case of mineral content, race M2 presented the highest or second highest values for ten out of eleven minerals, and calcium which was significantly lower than any other group. Race M1 presented a pattern similar to M2 for several elements but at a significantly lower level. Subraces D1 and D2 were significantly different for several elements: B, Ca, K, Mn and Na. However, race G presented the highest levels of iron and the lowest manganese among all races.

Conclusions

The significance of the races and sub-races that were distinguished by RAPD analysis was confirmed by comparison of molecular groups with the phenotypic data of the respective accessions. In bush beans, race M1 was superior in yield without fertility stress, as is widely recognized. However, within race D, sub-race D2 expressed superiority over D1, especially under P stress in which D2 was the best group of all. Sub-race D2 deserves particular attention in terms of its potential contribution to yield. Statistically significant differences in minerals and in reaction to several pathogens were also detected, suggesting where sources of resistance or superior mineral content might be detected. Race G stood out as a source of two important traits: iron content, and resistance to ALS. These results not only confirm that the differences detected by RAPD are real, but should also contribute to the better usage of genetic resources.

Collaborators

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1.1.7 Introgression from Mesoamerican bean to the Andean gene pool and its effect on phenotype

Background

Studies of DNA polymorphism have indicated that most Andean landraces of common bean have a narrow genetic base, although about 10% in the CIAT core collection presented evidence of introgression from other gene pools. The extent of introgression is not clear nor is the effect of such introgression known, either on agronomic or nutritive values. The objective of this study was to quantify the degree and to determine the effect of spontaneous introgression between gene pools on disease resistance, yield and nutritional characters using molecular markers. An attempt was made to relate the introgressed fragments to changes in these same characters through QTL analysis, and to map the markers on the CIAT genetic map.

Materials and Methods

This study involved 611 common bean accessions, drawn from the core collection based on typically Andean phaseolin types: 'T', 'H', 'Ca', 'Pa', 'C', 'To', plus other non-frequent phaseolin types of Andean America, such as 'K', 'Ko', 'TCA' etc. One Mesoamerican check (race M cv 'ICA Pijao', with small black seed) and one Andean check (race N cv 'Calima', with large seed) were included. DNA was extracted and RAPD reactions carried out using ten primers that offered a high degree of polymorphism.

To examine the relationship between the prevalence or rarity of markers and the agronomic performance of the cultivars bearing those markers, the frequency of each of 151 RAPD markers within the germplasm pool was computed as the number of cultivars in which it was present divided by the total number of cultivars. Then for each cultivar, we computed a "marker prevalence index" as the average of the frequency values of the markers present in that cultivar. A low index indicated a high degree of introgression of rare bands. The prevalence index was related to the phenotypic data (protein content, disease reactions, mineral contents, and yield) by regressing the variables on the prevalence index.
Subsequently, the effect of each individual RAPD was associated with the phenotypic data through the use of a t-test. This is fundamentally a QTL analysis of the simplest sort. Finally, the same ten primers were evaluated on the CIAT mapping population of G19833 x DOR 364, to locate the fragments that were used in the analysis. Since the fragments were largely introgressed from Mesoamerican germplasm to the Andean pool, and the two parents of the mapping population represent the same two gene pools, many of the same polymorphisms could be discerned for mapping.

Results

The 607 accessions within our sample had marker prevalence indices ranging from 0.44 to 0.90 with mean index of 0.75. The linear regression revealed highly significant relationships with significant F values ≤ 0.01 for ten quantitative traits (ALSand, ANTand, ALSmes. ANTmes, DTF, 100 seed weight, protein content, iron, sulfur and zinc); four traits (CBBpod, HP-clim.dar, DTM, and calcium) showed significant relationship with significant F values ≤ 0.05. No relationship was revealed between marker prevalence index and eight quantitative traits (CBBleaf, empoasca, Yield-LoP-bush.dar, Yield-LoP-clim.dar, Yield-HiP-bush.dar, Yield-HiP-bush.pop, Yield-HiP-clim.pop and phosphorus). The direction of the effect (which is to say, the positive or negative value of the regression coefficient) suggested that the rare bands did in fact proceed from the Middle American gene pool, since rare bands resulted in a phenotype more similar to the Middle American bean. For example, reaction to Mesoamerican races of anthracnose increased with more rare bands (reflected in a negative regression value), as would be expected with more Mesoamerican influence on plant phenotype.

It was possible to map 45 of the total of 151 RAPD generated on the germplasm accessions, using the population of G19833 x DOR 364. Several of these RAPD were associated with phenotypic traits, thus loci of agronomic and nutritional traits were

successfully mapped. In cases in which similar phenotypic data had been obtained on the mapping population and loci had been mapped (as for ALS and anthracnose resistance genes), several of the same loci were found, thus confirming the previous data and the validity of the present methodology.

In the case of yield, the overall effect of introgression was not significant. When individual loci were examined, some had a negative effect and some a positive effect, thus explaining why no consistent effect was detected through the prevalence index. Among individual loci that increased yield, most were also associated with either later maturity or smaller seed size, both of which are undesirable traits.

Conclusions

Although the Andean gene has an inherently narrow genetic base, it has been broadened through the spontaneous introgression from Mesoamerican beans that are cultivated in the Andean zone. This introgression has had a detectable effect on disease resistance and mineral content of the accessions. This has permitted us to mark genes and to map the loci that are responsible for several traits without recurring to populations developed specifically for this purpose. The effect of introgression on yield has been variable and QTL for higher yield have frequently been associated with later maturity or smaller seed size.

Table 3: Simple linear regression of trait scores on marker prevalence indices

Traits	R2	F values	Beta/r	Significance of F or T
ALSand	0.059	37.73	0.242	.0000
ANTand	0.032	20.26	0.180	.0000
ALSmes	0.025	15.52	-0.158	.0001
ANTmes	0.014	8.47	-0.118	.0037
CBBleaf	0	<.001	<.0001	.983
CBBpod	0.010	6.13	0.100	.0135
empoasca	0	<.001	<.0001	.9810
Days to flower	0.013	8.04	-0.115	.0047
Days to maturity	0.007	4.32	-0.085	.038
100 seed wt.	0.066	42.13	0.257	.0000
Yield-loP-bush.dar	0	.25	0.030	.617
Yield-loP-clim.dar	0	1.20	-0.061	.275
Yield-HiP-bush.dar	0.014	3.83	-0.116	.0513
Yield-HiP - clim.dar	0.014	4.57	-0.118	.0332

Traits	R2	F values	Beta/r	Significance of F or T
Yield-HiP bush.pop	- 0.006	1.56	-0.075	.213
Yield-HiP clim.pop	- 0	.032	0.01	.857
Protein cont.	0.026	16.34	-0.162	.0001
Calcium	0.010	5.35	-0.101	.021
Iron	0.025	13.49	-0.159	.0003
phosphorus	0.002	1.09	-0.046	.297
Sulfur	0.104	60.48	-0.323	.0000
Zinc	0.018	9.53	-0.134	.0021

Collaborators

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1.1.8 Evaluation of a core collection of *P. coccineus* and *P. polyanthus* for common bean diseases.

Background

Other species of Phaseolus genus express traits that are non-existent or poorly expressed in common bean, thus making them attractive for interspecific crosses for the transfer of traits. A core collection was formed three years ago to better determine the value of these species for the improvement of common bean. As a first step, some of the common bean diseases were evaluated on the core, to obtain a perspective of the contrast that these species might have with common bean. Thus, the core was evaluated for reaction to common bean anthracnose-ANT (Mesoamerican and Andean races); angular leaf spot-ALS (Mesoamerican and Andean races); and ascochyta blight-ASC.

Methods

Inoculations with mixtures of isolates of the ANT and ALS pathogens were carried out in screenhouses in the Popayán station of CIAT. Ten plants were established of each accession. Inoculations with ASC were carried out in the field in Popayán on five meter rows. Isolates of all three pathogens were obtained locally. ANT and ALS pathogens are known to display adaptation through co-evolution with the bean host, and the isolates used had been classified as Mesoamerican or Andean in reaction with common bean. All reactions were registered on a scale of 1 (immune) to 9 (totally susceptible).

Results

Both wild and cultivated *P. polyanthus* were universally and highly resistant to ASC. Although only two wild accessions of PP were evaluated, results with wild were nonetheless consistent with results with the cultivated. Thus it appears that the resistance may be an ancestral trait derived from the wild polyanthus and has not been substantially altered during domestication. On the other hand, both wild and cultivated *P. coccineus* present a range of reactions to ASC. The wild presented values from 1.0 to 5.5, and the cultivated from 1.3 to 7.0. Thus, the reaction of coccineus to ASC has not changed substantially with domestication either. However, within *P. coccineus* there is certain geographical stratification, such that Mexican and European accessions often present intermediate to susceptible reactions to ASC, while accessions to the south of Mexico tend to be more resistant. This could feasibly reflect the lateness of these latter accessions, as noted in last year's report.

The contrast between polyanthus and coccineus presents an interesting evolutionary question. It has been shown that the nuclear DNA of these two species presents greater similarity than either do with vulgaris, while the non-nuclear DNA of polyanthus is more closely related to vulgaris. This suggests that the nucleus of polyanthus was heavily introgressed by coccineus at some point in its evolution. Wild *P. polyanthus* is known to exist only in Guatemala to date, and two of these accessions presented ASC readings of 1.0. Wild Guatemalan coccineus presents ASC reactions from 1.3 to 4.8. If polyanthus has been heavily introgressed from coccineus, why does it present such a narrow range of ASC values? Does the uniformly high reaction of both the wild and cultivated polyanthus suggests that this introgression was limited to certain populations of wild coccineus, even among the several populations in Guatemala? Again, this should be taken within the context of the limited sample size of wild PP, and should be interpreted as a topic of future study and not as a conclusion.

Reaction of PP and PC to common bean ALS is variable but generally low. One might have speculated that Mesoamerican isolates of the pathogen could have attained greater adaptation to the PP/PC hosts than Andean isolates, since PP and PC are believed to have evolved essentially in Middle America. In fact, the Mesoamerican isolates were slightly more virulent on about 25% of the accessions, although disease reaction did not follow gene pool lines of the pathogen closely. The reaction of a given accession with the two types of isolates was similar in more cases than it was distinct. Curiously, in the few cases in which the Andean isolates gave a more virulent reaction, this was observed only with polyanthus. This suggests some subtle differences in the evolution of the two species in relation to the ALS pathogen. Could PP have experienced some stage of its evolution in contact with Andean isolates of ALS? This could be studied further with selected, virulent Andean isolates of the pathogen.

Reaction to ANT gave the least discrimination among accessions of the three pathogens studied. Both species are almost universally resistant to either Mesoamerican or Andean isolates of the pathogen. While a few individual plants were registered with reactions as high as 7, the highest average value for an accession was 4.1 in G35346, a Mexican

accession of PC inoculated with Mesoamerican isolates. The same accession presented a reaction of 3 with Andean isolates.

One accession of *P. costaricensis* was also included in the evaluations, and this accession was resistant to all three pathogens.

The core was dispatched to Puerto Rico in an attempt to evaluate resistance to BGMV, but disease pressure was unusually light and did not permit discrimination of differences among accessions.

Conclusions

The sister species of the common bean display high levels of resistance to the three fungal pathogens that were assayed. It is possible that out of the same interspecific crosses, resistance to several diseases could be transferred.

Collaborators

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1. SB-02 Project; 2. IP-1 Project

1.1.9 Investigation on Soil Bacterial Diversity

Introduction

The overwhelming majority of soil bacteria are not cultivatable. Traditional culture methods thus give little information about bacterial community structure. Ribosomal DNA has recently been used to study soil bacterial populations. Random cloning and sequencing studies have shown that the majority of the soil community is made up of previously unidentified species. We are developing ribosomal DNA based methods that can characterize the soil prokaryotic community to develop indicators of soil health and to determine how bacterial ecology varies over the landscape.

Ongoing Activities

Work this year has been around two activities, DNA extraction from soil and the development of bacterial community fingerprinting methods.

-DNA extraction from soil.

Developing a reliable extraction method was very important for further work. It is difficult to compare the results from soil molecular biology studies that use different DNA extraction methods. As we plan to look at how soil bacterial diversity varies over the landscape, a single extraction method that can be used on a wide variety of soils was

needed. The Humboldt institute is studying forest Andisols from Quindio, so it was particularly important that the method work for high organic carbon soils.

Much of this year was spent modifying a direct DNA extraction method from soil. We submitted a short article for publication in Biotechniques based on experience extracting DNA from eight contrasting Colombian soils. (Table 1).

Table 1. Properties of soils used for DNA extraction including yield and purity of extracted DNA samples (all results expressed relative to oven dried weight of soil)

Soil Unit	Site	Land Use	Clay %	Organic Carbon %	pH in H_2O	C.E.C Meq/100 g soil	DNA Extracted µg/g soil	A_{260}/A_{280}
Eutric Vertisol	CIAT Palmira	Leucaena	53.2	3.4	7.6	30	9.5	1.8
Haplic Kastanozem	CIAT Palmira	Bamboo Forest	33.5	3.3	6.3	22.6	3.5	1.8
Haplic Kastanozem	Rozo,	Tobacco Field	23.0	0.90	6.1	13.2	4.0	1.7
Urbic Anthrosol	CIAT Palmira	Garden	30.4	7.7	6.7	33.8	4.8	1.7
Humic Cambisol	Panse,	Secondary Forest	48.0	7.9	4.8	29.6	18.2	1.6
Eutric Leptosol	Cristo Rey,	Weed Fallow	21.2	7.3	7.1	35	11.5	1.6
Humic Andosol	Mondomo	Secondary Forest	48.4	5.8	5.0	26.4	2.5	(1.6)‡
Haplic Ferralsol	Carimagua	Native Savanna	18.3	1.1	4.3	2.0	2.3	(1.3)‡

‡ Estimates are not very accurate because of low DNA concentrations

Since then this method has been applied to Andosols from Quindio with organic carbon contents of up to 30 % and well as a pasture soil from Leticia.

-Development of Community Fingerprinting Methods.

Ampified rDNA-restriction analysis (ARDRA) is the first form of bacterial fingerprinting that is being tested. ARDRA involves PCR amplifying all or part of the bacteria's

ribosomal gene, digesting the amplification products with a restriction enzyme and separating the digestion products on a gel. The advantages of ARDRA are that it can use a wide range of primers and agarose gels (other fingerprinting techniques that have been used to characterize the soil bacterial community require polyacrilymide and gradient gels). The main disadvantage of ARDRA is that the bands it produces are difficult to match to phylogenic groups. The results of ARDRA will thus show us which phylogenic groups it is worth developing primers for more demanding fingerprinting techniques (DGGE or RISA).

Amplification products of the following primer sets are being tested for ARDRA:
-530F-1406R universal primers (as used by Borneman and Triplett 1997)
-141F-1314R NH_4 oxidising primers (as used by Smit et at 1997)
-63F-1387R Eubacterial primers (as designed and tested by Marchesi et al 1998)
Work has also started on optimizing PCR conditions for the archeal primers of Bintrim et al (1997). The Humboldt institute will also work with primers for actinomycetes and acidiobacteria.

The following restriction enzymes are being tested on the amplification products: AluI, HinfI, MspI and RsaI.

For the eubacterial primers we are using both samples of soil DNA and strains of *Xanthomonas* campestris donated by George Mahuku of the bean program. The results of the *Xanthomonas* fingerprints may show us how sensitive our ARDRA patterns are to minor genetic differences and may also be useful for the bean program. Results so far show little difference between the Colombian strains.

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Collaborators

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1. SB-02 Project 2. IP-4 Project

1.1.10 Morphological, biochemical and molecular characterization of promising Amazon species of the genus *Capsicum* for their conservation and use.

Introduction

Peppers are a major and ancient crop McNeish (1964) concluded that it was a part of the diet of ancestors of the Indigenous Americans since 7500 BC, and that it was first cultivated between 5200 and 3400 BC. Peppers originated in the New World, having different points of domestication and speciation. One of them seems to be the northern Amazon region, more precisely in Colombia (Debouck et al. 1996), as observed from the great variability of *Capsicum* species found along the equatorial line.

According to some authors the genus *Capsicum* (Solaneceae) consists of 22 wild and five domesticated species which are *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens*. There is a debate whether *C. annuum*, *C. frutescens* and *C. chinense* are truly species or one single polytypic specie. Peppers have been used as a spice, condiment and as a vegetable worldwide. Peppers are a staple food for indigenous Amazon people. The total world production of green chili peppers in 1997 was 16.393.000 tons, ten thousand of which came from Colombia. However, all of the Colombian production derived from imported seed. The objective of this study was to gather information on the genetic diversity of Colombian Amazon germplasm by molecular characterization using AFLPs. This work was developed at CIAT, while the biochemical and morphological characterization is being developed in the SINCHI Institute (Bogotá) and the Universidad Nacional de Palmira respectively.

Materials and methods

Seeds of *Capsicum* species were collected from indigenous communities of the Colombian Amazon. The collection sites covered the departaments of Guainía, Amazonas, Putumayo, Vaupés, Vichada, Caquetá and Guaviare. We visited the ethnic groups (indian tribes) Curripacos, Tucanos, Puinabes, Desanos, Kubeos, Piapocos, Sikuanes, Guahibos, Ingas, Camentsas, Cofanes, Uitotos, Tanimukas, Boras and Tikunas. Collected seeds were planted in the green house at CIAT and plants grown to maturity. More than 400 accessions were collected from which 81 have been analyzed these from the Amazonas, and Caqueta departments. As reference, we also grew seeds from five cultivated species, which were used for AFLP analysis.

DNA isolation was basically according to Dellaporta (1983). Radioactive AFLPs were performed as described by Vos et al. (1995) using the GIBCO BRL Kit. Four

polymorphic primer combinations were chosen: E+AGC / M+CAG, E+ACA / M+CTA, E+ACC / M +CAA and E+AAG / M+CAG.

We constructed a data matrix for the presence/absence of bands. The matrix was analyzed by Multiple Correspondence Analysis (MCA) using the CORRESP procedure (SAS Institute 1989) and by cluster analysis (UPGMA) based on the index of Nei and Li (1979)

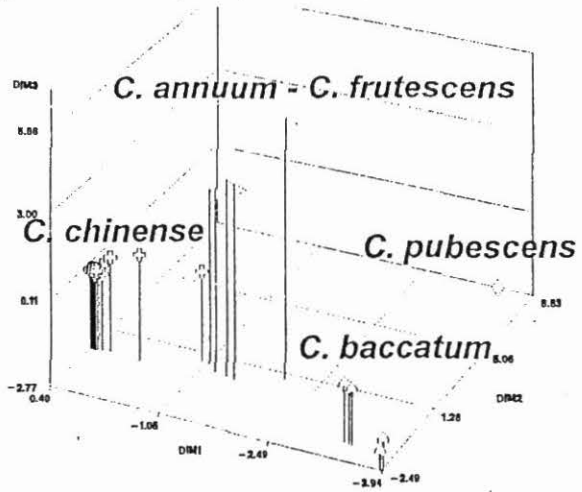

Fig. 1. Three principal axes of variation obtained by multiple correspondence analysis of AFLP among 81 accessions of *four groups*.

using NTSYS (version 1.8; Rohlf 1994). Gene diversity (His), as a measure of genetic variation within each cluster, was calculated according to Nei (1987) using the IML procedure of SAS.

Results and Discussion

The MCA analysis revealed four groups with a Rsq of 0.95, which represents 95% of the total variation (Figure 1). The reference accessions helped in group identification. The accessions collected fell into three major groups: 1. *C. chinense* (crosses in Fig.1); 2. the complex *C. annuum- C. frutescens* (triangles in Fig. 1), the third to the group of *C. baccatum* (see spades in fig. 1) and the fourth contain the reference accession of *C. pubescens* (see diamond fig. 1). The first dimension of MCA was effective in separating *C. chinense* gene group and the complex *C. annuum- C. frutescens* from *C. baccatum* and *C. pubescens* gene groups. The second dimension separated *C. baccatum* and *C. pubescens* gene groups. And the third dimension distinguished further between the gene group of *C. chinense* from the complex *C. annuum- C. frutescens*. Of these four groups, three contained accessions collected in the Colombian Amazon. Accessions CS 172 and CS 183 fell into the *C. baccatum* gene group, while accession CS 200 was within the complex *C. annuum- C. frutescens* and the rest fell into the *C. chinense* group.

The clustering was in agreement with the MCA analysis. Four major groups were found, which corresponded to the groups of *pubescens* (cluster 1), *C. baccatum* (cluster 2), the complex *C. annuum- C. frutescens* (cluster 3) and *C. chinense* (cluster 4).

The *C. chinense* group, containing the majority of the accessions, had the lowest gene diversity value (0.072). A possible explanation may be that they represent a recently domesticated group of landraces with a narrow genetic base. *C. baccatum* was more variable (0.127) than the previous group and the *C. annuum* - *C. frutescens* complex was the most variable (0.182). The latter group has a greater proportion of reference accessions of non-Amazon origin, thus increasing the diversity of the group due to their different geographical origin. Higher gene diversity values have been found in RFLPs studies of Mexican accessions (Prince et al. 1992) where the average gene diversity for *C. annuum* was between 0.262 and 0.275 (Northeast and Northwest region) and for *C. frutescens* 0.238. Most accessions of Prince study were wild which may explain the higher values. We must emphasize that the results previously described are based on the analysis of only 81 out of more than 400 accessions collected, which may not represent the actual genetic diversity among peppers in the Colombian Amazon.

Further activities

We selected a single primer combination (EAGC/MCAG) to screen the rest of accessions. Results of the clustering analysis (dendograms) with this single primer combination were very similar to those obtained using four primer combinations. We also standardized the silver staining technique for our AFLP gels, avoiding the use of isotopes. Finally, we initiated the database (pc Green; IPGRI 1998) to enter passport data for all accessions.

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Collaborators

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3: Tropical Forrages Project

1.1.11 Alexander von Humboldt Biological Resources Research Institute Molecular Biology Laboratory and Tissue Bank

Introduction

Within the philosophy of the institute of resource optimization by means of strategic alliances with other institutions, a cooperation agreement was established between the International Center for Tropical Agriculture and the Alexander von Humboldt Biological Resources Research Institute. This agreement created the Molecular Biology Laboratory and Tissue Bank presently located at the Biotechnology Research Unit (BRU). The first year of laboratory work, has begun with three research projects in molecular genetics of palms, molecular ecology of soil microorganisms and population genetics of high mountain plants. The Humboldt laboratory has supported collaborative works with Colombian and foreign researchers who have needed the facilities of the laboratory of the Institute and those of the BRU to conduct undergraduate, masters thesis, and doctoral dissertations related to the study of the Colombian biodiversity integrating molecular markers. In addition, researchers from the Sinchi Institute, that conducts biodiversity and sustainable development research in the Amazon basin, also utilize the Humboldt laboratory for the genetic characterization of wild and cultivated amazon *Capsicum* spp. The second main objective of the laboratory, the establishment of a biodiversity tissue bank in liquid nitrogen with capacity for 70,000 samples, is in the initial phase of the storage system setup. This tissue bank is unique in the country and is part of a national strategy for the conservation of biodiversity in Colombia.

Institute's projects

Project 1: Use of molecular techniques for both biodiversity and conservation studies in endangered palms

Activities

- Standardization of DNA palm extraction.
- Construction of enriched libraries in two (of four) different palm species.
- Sixty clones sequenced in total
- Thirty sequences designed

On-Going Activities

- Construction of two libraries in *A. amygdalina* y *W.hirsuta*
- Sequencing of positive clones in each library
- Primer design
- Characterization of these primers in each population

Project 2: Community structure of soil *Streptomyces* in remnant forest and agroecosystems from Quindio (Colombian Andes mountain)

Activities

- Standardization of culture methods
- Isolation and characterization of morphotypes
- Selection of primers and enzymes for ARDRA analysis
- Extraction DNA from soil bacterial community

On-Going Activities

- Amplification of the 16S rRNA with specific primers
- Restriction with restriction enzymes
- Visualization of products in PAGE
- Characterization of community structure

Project 3: Genetic population structure and morphological variation of two populations of *Espeletia Schultesiana*

Activities

- Evaluation of RAPDs as molecular markers for *Espeletia Schultesiana*
- Characterization of genetic structure

Project 4: Establishment of Tissue Bank

Activities

- Installation of liquid nitrogen storage system for conservation
- Elaboration of Plant and vertebrate tissue collection manual for molecular analysis
- Processing of ca. two hundred samples

On-Going Activities

- Implementation of the data base
- Elaboration of a manual for invertebrates and microorganisms tissue collection for molecular analysis
- Elaboration of samples request policies

Collaborative projects

Project 1: Geologic influences on Amazon *biodiversity* as revealed by genetic analysis of river dolphins

Activities

- Extraction of river dolphin DNA
- PCR of mitochondrial genes
- Sequencing of two mitochondrial genes, cytochrome *b* and cytocrome oxidase II

Project 2: Genetic effects of forest fragmentation in *Quercus humboldtii* Bonpl.

Activities

- *Quercus* DNA extraction standardization
- Evaluation of 24 microsatellite primers developed for Fagaceae
- Genetic analysis of pilot fragment consisting of 42 trees and 30 offspring

On-Going Activities

- DNA extractions of complete study site (ca. 600 trees and seedlings)
- PCR and PAGE genotyping of all samples

Project 3: Genetic variation in *Inia geoffrensis* and taxonomy evaluation with molecular methods

Activities

- DNA extraction of all samples of *Inia geoffrensis* of the two Colombian populations and one Bolivian population.
- Sequencing of D-loop region of mtDNA
- Statistical analysis

On-Going Activities

- Evaluation of microsatellites in *Inia geoffrensis* of all populations sampled

Project 4: *Rhizophora* species microsatellite development and evaluation from Buenaventura bay populations in Pacific Colombia

Activities

- Standardization of DNA extraction.
- Construction of enriched libraries.

On-Going Activities

- Sequencing of positive clones in each library
- Designing of primers
- Evaluation of microsatellite primers in the population

Project 5: Determination of grade of variability and genetic structure of iguanero mangrove (*Avicennia germinans*) from three zones in the Colombian Pacific coast

Activities

- Standardization of DNA extraction.
- Training in of AFLP's technique

Collaborators

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[1] SB-02 Project [2] Natural Sciences Institute; [3] Alexander Von Humboldt Biological Resources Research Institute; [4] National University of Colombia; [5] University of California, Berkeley; [6] University of Valle; [7] University of Washington, St Louis; [8] University of Missouri, St Louis.

ACTIVITY 1.2 Identification and mapping of useful genes and gene combinations.

MAIN ACHIEVEMENTS

- A region of the cassava molecular map associated with resistance to the cassava mosaic disease (CMD) has been identified, opening the way to the introgression of CMD resistance into Latin American germplasm. On the other hand, genetic markers linked to whitefly resistance have been located in cassava.

- QTLs involved in resistance to cassava bacterial blight (CBB) have been identified. Association of a linkage group to greenhouse and field resistance suggest its role in horizontal CBB resistance. New populations segregation for CBB resistance have been mapped using RFLPs and SSRs.

- Resistance gene analogs (RGAs) were identified using degenerate primers and linked to resistance to the bean golden mosaic virus (BGMV) in common bean. On the other hand, characterization of RGAs for rice blast resistance continued using degenerate primers.

- Identification and mapping of specific genes expressed in cassava during post harvest physiological deterioration (PPD) was carried out as part of work on the search of wound response genes involved in PPD.

- QTLs with large yield effects were identification in common bean under low and high P fertility conditions. These should be complementary to the QTLs for root traits involved in P uptake, reported last year. In order to expidite the use of these and other disease-resistance related QTLs in bean breeding, the development of SCAR markers was initiated.

- A marker assisted selection (MAS) scheme was succesfully implemented using a SCAR marker for BGMV resistance. Results were provided to the breeder 34 days after planting, and multiple crossess were made using only plants harboring the resistance marker. Breeding effort was reduced in about 60% due to the use of MAS. A similar MAS scheme on *Brachiaria* breeding in underway.

- Following a pre-distribution/test, a GIS-based Flora Map tm, has been refined and prepared for sale/distribution this year. The system has been, and will be, used to guide plant collection, to investigate taxonomic and genetic variation, and to map crop pests and their potential predators.

1.2.1 Molecular Mapping of Genes Conferring Resistance to the Cassava Mosaic Disease (CMD) in African Cassava Germplasm

Introduction

Cassava, considered the most important food crop in sub-Saharan Africa has as its most important production constraint the cassava mosaic disease (CMD) . This disease is also becoming a growing threat to cassava production in Asia and Latin America, due to the alarming rate of spread of the biotype B of *Bemisia tabaci*, the only known vector of CMD. Epidemics of CMD have been known to occur in a cyclic fashion in the sub-Saharan African region. The most recent epidemic, in Uganda, which began in 1986, has wiped out cassava production in more than 70% of the country. Responding to the epidemic, mainly through the distribution of resistant material, has been slow due to the time lag required for resistance breeding, and multiplication of resistant genotypes. The objective of the CMD resistance mapping project, with funding from the Rockefeller Foundation, is to identify molecular markers tightly linked to different sources of CMD resistance for efficient and cost-effective pyramiding of resistance genes, and to ultimately clone the resistance gene(s) for rapid deployment. The project is a collaborative effort between the International Institute of Tropical Agriculture, (IITA), Ibadan, Nigeria, and the International Center for Tropical Agriculture (CIAT, its Spanish acronym), Cali, Colombia, and activities were conducted at both institutions. This report presents the activities in CIAT in the past one year and also concludes the first phase of the project. Funding for a second phase is being requested.

Materials and methods

Molecular marker genotyping of CMD mapping populations

A total of 250 RFLP, 186 SSR, 100 RAPDs, 10 ESTs and known genes, and 3 isozyme markers, developed in the cassava mapping project at CIAT, were available for marker-aided analysis of resistance in the half-sib BC_1 population.

Genetic mapping of a CMD resistance gene

Parallel to the BC_1 mapping effort, a bulk segregant analysis (BSA) approach (Michelmore et al., 1988) was conducted to quickly identify markers linked to CMD resistance. The entire set of 186 SSR markers were screened on bulked DNA from 10 resistant, and 5 susceptible genotypes, from a single family of the half-sib BC_1 population.

Results and discussion

More than 70 markers from the frame work map of cassava have been transferred to the half-sib BC_1 population. Enzyme/probe combination, southern hybridization, and linkage analysis, are as described for the female-derived map of cassava (Fregene et al., 1997). Currently, other markers from the 186 Cassava MapPairs SSR markers developed at CIAT, are also being placed on the map to complete the BC_1 frame work map. Ten SSRs were found to be polymorphic in the bulks. The markers were then evaluated in individuals of the bulks, and 1 SSR marker, CSY1, was polymorphic between the resistant and susceptible genotypes, with one recombinant (Figure 1).

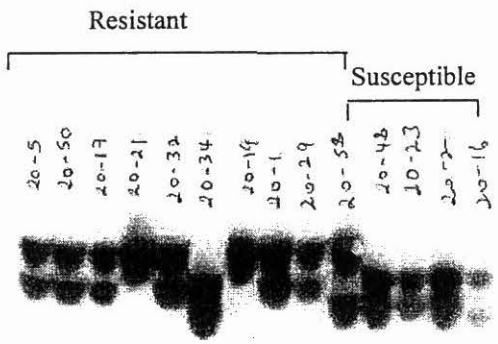

Figure 1. Silver stained PAGE gel of SSR CSY1 PCR amplification of DNA from extreme resistant and susceptible genotypes from BC_1 family 8820. Note the recombinant 8820-34.

Segregation and linkage analysis of CSY1 in F_1 cassava map progeny placed the marker on linkage group D of the female-derived molecular genetic map of cassava. A considerable portion of linkage group D is thought to be an introgression from *Manihot glaziovii,* as evidenced from a large number of polymorphic markers, significantly reduced recombination, rare alleles (Fregene et al. 1997). Genetic mapping of CMD resistance genes was by single marker, and interval marker analysis, using the QGene (Nelson,1997) software. CMD resistance data of the half-sib back-cross family, at 6 months after planting, was regressed on the marker genotype classes of SSR marker CSY1, and other RFLP markers on Linkage group D. Results reveal that an RFLP marker, GY59, and CSY1 marker flank a region of group D that explains 50% of the phenotypic variance of CMD resistance ($P<0.0001$). These markers, GY59 and CSY1, are separated by a distance of 15cM, and no other markers have been found between

them. To identify more markers in this region, 64 AFLP primer combinations were evaluated in bulks from the genotype classes of the co-segregating markers, and 2 AFLP markers were found lined to the SSR marker, these markers are being analyzed in the F1 mapping progeny. The frame work map of the region of linkage group D, based on segregating of RFLP and SSR markers in the BC1 half-sib is in preparation. These results support earlier observations (Calvert, 1998 pers comm; Hahn, 1980) that at least one major component of resistance to CMD is due to a recessive gene.

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Future Activities – Extension Request

1. Saturation of the region of the cassava genome bearing the CMD resistance gene with more markers; development of a larger BC_1 cross for fine mapping.

2. Development of a marker-assisted selection scheme to introgress resistance into Latin American and Asian cassava gene pools. This involves developing populations adapted to CMD for the different ecological zones of CIAT's mandate in Latin American and Asia. Genotypes developed by marker-assisted selection will be field tested in Africa to confirm fidelity of the marker.

3. Positional cloning of the resistance gene, using a recently constructed bacterial artificial chromosome (BAC) library.

4. Controlled green house screening of CMD mapping populations, to complement field screening and refine gene mapping results.

Collaborators

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1. SB-02 and Cassava Project; 2. SB-02 Project

1.2.2 Identification of molecular markers liked to genes conferring to whitefly in cassava

Introduction

Whitefly (*Aleurotrachelus socialis*) is one of the most serious pests and disease vectors that affect agricultural production in the world. There are almost 1200 species with a wide range of hosts like legumes, fruit trees and ornamentals where this insect causes major economic losses.

In cassava (*Manihot esculenta* Crantz), whitefly causes between 70 to 80 percent of economical losses. The principal symptoms in the plant are: total chlorosis of the leaves, curling of the apicalk leaves; yellowing and drying of the basal leaves; and stoppage of the plant's development.
The adult insects are found preferentially in the apical zones of the plant where they extract large quantities of the sap from the conductive vessels, causing a considerable damage by loss of vigour. This of course leads to reduced yield. The honeydew which they excrete as a result of the copious sap intake serves as a substrate for sooty mold fungi, which can also damage hosts by preventing photosynthesis.

Materials and Methods

Different sources of resistance to Whitefly has been reported (CIAT, 1995). The most important sources of the resistance genes are: MBra-12 and Ecu-72. These have been used as parentals in the generation of new genotypes. One of the offsprings, CG489-34 has shown the highest resistance to this pest. Some of the very susceptible genotypes were MCol 2026 and MCol 2246. Different breeding populations have been obtained from crosses between the resistant and susceptible genotypes. The cross CG489-34 X MCol-2026 produced 131 individuals

We selected the more contrasting individuals (resistant and susceptible clones) in the field for each family. DNA was extracted from the different individuals and each group with the parental was mixed in a bulk.

We are using molecular markers Amplified Fragment Length Ploymorphisms (AFLPs) and Simple Sequences Repeat (SSR) to find markers associated to resistance for mapping and ultimately cloning of the resistant genes. We are using silver staining to visualize the allelic segregation of the markers.

Results

The resistant and susceptible bulks were evaluated using AFLPs markers. Sixty-four different combinations of AFLPs Gibco Kit were evaluated, and 15 combinations were polymorphic. Among these, 22 polymorphic bands were obtained, i. e. they were only found in the resistant bulk. When the bulk was opened, the bands segregated in all the resistant clones (Fig.1 and 2). Every combination was evaluated in the progeny. These data will be compared with the data from the glasshouse and the field in order to establish a correlation between phenotypic and molecular data. These bands were eluted for sequencing later.

The resistant parentals Ecu-72 and CG489-34 and susceptible parentals MCol 2026 and MCol 2246 were evaluated with 186 cassava SSR markers (Mba et al, In preparation) (Fig. 3, 4 and 5). Approximately 90 SSRs were polymorphic. Several SSR's have been evaluated with F1 (Fig.6) population to confirm its association with the resistance gene and for linkage mapping.

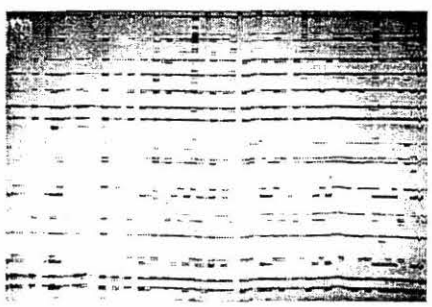

Fig. 1 AFLP AGG-CTC in the F1

Fig. 2 AFLP AAC-CTA in bulks resistant and susceptible

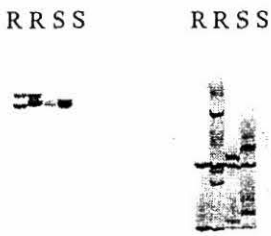

Fig.3 SSR 215 and 300 in parentals

Fig.4 SSR 338 in parentals

Fig.5 SSR 352 in parentals

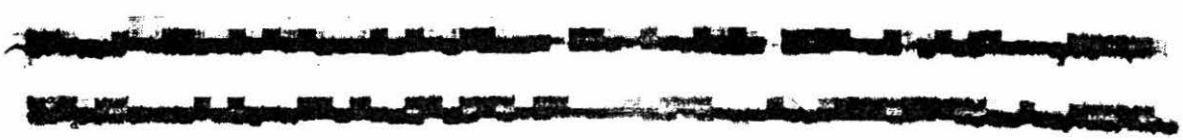

Fig.6 SSR 215 in the F1

⇑ ⇑ ⇑ ⇑
1 2 3 4

1: Ecu-72 ; 2: Mbra12 ; 3: CG489-34 ; 4: Mcol2026

Conclusions and Ongoing Work

- Using AFLPs, we found bands associated with resistance to white fly. These materials will be sequenced. It could be used to generate a Scar for the identification of resistant materials for use in breeding programs. Also with the sequence of the genes of resistance we could establish homologies with the reported ones for other crops, to understand its expression patterns.

- It is planned that with the segreagation data from the SSR evaluations, a linkage map for resistance to white fly will be constructed.

- Another cross using Ecu-72 as the resistant parent and MCol 2246 as the susceptible parent will be made. MCol 2246 has such good attributes as tolerance to other pests like mites and thrips, its flowering is good but is very susceptible to white fly.

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Acknowledgments

We acknowledge the valuable contribution of Fernando Calle in the establishment of plants in the field and glasshouse. We thank Bernardo Arias, for evaluations in the field in CIAT and Nataima and in the glasshouse.

Collaborators

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1.2.3 Mapping of root quality traits and morphological characters in cassava

Introduction

The QTL mapping project of root quality and other agronomic traits at CIAT is a continuation of activities in the construction of a molecular genetic map of cassava and its application to cassava breeding. A molecular linkage map of cassava was developed at CIAT on an F_1 mapping population using RFLPs, RAPDs, and isozyme markers (Fregene *et al.*, 1997). On going efforts are being intensified to saturate the linkage map with microsatellite markers. Their high polymorphism makes microsatellite suitable for the development of linkage maps (Broun and Tanksley 1996). The objective of this study is to identify markers tightly associated with genes controlling root quality, and morphological traits of interest to cassava breeding, as a starting point for marker assisted selection of these traits to increase the efficiency and cost effectiveness of cassava improvement.

Materials and Methods

Field trials and phenotypic evaluation - The F_1 mapping population comprising 144 genotypes, was established in January 1998 at Palmira and Quilichao in a replicated trial, using a partially balanced triple lattice design with three replicates of 12 blocks each. Plots were made up twenty plants per genotype, per replication. A second year trial of the same population using the same design was planted in January, 1999 at Quilichao and Palmira. The early bulking trial to investigate initiation and rate of starch accumulation was set up in December 1998 using 80 genotypes selected from the F_1 population. Based on the result from the first year QTL mapping trial at Palmira in 1998, top forty genotypes and lowest forty genotypes for dry matter yield at 7 MAP were selected. These genotypes were planted in a randomized complete block design with two replications. Starting from 6 WAP, sequential harvesting was done at three-weekly interval up to 30 WAP. Morphological characters (plant height, branching levels height of first branch, leaf shape and length of stem with leaves) were evaluated in October 1998 on 6 non-border plants at 10 MAP. Other traits, including dry matter percent, dry matter yield, starch content, culinary quality, post harvest deterioration were evaluated at 11 MAP using the six central plants of each plot. Post-harvest physiological deterioration was evaluated at 5, 10, and 15 days, scored on randomly selected storage root. Leaf morphology was assessed both as qualitative and quantitative trait. TMS 30572, the female parent of the F_1 progeny has elliptic shaped leaves, while the male parent, CM 2177-2 is linear shaped. Leaf morphology was scored qualitatively based on shape. Quantitative measurement was done using the width/length ratio of the central leaf lobe as trait value. The early bulking trial was evaluated for dry foliage yield, storage dry matter yield, harvest index, storage root diameter, leaf area index, root number, and starch initiation.

Genetic analysis and mapping of agronomic traits in the F_1 population -Analysis of variance of raw data for all traits in each environment was computed with trait values averaged over replications per genotype, considering all effects as random. Broad sense heritability estimates were calculated from variance components. Correlation coefficients across environments for traits were also calculated. For early bulking studies, correlation and multiple regression of yield with other growth related parameters were also done to identify the most paramount characters influencing early bulking. To detect association between markers and QTLs, adjusted trait means, from the ANOVA were used in a simple linear regression of phenotypic data on marker genotype classes, as independent variable, using the computer package Q gene (Nelson, 1997). A region of the genome was considered to be associated with a QTL for any trait if $P < 0.005$.

Results and Discussion

Broad sense heritability estimates (H^2) were high for plant height, first branching height, and branching levels, 78, 91, and 68% respectively, and low for length of stem with attached leaves (48%). Broad sense heritabilty estimates for early bulking, measured as dry matter yield at 7MAP, were 0.6 in Palmira and 0.64 in Quilichao respectively. Three regions of linkage group D were found to bear the most important QTLs for dry matter yield at 7MAP and 11 MAP, dry matter percentage, starch content, branching levels and cooking quality. Together they explain 49 and 37% of dry matter at 7 and 11 MAP respectively in Quilichao. The low level of recombination on linkage group D, relative to the rest of the genome, and the large number of markers on this linkage group suggest it might be an introgressed segment from *M. glaziovii*. A QTL controlling cooking quality was found in the same region as the AGPase small sub-unit gene, suggesting a role for genes involved in starch biosynthesis in cooking quality. Three QTLs linked to RFLP markers GY 120, CDY 131 and GY 138 on linkage groups L, X and U respectively accounted for 8-12% of phenotypic variance of post harvest physiological deterioration. Single point marker analysis revealed a single region of the genome with strong association ($p < 0.0001$) between phenotypic variance for leaf morphology in the F_1 cross and RFLP marker rGY99 on linkage group H of the male-derived linkage map (Fig. 1). The association accounted for 79% of the phenotypic variance. Qualitative scoring revealed that linear:elliptic leaf shape segregated in the F_1 population in the ratio of 1:1. Result suggests that a single major gene control leaf shape in cassava linear shape being dominant to the elliptic shape as in earlier findings (Hershey & Cesar, 1989).

In early bulking studies, for each harvest, correlation coefficient of storage root weight was highly significant ($P < 0.002$) with root diameter, foliage, dry weight, plant height, harvest index, vigor, starch initiation and root number. Multiple regression of dry root yield with these related traits, showed that, only the regression coefficients for foliage and HI index were significant, indicating these two traits as the most highly associated traits influencing bulking, and are therefore of great primary importance for early bulking improvement in cassava.

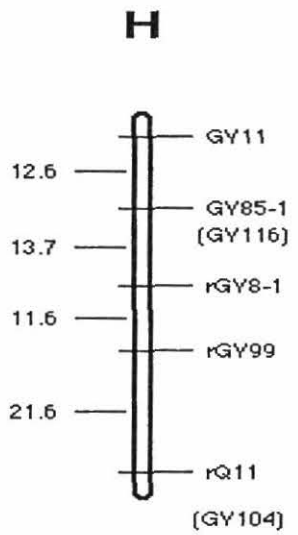

Fig. 1. Linkage group H showing RFLP marker rGY99 linked to leaf morphology

Single regression analysis identified three markers associated with foliage, rCDY74, CDY76 and CDY 131 on the female derived map explaining 25-36% of phenotypic variance (Table 1). For harvest index, markers GY 153 and GY 34 and rBEST-2 (accounts for 15-31% of phenotypic variance) were found associated with this trait (Table1). Markers GY 34 and CDY 131 were also associated with dry matter yield at 7 MAP for dry matter yield in Palmira in 1998. The significant relationship of foliage with yield, underscores the importance of top growth, and thus photosynthetic activity, in assimilate production, while harvest index is a direct measure of assimilate partitioning between the top (source) and the sink strength (bulking). Markers GY 202 and rP3 from the female map were significantly linked to QTLs controlling bulking rate (Table 1).

Table 1. Markers on the genetic map of cassava showing significant linkage to QTL effects for foliage, harvest index and bulking rate from early bulking studies at Palmira, 1999.

Trait	Marker	Chrom	N	F	RSq	P	AA	SE	Aa	SE
Foliage	RCDY74	NgJ	34	18.43	0.3655	0.0002	197.8	25.26	376.74	32.89
	CDY76	NgJ	40	18.42	0.3265	0.0001	210.2	23.49	373.11	30.30
	CDY131	NgX	36	11.54	0.2534	0.0018	371.69	35.35	224.93	23.90
HI	GY153	NgS	77	18.18	0.1951	0.0001	0.13	0.01	0.20	0.01
	GY34	NgJ	64	11.76	0.1594	0.0011	0.40	0.02	0.50	0.02
	RBEST-2	CmA	41	18.14	0.3175	0.0001	0.35	0.02	0.23	0.02
Bul. rate	GY202	NgL	78	12.19	0.1382	0.0008	23.41	1.66	16.71	1.0
	rP3	NgQ	39	10.95	0.2284	0.0021	24.47	2.04	15.44	1.82

On-going activities

- Evaluation of the second year trial of F_1 population for morphological and root quality traits.
- Selection of parent lines for use in crosses to developing plant population for marker fidelity studies.
- Multiplication and evaluation of BC1 for mapping of traits.
- Analysis of generated data.

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Collaborators

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1.2.4 Genetics of field resistance to Cassava Bacterial Blight

Introduction

Cassava has a long breeding cycle (up to 2 years for sexual seeds to sexual seeds). The development of improved cultivars resistant to specific constraints is delayed and the ability of breeders to respond to changes in host pathogen interaction is limited (Lozano and Belotti 1979, Lozano *et al.*,. 1980). Analysis of pathogenic characteristics of *Xam* strains collected from three major ecozones has lead to the definition of different pathotypes, specific to each ecozones in Colombia (Restrepo *et al.*, 1999). These results highlight the importance of developing adequate resistance for the different ecozones.

A previous work reports the mapping of resistance based on evaluation of F_1 population *Xam* symptoms in greenhouse (Jorge *et al.*,. 1999). The aim of this work is the evaluation of CBB resistance under field conditions, taking into account knowledge of the pathogen population structure present in the field and identify the most significant QTLs for marker assisted selection (MAS) and gene cloning.

Material and methods

Plant material and field design. Plant materials were a F_1 progeny of 150 individuals from a cross between TMS 30572 (the female parent) and CM 2177-2 (the male parent) employed in generating a genetic map of cassava (Fregene et al.,. 1997). Plants were first multiplied at CIAT-Palmira, a CBB free area and established at CIAT-Villavicencio during the rainy season successively in 1997 and 1998 in a complete randomized blocks design.

Evaluation of disease resistance. Disease evaluations were conducted during 1997 and 1998. Each plant was evaluated at four and 7 months after planting using a scale from 1 = no symptoms to 5 = dieback of the whole plant. For each genotype, 18 plants were evaluated and the proportions of resistant plants were used for QTL analysis.

Sampling, isolation and characterization of *Xam* strains. A total of 80 strains was isolated from distinct plants and varieties at 7 month after planting in 1997 and in 1998. All isolates were evaluated for aggressivity on the susceptible cassava cultivar MCol 1522 and characterized with a RFLP probe *pth*B. Each unique banding pattern generated by the probe was regarded as an haplotype.

QTL analysis. Two framework maps were used for QTL analysis, female and male derived map. Association between molecular markers and resistance was evaluated comparing the phenotypic means of the two marker classes using a single locus analysis of variance. Total r^2 values from each QTL were calculated.

Results and discussion

Eleven QTLs were detected, but in each evaluation, two to five QTLs were involved in resistance showing that resistance to CBB is polygenic. Changes in QTLs detection between 1997 and 1998 are observed except for one QTL (group D) that remains constant. These changes can be correlated to a change in the *Xam* population structure specially virulence. We demonstrated, in a previous work, where the same mapping population was evaluated in greenhouse inoculating characterized strains (Jorge *et al.*, 1999), that different QTLs control resistance to distinct strains. Resistance evaluation in field confirms these previous results. Special emphasis has to be made on linkage group D. This group were found associated with resistance to two strains in greenhouse (Jorge *et al.*,. 1999), and with resistance in field (1997 at 7 months and 1998 at 4 months after

planting). We think that QTL on group D can be a factor that control horizontal resistance, selected from the initial interspecific cross *M. esculenta*×*M. glaziovii*, original parents in the pedigree of the female parent, TMS 30572.

Although both female parent (TMS 30572) and male parent (CM2177-2) have been reported intermediate resistant, in this study, CM2177-2 shows more resistant plants than TMS 30572. This observation is correlated with the finding of highly significant QTLs associated to molecular markers of the male framework map. Furthermore, the changes in resistance behavior suggested that an interaction between pathogen and host plant may exist. This implied that a or several specific resistance factor against particular *Xam* strains are not effective with other strains, so resistance breakdown when the pathogen population change.

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Collaborators

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1.2.5 Mapping genetic resistance to Cassava Bacterial Blight using a backcross population

Introduction

Genetic of resistance to cassava bacterial blight has been recently studied using a F1 population from a cross between two elite varieties (TMS 30572 and CM 2177-2). It appears to be controlled by several genes and to be strain specific (Jorge *et al.*, 1999). A half sib family of over 200 seeds derived (BC1) from a backcross of five fertile hybrids from the F1 cross, to TMS 30572 was produced at CIAT in 1995. The objective of the present study was to characterize the BC1 progeny at molecular (RFLP and microsatellites) and resistance level. BC1 population will provide additional opportunities for recombination betweeen markers and resistance genes, and among genes of resistance.

Material and methods

Material. The plant material used in this study was BC1 population which pedigree is as follows:

Original mapping population	*no. of genotypes*
CM 7857 (TMS 30572 x CM 2177-2)	150
Backcross population BC1	
CM8820 (TMS 30572 x CM7857-04)	90
CM8870 (TMS 30572 x CM7857-10)	15
CM8872 (TMS 30572 x CM7857-51)	15
CM8873 (TMS 30572 x CM7857-77)	81
CM8877 (TMS 30572 x CM7857-115)	11

Five strains of *Xam* belonging to different haplotypes and collected from different ecozones were used.

Molecular characterization. We selected RFLP markers from the framework map developped with F1 population (Fregene *et al.*, 1997) for genotyping. DNA extraction, southern blot and hybridization were as previously described (Fregene *et al.*, 1997))

Evaluation of resistance. In greenhouse, eight plants per genotype and per strain were evaluated at seven, 15 and 30 days after inoculation by puncture following a scale previously described. Disease ratings at the three dates were used to calculated area under the progress disease curve.

Results and conclusions

A total of 56 RFLP probes has been hybridized on BC1 progeny. Construction of framework map is underway using computer program Joinmap.

Distribution of disease reaction of BC1 progeny to the five *Xam* strains is presented in figure 1. Distribution are quite normal except for ORST X-27 strain. The continous variation of AUDPC values shows that a large number of genetic factors controlling resistance are segregating.

Correlation between resistance values to different strains are shown in table 1. Values are not high suggesting that BC1 progeny is reacting differently to the different *Xam* strains. This difference has been also observed in the mapping of CBB resistance using F1 progeny.

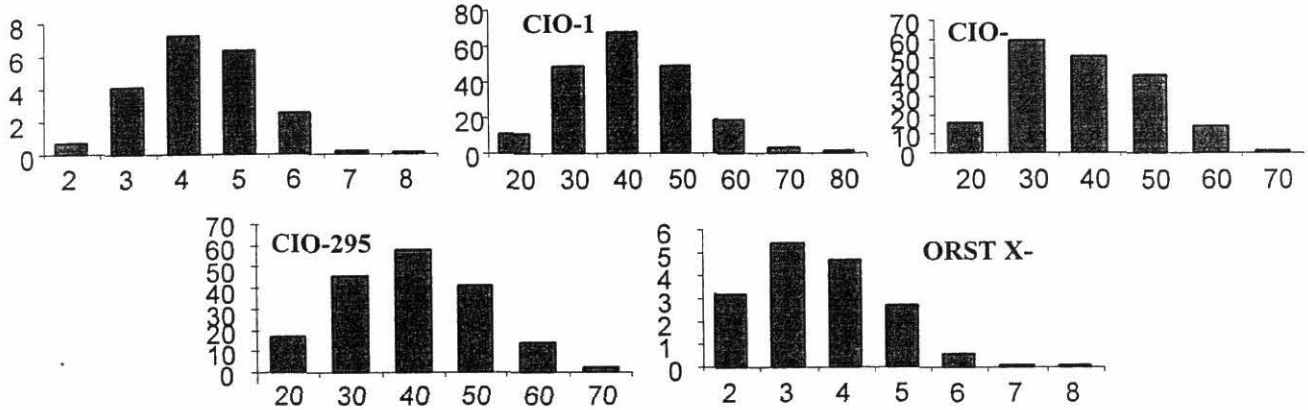

Fig 1. Distribution of AUDPC values for the BC1 progeny

Table 1. Correlation coefficients calculated between AUDPC value for each strain inoculated

	CIO-84	CIO-1	CIO-136	CIO-295
CIO-1	0.388592			
CIO-136	0.425779	0.379284		
CIO-295	0.279316	0.389862	0.396563	
ORSTX-27	0.3726	0.314638	0.317532	0.460686

Future activities

These will include, first, the construction of a new framework map analyzing the segregation of RFLP and microsatellites markers and, second, the mapping of the CBB resistance with this backcross population.

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Jorge V, Fregene MA, Duque MC, Bonierbale MW, Tohme J and Verdier V (1999). Theor Appl Genet, *in press*

Collaborators

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1.2.6 Characterization of a new segregating population CM 6744 based on resistance to *Xanthomonas axonopodis* pv *manihotis*

Introduction

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is a major disease of cassava. Planting resistant varieties is the preferred method of disease control. Genetic mapping of an F_1 cross (TMS 30572 × CM 2177-2) led to the construction of the first molecular genetic map of cassava. Recenlty, Jorge *et al.,*. (1999) report molecular markers associated to putative genes that control CBB resistance in cassava. Individuals of the F_1 cross were evaluated for CBB resistance to five strains of *Xam*. Eight QTLs (quantitative trait loci), located on linkage groups B, D, L, N, and X of the female-derived framework map, were found to explain 9 to 20% of the phenotypic variance. With the male-derived framework map, four QTLs on linkage groups G and C explained 10.7 to 27.1% of the variance. They suggested that several genes are probably required for resistance to CBB. Another study mapped a cassava homolog gene from the *Xa21* gene in rice, and it showed significant association with resistance to one *Xam* strain (Bonierbale *et al.,.*, 1997). The objectives of this study were to add more polymorphic markers associated with resistance to CBB to the actual cassava map using a new cross (CM 6744) and to characterize the genes involved in resistance.

Materials and Methods

Plant materials. Plant materials used in this study were the 115 individuals of the F_1 progeny (CM6744) derived from the cross (MCOL1522 female, and SG107-35 male). The F_1 progeny was multiplied *in CBB-free fields at CIAT (Palmira, Colombia) in 1998.*

Evaluation of resistance. Five strains of Xam (CIO 1, CIO 84, CIO 136, CIO 295 and X-27) that were previoulsy described (Restrepo et al.,., 1997) were selected to assess the resistance of the F1 progeny and the parents. The inoculation was made by stem puncture and three plants by individual were inoculated with each strain. Disease severity was rated on a 0 to 5 scale. Plants with a mean disease reaction of ≤ 3 were grouped as resistant, while those with a disease reaction of > 3 were classified as susceptible.

DNA Extraction. The DNA was extracted from young leaves triturated in liquid nitrogen, following the Gilbertson-Dellaporta protocol (Gilbertson-Dellaporta *et al.,.*, 1983) with some modifications. The DNA is quantified using a Fluorometer and diluted to 10ng/uL. DNA was extracted from the parents and from a bulk of 9 resistant and 9 susceptible F1 individuals.

Polimerase Chain Reactions and PAGE's. The PCR was held in a final volume of 25 uL, and the conditions for each reaction were:

Buffer (1X), $MgCl_2$ (1.5mM), dNTP's (0.05mM), Forward Primer (0.12uM), Reverse Primer (0.12uM), Taq polimerase (1 unit). A bulk of resistant and a bulk of susceptible progeny, along with the parents, were amplified with 186 different pairs of primers designed for microsatellites in cassava, Cassava MapPairs (Mba,C., unpublished). Each reaction was run in a polyacrilamide gel electrophoresis (PAGE), for 1 hour and 45 min; and stained using the silver staining process.

Results

The results from the resistance test showed MCOL1522 as the susceptible parent and SG107-35 as the resistant one. 8 individuals in the F1 progeny were resistant to all strains, while 25 were susceptible to all strains. We chose randomly 9 from the susceptible ones and all the 8 resistant plus another that was resistant to four Xam strains. Bulk of resistant and susceptible genotypes were used to amplify with the microsatellite primers, as a preliminary test.

Among the 186 pair of primers only two showed polymorphism between the two parents and also between the bulks of progeny. The pattern composed of 6 to 10 bands, were identical for both the resistant parent and resistant bulk. Same results were obtained with the susceptible ones (parent and bulk). We found 34.4% of polymorphism between the parents, but these polymorphisms did not necessary applied to the bulks of the progeny. This polymorphisms can be use to build a map for this new population, but only the ones that presented marked differences between the bulks showed strong association with the phenotipic character of resistance to *Xanthomonas axonopodis*.

Ongoing activities

We are extending the microsatellites analysis to the entire progeny with the primers that showed interesting polymorphism in the preliminary test.

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Collaborators

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1.2.7 Characterization of Resistance Gene Analogs (RGA) amplified with degenerate primers in Cassava

Introduction

Cassava (*Manihot esculenta* Crantz) is a major food crop for most of the tropical regions of the world. One of the most serious diseases of this crop is Cassava Bacterial Blight (CBB) which is caused by *Xanthomonas axonopodis* pv *manihotis*. Some improved cassava varieties are highly to moderately resistant to the disease, suggesting the presence of resistance genes (R). In an attempt to isolate R-gene candidates, we have used PCR amplification with degenerate primers designed from the conserved domains: a) NBS (Nucleotide Binding Site) of the resistance proteins L6 (Flax), RPS2 (Arabidopsis) and N (Tobacco), and b) LRR (Leucine Rich Repeats) and PK (Protein Kinase) from the resistance gene *Xa*21 of rice.

Materials and Methods

DNA was extracted from leaf tissues of two genotypes, NGA-2 and CM2177-2, both of them showing partial resistance to the disease and the F_1 individuals from this cross.
PCR reactions were performed using different degenerate primer combinations (Table 1). PCR-purified products were cloned into the pGEM-T easy vector system (Promega) and transformed into *E. coli* electrocompetent cells. Seventy clones of each combination were randomly selected and grouped based on restriction patterns. About two clones of each group were hybridized as RFLP probes in the parentals (NGA-2 and CM2177-2). Polymorphic probes were evaluated for segregation in the F_1 individuals from this cross. At least two clones of each group were sequenced using the Big-Dye Terminator Cycle Sequencing kit and Applied Biosystems Prism 377 DNA Sequencer of Perkin-Elmer, USA.

Results

With primer combinations I and II (Table 2), it was possible to amplify a single band of about 500 bp, that shows high homology with RGA from Arabidopsis and sunflower. We obtained four different classes of RGA from both combinations using two criteria: sequence homology percentage and hybridization pattern on DNA blots. None of the RGAs showed polymorphism so they could not be mapped.

Using primer combination III (Table 2), we amplified two bands, 450 bp and 400 bp respectively, showing homology with RNA helicases but not with resistance genes. Seven groups were formed according to restriction patterns. We chose one member of each group that showed polymorphism (four in all) when evaluated by RFLPs in parental blots, to map the group. Two of them mapped in linkage group **V**, near markers associated with root quality and earliness. The other two probes mapped in group **B** linked to a CBB resistance marker to the *Xam* strain Cio-136. Although these probes show no homology to RGA, they were useful as new markers in saturating the cassava molecular genetic map.

Table 1. Primers designed from the conserved domains of resistance genes L6, N, RPS2 and Xa21.

Domain	**Primer name**	Sequence peptide/nucleotide	Reference
		G G V G K T T	
NBS	S1	GGT GGG GTT GGG AAG ACA ACG	
	S2	GGI GGI GTI GGI AAI ACI AC	Leister et al., 1996
		G L P L A L	
	AS1	**CAA CGC TAG TGG CAA TCC**	
NBS	AS2	**IAA IGC IAG IGG IAA ICC**	
	AS3	**IAG IGC IAG IGG IAG ICC**	Leister et al., 1996
	AS4	**IAG IGC IAG IGG IAA ICC**	
	AS5	**IAA IGC IAA IGG IAA ICC**	
		I S/P N/L N/L F/S S/L G/S H-Q I-M	
	LRR1	**TCA AGC AAC AAT TTG TCA GGI CAI ATI CC**	Ronald,P
LRR	LRR2	**CTC GCG CTG CTC TCT CTT TCA A**	Taylor, N
	LRR3	**P N S F S N L F**	Taylor, N.
		TGA AAA GAT TGG AAA ACG AAT TAG G	
		Q-H R D/E N/K L S S N V L L	
PK	PK1	**TTA CAG CAC ATT GCT TGA TTT IAI ITC ICG ITG**	Ronald, P.
	PK2	**TAA CAG CAC ATT GCT TGA TTT IAI ITC ICA ITG**	
	PK3	**TAA CAG CAC ATT GCT TGA TTT IAI ITC ICT ITG**	

Amplification products from combinations IV and V (Table 2) show no homology with the domains from which the primers were designed. Besides, they were monomorphic when evaluated in DNA blots from parentals.

Table 2. Successful amplifications

	Combination	AT (C°)	Bandsize (bp)	Homology
I	S2-AS3	46	500	RGA
II	S2-AS4	46	500	RGA
III	S1-AS2	42	450-400	RNA Helicase
IV	LRR2-LRR3	42	650	Ribosomal protein
V	LRR1-PK1	42	750	Reverse transcriptase

On going activities

1) Mapping RGAs using blots with different restriction enzymes in order to obtain polymorphism.
2) Evaluation of new primer combinations from different conserved domains (TIR, Motif1) in known resistance genes.
3) Use of a cDNA library to get full coding sequence of isolated RGAs.
4) Use of a BAC library to do physical mapping of RGAs.

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Collaborators

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1.2.8 Implication of Wound-Response Genes the Postharvest Physiological Deterioration (PPD) of Cassava *(Manihot esculenta* Crantz*)*

Introduction

Post-harvest physiological deterioration (PPD) of fresh roots once detached from the plant is rapid (24-48hrs). The process of deterioration begins 2 or 3 days after the harvest, followed by a microbial deterioration between the next 5 to 7 days. The mechanism of response of plants to lesions and attacks of pathogens include several biochemical responses, which are common to a wide range of plants. These include an increase in the synthesis of some proteins, changes in the composition of the membrane lipids, and production of ethylene (Beeching et al. 1997). The correlation of post-harvest deterioration in cassava with these genes will allow indirect selection in breeding programs for different traits related with stress.

The objective of this collaborative project with Bath University and CIAT is to transfer the existing knowledge and the molecular tools developed in other species to cassava, so making use of the linkage map developed at CIAT to identify molecular marker linkages to QTLs for PPD.

Methodology

An F_1 population developed at CIAT for molecular linkage map of cassava (Fregene et al. 1997) was used for the evaluation of PPD in replicated trials and across environments, and for QTL analysis of PPD. The mapping population of cassava consists of 150 F_1 plants from a cross between "TMS 30572" and "CM2177-2". The phenotypic evaluation for PPD was carried out in two different environments: CIAT Palmira and the CIAT Station at Quilichao. These evaluations are based on the methodology of Wheatley (1982). The cDNA probes for 12 specific genes expressed during wound response have been used for hybridization.

For RFLP analysis the genomic DNA was isolated from fresh, young tissue of cassava leaves from parents and the 150 individuals of mapping population, the extraction was carried out according to Dellaporta et al. (1983). The restriction analysis, southern blotting and hybridization with parents and progeny were performed as Fregene et al. (1997).

Only those polymorphic RFLPs that were obtained from Southern hybridization of the probes in the parents were used for linkage analysis and scored in the progeny. Markers were placed onto the existing map of cassava using the linkage analysis computer package MAPMAKER (Lander et. al.1989) on a Macintosh G3 computer.

Results and Discussion

Phenotypic evaluation was carried out in two environments, CIAT Palmira and the CIAT station at Quilichao. The frequencies of distribution for the phenotypic values are shown in Figure 1. For Quilichao the observed distribution is asymmetric on the left, presenting a high frequency of low values between 0- 5%. A normal distribution was found for this cross from similar data collected in Palmira. The data sets from Quilichao were adjusted to a normal distribution for the ANOVA and heretability analysis through a log transformation.

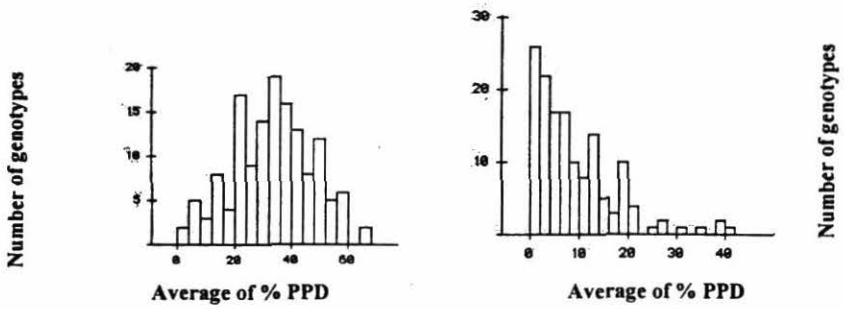

Figure 1 Histogram of average for PPD by genotype in CIAT Palmira (left) and CIAT Quilichao (right) 5 days after.

Of the 12 PPD genes surveyed in the parents of the mapping population, 9 namely: HRGP1, GLU, MEPX, ACCOX1, RNA, MEPAL, PAL, PK, CPI and ASP were polymorphic with at least one of the five enzymes employed in the parental survey. Only 7 segregate in progeny and have been include in the map. The linkage analysis for placement on the existing map of cassava has been by the MAPMAKER program (Lander et al. 1989). Phenotypic evaluation of PPD data was regressed on the genotypic classes of the molecular markers using the single point and interval analysis of the computer programs: Q-Gene (Nelson, 1995) and significant QTLs are present in table 1.

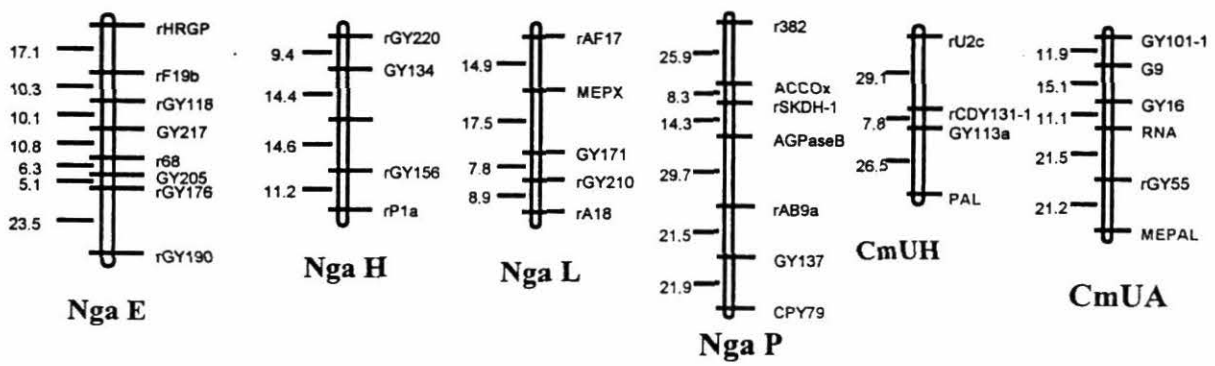

Figure 2 Linkage groups with new molecular markers for specific genes expressed during PPD.

Table 1 Markers associated with PPD according to analysis in SAS and QGENE

Marker	ent	Linkage Group	Parent Source	R^2	Probability	Value
GY138	Quilichao	NgU	Nga2	0.12	0.0000	5.36
rGY164	Quilichao	NgU	Nga2	0.09	0.0004	4.97
rNI1.C2	Quilichao	CmC	Nga2	0.13	0.0008	4.94
rSSR83	Palmira	NgP	Nga2	0.09	0.0012	11.18
CDY131	Palmira	NgX	Nga2	0.13	0.0025	13.15
rK16d	Quilichao	NgG	CM2177-2	0.11	0.0026	-5.47
GY120	Palmira	CmL	CM2177-2	0.11	0.0028	-11.91
SSR6	Quilichao	NgU	Nga2	0.08	0.0032	4.11
SSR6	Palmira	NgU	Nga2	0.07	0.0045	9.29
GY202	Palmira	NgL	Nga2	0.05	0.0057	8.02
rM5a	Palmira	NgX	Nga2	0.10	0.0070	11.73
rGY22-1	Palmira	CmA	CM2177-2	0.09	0.0078	-11.2
AC-1	Palmira	NgM	CM2177-2	0.10	0.0082	-10.95
rGY26	Quilichao	CmC	Nga2	0.09	0.0082	3.89
rE14b	Palmira	NgJ	CM2177-2	0.10	0.0091	-11.76
rHRGP	Palmira	NgE	CM2177-2	0.05	0.0094	-7.83
CDY123a	Quilichao	CmE	CM2177-2	0.09	0.0094	-5.1

Ongoing Work

- *In situ* detection and localization of reactive oxygen species (ROS)

  a. SUPEROXIDE DETECTION –timecourse experiment on triplicate root samples of cultivars Mcol22, MNga2, CM2177-2, MDom5. Experiment indicates a transient oxidative burst in response to wounding.
  b. HYDROGEN PEROXIDE DETECTION –timecourse experiment on replicate samples of cultivars Mcol22, MNga2, CM2177-2, MDom5. Timecourse was carried out over 5 days in order to study H2O2 production during PPD.

- TISSUE PRINT DETECTION + LOCALIZATION OF ENZYME ACTIVITY
  a. Catalase
  b. Peroxidase

  Timecourse experiment carried out on triplicate root samples of cultivars Mcol22, MNga2, CM2177-2, MDom5.

- RNA EXTRACTIONS. RNA extracted from cultivars Mcol22, MNga2, CM2177-2, MDom5 as below:
  1. Young leaf
  2. Mature leaf
  3. PPD time course of roots for the first 6 days after harvest.

4. Roots from pruned plants (pruning 2 weeks before harvest) and control non-pruned plants harvested at the same time.
5. Roots of each cultivar treated with Ethephon (Ethylene generating compound) salicylic acid +Jasmonic acid.

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Collaborators

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1.2.9 Marking of QTL for low P tolerance in bean

Background

Over the past ten years we have identified germplasm with superior yielding ability under low phosphorus (P) stress. The utilization of the genes in this superior germplasm would be facilitated greatly by the existence of molecular markers that permit the recognition of favorable alleles in segregating populations or advanced lines. The present work was undertaken to find markers for yield genes under P stress and also under P-nonlimited conditions.

Materials and Methods

A population of Recombinant Inbred Lines (RILs) was developed from the cross of BAT 881 x G21212. BAT 881 is a bred line with very high biomass production but poor yield under P stress. G21212 presents excellent yield under P stress and appears to have good partitioning of dry matter to seed in these conditions. A total of 96 RIL were evaluated for yield in two years. In 1997 and 1998, the population was planted under P-nonlimited conditions, and in 1998 under P-stress as well. Three repetitions were used in a 10 x 10 lattice design. RAPD were run on the RILs, first on the extreme lines (best and worst yielding under P stress), and then on the entire set of RILs using primers that generated RAPD that appeared to be associated with one or the other group of extreme lines. Linkage groups were established using MapMaker and QTL analysis was carried out with the Q-gene program.

Results

QTL for yield were found for both the high and low P treatments, although in most cases these were not the same. That is, independent genes controlled yield at different P levels. At high P BAT 881 contributed more genes to yield, while at low P genes from G21212 were more important. Linkage group 3 was particularly important at low P, and in fact, a long segment in group 3 of more than 80 cM appeared to carry QTL for yield. The entire segment taken as a whole accounted for more than 300 kg/ha at low P - a remarkable effect in very difficult production conditions. On the other hand, QTL from BAT 881 were not only identified but confirmed in two seasons. These should be pursued for their possible contribution to yield potential under optimal production conditions.

Table: QTL for yield at high and low P in a population of RIL of the cross BAT 881 x G21212

			Phenotypic value of marker (kg/ha)		
Marker	Source of positive allele	Linkage group	High P, 97	High P, 98	Low P, 98
E406	BAT 881	4	324	458	NS
V401	G21212	15	235	461	141
AK1201	BAT 881	Unlinked	218	411	NS
Q1702	G21212	3	NS	NS	237
H1801	G21212	11	NS	NS	153
L1102	BAT 881	13	NS	NS	131

Conclusions

QTL with quite large effects were identified in both BAT 881 and in G21212. However, different QTL were expressed under different P levels. This implies the need to stratify MAS for different production systems. The effects of stress-yield QTL from G21212 were surprisingly large, and these QTL need to be pursued to the benefit of resource poor farmers. Last year we reported on QTL for root traits that affect P uptake, and those QTL should be complementary to those that we now report. Combinations of QTL should offer a significant advance in abiotic stress tolerance. The QTL for high input agriculture

were also substantial in their effects and could make a significant contribution to yield potential.

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Collaborators

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1.2.10 Progress towards the development of SCAR markers for multiple traits: root phenotype, disease resistance, low phosphorous tolerance, biological nitrogen fixation and yield.

Rationale

Last year we reported the location of QTLs for root traits, BGMV reaction and resistance to other diseases on the CIAT mapping population (DOR364 x G19833). In addition we reported the tagging with RAPDs of QTLs for low phosphorous tolerant biological nitrogen fixation (BNF) and yield under phosphorous deficiency in two populations of recombinant inbred lines (DOR364 x BAT477 and DOR364 x G3513). The utilization of these QTLs would be facilitated greatly by the existence of easy-to-use PCR based molecular markers such as the sequence characterized regions (SCARs) that were developed previously at CIAT for selection of resistance genes. The purpose of the present research was to clone the RAPD bands that were linked to the traits identified in the previous studies and to sequence the resulting plasmids, in preparation for the development of primers to amplify SCAR markers.

Materials and Methods

A total of 21 RAPD bands were targeted for cloning (Table). The appropriate RAPD reactions were run for the parents of the populations of interest. The selected bands were excised from low melting point agarose and purified. Where the polymorphism was dominant a single band was cloned, while two bands were cloned for co-dominant polymorphisms. The excised bands were re-amplified with the original primers, run in a preparative gel and purified again, before cloning into the pGEMeasy plasmid vector (Promega). The ligation reaction was transformed into electro-competent host cells (DH5alpha) and the bacteria were plated onto selective media. For each cloning reaction five white colonies were picked and minipreped. The miniprep DNA was sized with standard technique and used in PCR reactions with the original RAPD primer. Clones that amplified a correctly sized band with the RAPD primer were selected. Any positive clones that had similar size inserts, were further characterized by fingerprinting with frequent-cutter (4 bp recognition site) restriction enzymes and grouped based on the pattern of restriction fragments. Duplicate clones based on these assays were discarded. The unique clones were selected for sequencing. Clones were prepared as bacterial stabs and sent to Cornell University. There, they were grown overnight in LB media containing the appropriate antibiotic. Plasmid DNA was extracted from the cell pellets by a Qiagen robot and sequenced on an ABI377 automated sequencer with standard chemistry. The plasmid inserts were fully sequenced by using the T3, T7 or Sp6 universal primers. The software DNAsis was used to join the overlapping portion of the two end sequences of a given clone and to search for homology of the sequences to each other using so as to determine which were duplicates. In addition the sequences were compared to entries in the public database using the BLASTn and BLASTx searches at the NCBI website.

Band	Mol. Weight	Origen	Chrom.	Inheritance	Trait selected for
A0402	350	G3513	DG1	Dominant	Yield
A0401	450	BAT477	DB2	Dominant	Yield
U1801	500	BAT477	DB8	Dominant	Biological Nitrogen Fixation
X0903	500	BAT477	DB4	Dominant	Biological Nitrogen Fixation
U1402	590	DOR364	DB9	COD	Biological Nitrogen Fixation
U1403	595	BAT477	DB9	COD	Biological Nitrogen Fixation
P0302	620	DOR364	DB2	Dominant	Yield
U1304	640	BAT477	DB4	COD	Biological Nitrogen Fixation
U1303	650	DOR364	DB4	COD	Biological Nitrogen Fixation
AA1906D	610	DOR364	B11J	Dominant	BGMV
AN0107D	495	G19833	B04A	COD	BGMV
AN0108D	490	DOR364	B04A	COD	BGMV
G161G	1500	G19833	B10I	Dominant	ALS
G185D	1350	DOR364	B04A	Dominant	BGMV
H201G	1230	G19833	B10I	DOM	Phosphorous
H204G	510	G19833	B10I	DOM	ALS
P076G	300	G19833	B03C	DOM	Anthracnose
V103G	520	G19833	B09K	DOM	Phosphorous

Results

We successfully cloned most of the RAPD bands that were targeted, resulting in a total of 46 different clones being isolated. The sizes of the cloned bands ranged from 350 bp to 750 bp. Three pairs of co-dominant bands were cloned from both parents of a given population, while the remainder of the bands were dominant and could only be cloned from a single parent. Of these, clones have been fully sequenced. Two sequencing reactions, using universal primers, were sufficient to get full-length reads on all the clones. A few of the clones from the same ligation reaction proved to be duplicates, while in other cases, clones with the same size insert had different sequences. In BLAST searches, none of the sequences had homology to enzyme encoding genes. However a few of the cloned sequences contained parts of a putative pol/gag polyprotein found in *Arabidopsis thaliana* and in one case a segment of the 26S ribosomal rDNA genes. The homology of RAPD bands with the polyprotein, a typical component of plant retrotransposons, was interesting because by chance one would not expect to find this sequence represented in more than one or two clones. It is more likely that RAPD primers target retrotransposons preferentially. This could occur, because the inverted repeat sequences of nested retrotransposons, which are common intra-genic elements in the genomes of higher plants, provide the template for RAPD amplification to occur. RAPD primers require oppositely oriented, repetitive sequences within a distance that can be amplified by a typical PCR reaction and this situation occurs perfectly with nested retrotransposons. This would explain why some RAPD primers amplify many bands or many polymorphisms while others do not – because each primer may identify a different family of retrotransposons. This would also explain why RAPD polymorphisms tend to differ between gene pools – also because retrotransposon sites are ancestral and divergent between gene pools. The cloning and analysis of a large number of bands will allow us to determine the extent of the relationship between RAPD markers and retrotransposons and the evolutionary significance of this association.

Conclusions and future work

The RAPD bands that were cloned and sequenced in these experiments will be used to develop primer pairs that amplify SCAR markers. Where various sequences were found for a given band and in the case of the co-dominant bands more than one primer pair will be made for a given SCAR marker. We have approximately ten more RAPD bands that have been targeted that will undergo a similar process. Once primers are developed for these clones they will be tested for amplification and mapped in collaboration with Paul Gepts at University of California – Davis during the upcoming year. We will confirm the mapping at CIAT using the original population from which the bands were identified to see whether the SCARs map to the same position as the original RAPD bands from which they were derived. We hope to have at least 30 new SCARs by the end of the grant period. The final goal of this project is to disseminate and use these SCAR markers for marker-assisted selection in the Mesoamerican and Andean breeding programs at CIAT and in the bean programs of the NARs. Because SCARs are single copy markers that produce a single band for each allele reflecting a single occurrence of that sequence at a

single locus in the genome, they are very useful for marker assisted selection of linked traits. .

Collaborators:

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1.2.11 Pilot project to establish a high througput marker assisted selection facility at CIAT

Introduction

In 1994 the molecular marker lab was established within CIAT as a service to commodity research under the philosophy of maximizing the utility of investment in laboratory infrastructure through sharing a common facility which was esentially under the auspices of the users. This was a departure from the model used in other institutions in which biotechnology is isolated from users both administratively and technically in an independent unit. This model has been productive in bringing biotechnology to bear on individual crops, and we feel that we have been successful in our effort at gene (s) tagging and fingerprinting. Molecular markers have already been identified as possible markers linked to several agronomical important traits. Some of these markers are already being used by breeders in the private sector.

Several meetings were organized this year to discuss marker assisted selection (MAS) activities at CIAT. All relevant CIAT breeders, geneticists and the respective project leaders attended the meetings. A detailed analysis was made of the progress achieved and the future activities as it related to the four CIAT crops. A list was prepared of the different steps needed to advance from the stage of gene tagging to one of fully operational markers assisted selection programs at CIAT.

Based on the analysis made by the group, we initiated this year, with the help of the strategic fund ,a pilot project to establish of a "factory - assembly line" routine molecular markers lab for CIAT projects. The pilot project started this year mainly on bean and to some extend on Brachiaria during the last quarter of 1999

Bean Marker Assisted Selection

Bean golden mosaic virus (BGMV) is a devastating disease of common bean (*Phaseolus vulgaris* L.) in Latin America. The disease is caused by a geminivirus transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Gálvez and Morales, 1989). Most common

symptoms include a brilliant yellow coloring starting in leaf veins, a marked rugosity and distortion on the leaves, a malformation in pods and reduction in seed size and weight. Highly resistant breeding lines have been generated and genetic resistance to BGMV has been effective in controlling the disease (Beebe and Pastor-Corrales, 1991). The most important gene for the resistance to BGMV is the recessive gene *bgm-1* derived from the dry bean cultivar 'Garrapatos'. A RAPD marker tightly linked to *bgm-1* has been identified in this Mexican landrace (Urrea et al., 1996). This RAPD was converted to a *SCAR* at Ciat (S. Beebe and F. Pedraza, personal communication) and was designated DR21. The RAPD marker generated by the primer R2, is codominant, and generates two DNA fragments, a 570bp and a 530bp. The latter is found in highly resistant sources such as Garrapatos, A429, Tío Canela 75 and FEB 212. The main objective of this work is to use marker assisted selection as an efficient tool for breeding improvement.

Materials and Methods

All the methods, such as DNA extraction, PCR and electrophoresis were adjusted in order to screen 3000 samples groups before flowering time. This was accomplished in three separating plantings in February, June and July 1999.

Plants were labeled in the field approximately 16 days after planting (DAP). Young leaves were placed in plastic envelopes (17-18 DAP) and stored at 4°C for no more than one week.

A 6 mm (5mg) diameter leaf tissue disc per plant has been cut and organized into microtiter plates and then stored at –20°C. It was important that samples fit down into the bottom of a well of the microtiter plate for the next steps. An alkali treatment was used for large scale DNA extraction (Klimyuk, 1993) as follows: 40µl of 0.25M NaOH were added to each sample and then incubated in a water bath for 30 sec at 100°C. The tissue was macerated with polypropylene pellet pestles and then neutralized by the addition of 40 µl of 0.25M HCl and 20 µl of 0.5M Tris-HCl pH 8.0 before boiling for 2 additional minutes. 5µl of a 1:1 dilution in sterile water were used for PCR (1h15min duration). Samples were loaded four times per gel in order to save time. After electrophoresis, the presence of DOR 21 was scored in F_1 plants.

Results

A total numbers of 8000 plants derived from 112 F_1 multiple crosses and 800 advanced lines were screened for *bgm-1* marker during 6 months. For this purpose the nursery was divided in three groups, since the most that we could handle was 3200 plants at a time.

The *bgm-1* SCAR was successfully used in indirect selection for resistance to BGMV (Figure 1) and marker segregation was according to the expected ratios in each cross.

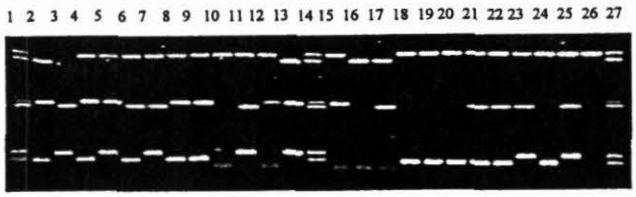

Fig. 1. Occurrence of bgm-1 SCAR in F1 multiple cross plants. Lanes 1,14 and 27 correspond to PCR products of a susceptible (DOR21570) and a resistant (DOR21530) variety used as size market. *In the third row bands* appear in lane 24 from Tio Canela 75 (Bred line resistant to BGMV) and lane 25 from Orgulloso (BGMV susceptible variety

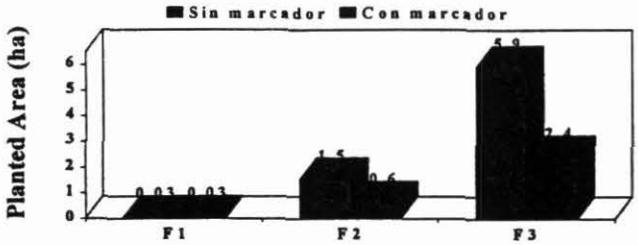

Figure 2. Efficiency of screening for BGMV with and without a molecular marker. Estimate of saving in terms of planting area when comparing the advances of populations without and with molecular marker selection using the SCAR for *bgm-1* .

Results were given to the breeder approximately 34 DAP and multiple crosses were made using only the plants having the *bgm-1* marker. Therefore breeding effort was reduced approximately in 60% due to marker assisted selection. For the advanced lines, 194 lines (24%) expressed the SCAR. Lines to de sent to Central America were selected among this reduced group (Fiure 2)

Table 1 shows the steps followed in *bgm-1* screening and the time spent on each one. Data correspond to the screening of 3000 plants. A comparison can be made between two trials in terms of the number of persons involved in each task and the time they spent. Briefly, 3000 plants were screened in the second trial in 16 days, four days less than in the first trial. This was basically due to having planted populations by hand at a regular space. Also we have found that the extracted bean DNA held at –20 °C is stable for at least three months.

Table 1. Large scale screening of *bgm-1* SCAR.

Task	1st Trial		2nd Trial	
	No. Persons	Time (days)	No. Persons	Time (days)
Preparation of stickers, envelopes, labels	4	2	4	2
Labeling plants in the field	6	1	3	2
Field sampling	12	1	10	0.4
Samples organization in Elisa plates	2	5	3	2
DNA extraction	2	3	2	2
DNA dilution	2	1.5	1	2
PCR	2	2	1	2
Electrophoreses	2	3	2	3
Reading gels	2	1	1	0.5
Total Time		19.5		15.9

On going activities in bean

- The cross ICTA OSTUA x (VAX 1 x TIO CANELA 75) was selected for the confirmation of the usefulness of marker assisted selection. Two hundred F_2 plants were screened for *bgm-1* SCAR. F_3 families will be tested for resistance to the virus in the greenhouse in order to compare the reaction of the families with and without the marker.

- Attempts to use marker assisted selection for other crops are being carried out. A *Brachiaria* breeding population is in screening for a SCAR linked to the apomixis locus. Some effort in the development of a rapid DNA extraction is currently been done.

On going activities in *Brachiaria*

5. Develop a rapid DNA extraction protocol for *Brachiaria*

6. Validate, in a "real life" *Brachiaria* breeding population, the SCAR marker derived from RAPD N14, which is linked to the apomixis locus.

7. Screening a population of approx. 95 hybrid clones obtained from crosses between clones selected in 1997 from the sexual breeding population and elite apomictic genotypes including both accessions and hybrid-derived clones. We plan to transplant them to a space-planted field nursery here at CIAT. This nursery will supply: leaf tissue for determination of the SCAR marker; spikelets for embryo sac analysis (ESA); and open-pollinated seed for a subsequent progeny trial.

8. Determination of reproductive mode phenotype by ESA combined with progeny testing

Collaborators

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Acknowledgments

We acknowledge the valuable contribution of Henry Terán in the establishment of plants in the field and the coordination of planting and sampling.

1.2.12 Identification of putative resistance genes in common bean

Background

The use degenerate primers designed based on sequence similarity of conserved motif between cloned disease resistance genes allowed us to isolate NBS-like sequences (BRU Annual Report, 1998). We have pursued this strategy by using new primers designed by Leister et al. (1998) to amplify the same NBS domain. We have also designed a downstream NBS primer (Motif1) and another primer combinations targeting the TIR (Toll-Interleukine 1-Receptor) domain that has not been used to this purpose before.

Materials and Methods

DNA was extracted as described earlier (Tohme et al., 1996) from leaf tissues of two genotypes, G19833 and DOR364. G19833 is highly resistant to anthracnose and some strains of angular leaf spot but susceptible to BGMV, whereas DOR364 is susceptible to angular leaf spot and anthracnose, but is resistant to BGMV. 87 RIL-F9 plants from the cross of G19833 by DOR364 were used as a mapping population.

The NBS primers were the same reported before (BRU Annual Report, 1998, fig 1). Primers LRR and Protein Kinase (PK) were designed based on these conserved domains present in the *Xa*21 rice resistance gene (P. Ronald, personal communication). We also designed a primer (motif1) from a conserved sequence downstream of the region used by Leister et al., allowing the amplification of a longer and more specific fragment. Finally, two primers (TIR1 and TIR5) designed at CIAT were used to target the TIR domain that is upstream of the NBS in *L6*, *RPP5* and *N* resistance genes.

After the PCR reactions, products were separated and each band was purified, cloned and transformed into *E.coli* electrocompetent cells. Groups of sequences for the different bands were established using restriction patterns of the picked clones. At least three clones of each group were tested as RFLPs in the parental lines; polymorphic probes

were hybridized to DNA from the 87 F_9 plants to evaluate segregation. At least one clone of each group was sequenced using the Dye Terminator Cycle Sequencing Kit and an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer).

RGAs linked to resistance loci were used to screen a BAC library containing bean genomic DNA. Identified BAC clones were mini-prepared by standard alkaline lysis procedure.

Results and discussion

Three classes of RGAs were isolated last year using degenerate primers targeting NBS sequences (BRU Annual Report, 1998, fig 2 and 3). Two of them mapped in a region of linkage group B11 where a highly significant QTL for anthracnose resistance has been identified (CIAT unpublished data, fig 4). A *Xba*I fragment of class 2 representing a different member of the family mapped linked to group B04 near a QTL associated with BGMV resistance. Class3 could not be mapped due to the lack of polymorphism between the parentals evaluated.

Table 1. Primers used in the present study

Domain	**Primer name**	Sequence peptide/nucleotide	Reference
NBS	S1 S2	**G G V G K T T** GGT GGG GTT GGG AAG ACA ACG GGI GGI GTI GGI AAI ACI AC	Leister et al., 1996
NBS	AS1 AS2 AS3 AS4 AS5	**G L P L A L** **CAA CGC TAG TGG CAA TCC** **IAA IGC IAG IGG IAA ICC** **IAG IGC IAG IGG IAG ICC** **IAG IGC IAG IGG IAA ICC** **IAA IGC IAA IGG IAA ICC**	Leister et al., 1996/1998
LRR	LRR1 LRR2 LRR3	**I S/P N/L N/L F/S S/L G/S H-Q I-M** **TCA AGC AAC AAT TTG TCA GGI CAI ATI CC** **CTC GCG CTG CTC TCT CTT TCA A** **P N S F S N L F** **TGA AAA GAT TGG AAA ACG AAT TAG G**	Ronald,P Taylor, N Taylor, N.
PK	PK1 PK2 PK3	**Q-H R D/E N/K L S S N V L L** **TTA CAG CAC ATT GCT TGA TTT IAI ITC ICG ITG** **TAA CAG CAC ATT GCT TGA TTT IAI ITC ICA ITG** **TAA CAG CAC ATT GCT TGA TTT IAI ITC ICT ITG**	Ronald, P.
TIR	TIR1S	**D/E V F L/P S F R/S G** GAI GTN TTY TTI TCI TTY AGI GG	CIAT, 1999
TIR	TIR5AS	**P/V F Y M/D V D P** IGG GTC IAC GTC GTA GAA IAC IGG	CIAT, 1999
MOTIF1	MOTIF1 AS	**F L/K D/C I A C F** GAA GCA IGC GAT GTC IAG GAA	CIAT, 1999

The isolation of new RGAs was achieved using primer s2 in combination with our designed primer Motif1. The amplification yielded the expected band of 680 bp in both parentals. Of 30 individualized clones sequenced, 26 showed homology with resistance genes and other RGAs. Fourteen has ORF and were classified in five new classes different between them and with those previously obtained. Class 8, similar to class 3, was monomorphic between the two parentals when assayed by RFLPs and could not be mapped. Class 6 mapped in linkage group B04 near the *XbaI* fragment of class 2. The location is near the region detected with a QTL for anthracnose and BGMV resistance. We are currently working om mapping the other classes.

RGA classes contained clones from both parentals, suggesting they share conserved sequences involved in common defense events to different pathogens. The presence of classes with clones from only one parental may indicate that they are sequences specific for some strains of pathogens and/or take part in initial signal transduction events.

Classes 1 and 2 have been used to screen the bean BAC library. We detected 17 and 36 clones, respectively. This is in accordance with the multicopy character of this classes and the 3 to 4 genome equivalents covered by the library. BAC inserts of these clones has been isolated.

Various bands with sizes between 700-1300 bp were isolated using primers targeting LRR and PK domains. Sequences from these bands did not show homology with R-genes. So they were no further analyzed.

TIR-containing sequences of about 280 bp were successfully identified by means of the primers designed for this domain (TIR1-TIR5). They showed high homology with resistance genes.

On going activities will concentrate on:

5) Mapping RGAs classes 4, 5 and 7.
6) Using new resistance-linked RGAs to screen the bean BAC library.
7) Constructing contigs of mini-prepared BAC clones of classes 1 and 2.
8) Using all resistance-linked RGAs to screen a bean cDNA library in order to get their full coding sequence.

Collaborators

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1.2.13 Characterization of Disease Resistance Gene Analogs (RGAs) Amplified with Degenerate Primers in Rice variety IRAT13

Introduction

As part of the study on blast resistance initiated last year, we have pursued the identification of resistance gene analogs of rice with a new set of degenerate primers in addition to those previously used.

Materials and Methods

DNA templates were used from the varieties Fanny and IRAT13, susceptible and resistant to some blast lineages, respectively. The same strategy described for the identification of RGA in bean (BRU report, 1998, 1999) is being followed with some modifications. Initially, we used degenerate primers designed by Leister et al. (1996) (Table 1). Additional antisense primers (AS4 y AS5) to this domain were taken from Leister et al. (1998). We have not used LRR and PK primers. Primers designed at CIAT and others obtained from Silvia Peñuela, University of Minnesota (PRS3) were assayed (table 1).

When clones of different combinations were individualized and discriminated by restriction pattern, about three clones of each group were sequenced before testing them using RFLPs as described for bean. Only different classes of sequences were used as probes, allowing considerable save of time.

Table 1. Primers used in the identification and detection of RGA in rice

Domain	Primer name	Sequence peptide/nucleotide	Reference
		G G V G K T T	
NBS	S1	GGT GGG GTT GGG AAG ACA ACG	
	S2	GGI GGI GTI GGI AAI ACI AC	Leister et al., 1996
		G L P L A L	
	AS1	**CAA CGC TAG TGG CAA TCC**	
NBS	AS2	**IAA IGC IAG IGG IAA ICC**	
	AS3	**IAG IGC IAG IGG IAG ICC**	Leister et al., 1996/1998
	AS4	**IAG IGC IAG IGG IAA ICC**	
	AS5	**IAA IGC IAA IGG IAA ICC**	
TIR	TIR1S	**D/E V F L/P S F R/S G**	This work
		GAI GTN TTY TTI TCI TTY AGI GG	
TIR	TIR5AS	**P/V F Y M/D V D P**	This work
		IGG GTC IAC GTC GTA GAA IAC IGG	
MOTIF1	MOTIF1AS	**F L/K D/C I A C F**	This work
		GAA GCA IGC GAT GTC IAG GAA	
PRS3	PRS3	**GGR AAI ARI SHR CAR TAI VIR AAR C**	S.Penuela (pers.comm.)

Results and discussion

We have performed PCR-amplifications with primer s2 in combination with all primers except as5. Only bands of the expected size (500 bp) were purified for cloning. A total of 51 clones were sequenced. 39 were identified as RGAs for their similarity with the reported NBSs RGAs and known resistance genes. They were classified in ten classes according to their percentage of nucleotide identity. All but two classes include members with Open Reading Frame (ORF). Initial use of these classes as RFLP probes has shown only one polymorphism between the parentals. Construction of new parental DNA blots with a set of different enzymes is underway to identify polymorphism for mapping of these RGAs.

Amplification using our designed primer Motif 1 did not yield the expected band (680 bp), but one of 450 bp that was cloned. Ten sequences were obtained. Only one was an RGA whose predicted amino acid sequence has multiple stop codons. This sequence may constitute a pseudogen with a long deletion.

Similar results were obtained using primers targeting a putative TIR domain in rice. Amplified band was 180 bp and the expected was 280 bp. Sequences of this did not show homology with any resistance gene; and will not be analyzed further. Maybe, rice does not contain R-genes with TIR domains or more degenerate primers are needed to amplify them if present.

The primers from University of Minnesota annealed a region near our Motif1. And amplified the expected band (700 bp). The sequences obtained at CIAT showed high homology with R-genes. PRS3 is a very degenerate primer, so it could anneal in rice R-like sequences, while our Motif1 did not.

On going work

- Mapping of all the RGAs in the cross Fanny x IRAT13. Use of these RGAs in other mapping populations, like Fanny x Oryzica Llanos 5.
- Screening of rice BAC and cDNA libraries with those resistance-linked RGAs.
- Assaying more degenerate TIR primers in rice.

Collaborators

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. 1.2.14 Release and distribution/sale of FloraMap™ BetaVersion 1.1

Highlights

- March 1999 saw the distribution of the review copy of FloraMap™ to over 20 germplasm scientists throughout the world. Enthusiastic responses have been received from many, with particularly useful hints on software requirements and suggestions for clarifying the manual.

- The manual has been rewritten and re-edited to incorporate new software tools and to clarify explanations where needed.

- A new software tool, the Climate Diagram has been incorporated, and comprehensive Help files have been added.

- A start has been made to modularise the software. The Climate Diagram and the Clustering Tool are now available as Active X modules and may be simply incorporated in other software packages.

- We have started the procedure for registering FloraMap™ as a trademark

- FloraMap™ Beta version 1.1 is scheduled for commercial release late in October 1999

Results

FloraMap is a system for producing the predicted distribution or the areas of possible adaptation, for natural organisms when little or nothing is known of the detailed physiology of the organism. It is assumed that the climate at the point of collection of a set of individuals is representative of the environmental range of the organism. In the case of plants, these are usually germplasm collection accessions or herbarium specimens.

The climate at these collection points is used as a calibration set to compute a climate probability model. The method uses a Fourier transform to standardize climatic timing and a principal components analysis (PCA) to produce a probability distribution in multiple dimensions. The principal component scores are used to map the probability surface from a set of interpolated climate surfaces. The system has been used to guide plant collecting, to investigate taxonomic and genetic variation, and to map crop pests and their potential predators.

The software has been comprehensively overhauled and a new manual produced. The feedback from the review team has been used to streamline the use of the windows and software tools. A new tool is the Climate Diagram tool which can display the climate for any accession point of for any point on the continental climate grids. It can display rainfall, temperature and diurnal temperature range as a histogram and graph in Cartesian

coordinates, or in polar coordinate. It can display the data in it's natural form numberg dates forward from 1st January or in their date standardized, rotated form.

New climate grids have been added with pixel sizes of 5 arc minutes for Asia and the continental USA. The latter with the kind permission of Dr. Chris Daly of Oregon State University.

Measurable/Expected Impact

Judging by the interest created by the review copy there will be a healthy demand for the first (Beta) release of the software. We are planning to produce 500 CD-ROM/Manual packages.

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

MAIN ACHIEVEMENTS

- In cassava, 172 SSR markers have been generated, out of these 36 have been evenly distributed in the 18 linkage groups comprising the cassava map. In common bean, primer pairs of a number of SSRs have been amplified for mapping. Work on *Brachiaria* SSRs is progressing.
- AFLPs and two new techniques (SAMPL and RAMP) have been implemented for the study of genetic variation in cassava bacterial blight pathogen (*Xam*). While RFLP showed extreme usefulness for population structure analysis, SAMPL and RAMP are recommended for searching *Xam* pathogenicity factors.

1.3.1 Micro-Satellite Markers To Facilitate Use of the Cassava Molecular Genetic Map and Provide New Genetic Information

Introduction

A molecular genetic map of cassava was constructed from segregation of predominantly RFLP markers in a F_1 intra-specific cross (Fregene et. al., 1997), as a first step towards marker-assisted genetic analysis of traits of agronomic importance. The usefulness of this map and the RFLP markers are however limited to research centers that can afford the technology required for RFLP markers, limiting the use of marker technology to these centers, which account for a small percentage of trained breeders working on the crop. In an attempt to make marker technology widely available in cassava, an effort was embarked upon to place on the cassava map simple sequence repeat (SSR) markers, markers that are PCR-based and highly polymorphic, and best meet the criteria required for marker technology transfer to developing country research systems. A project aimed at the development and mapping of at least 200 SSR's was initiated with funding from the Swiss Development Cooperation (SDC) and the International Development Agency (SIDA). A cassava breeder from National Root Crops Research Institute, Umudike, Nigeria is the principal investigator in this project.

Materials and methods

Two enrichment experiments, "Enrichment A" (after Karagyozov et al., 1993, as modified by Stephenson et al. unpublished data) and "Enrichment B" (after Edwards et

al., 1996), were conducted using total genomic DNA from two elite cassava clones, TMS 30572 (IITA, Nigeria) and CMC 40 (CIAT's core collection), respectively. Forward and reverse strands of all positive clones were sequenced using the M13 universal and reverse primers (New England Biolabs, USA and Microsynth, Switzerland) on an automated sequencer (Perkin Elmer/ Applied Biosystems models ABI 373 and 377). Primer design for cleaned and unique DNA sequences was with "Primer3" picking software found at http://waldo.wi.mit.edu/cgi-bin/primer/primer3 (Whitehead Institute for Biomedical Research). Oligonucleotide primers were synthesized by Research Genetics, USA and designated "Cassava MapPairs". The female (TMS 30572) and male (CM2177-2) parents of the F_1 cassava mapping population were evaluated with all the 172 SSR markers identified, using non radioactive PCR amplifications. The PCR products were electrophoresed on 5% ethidium bromide stained Metaphor agarose gels or on 6% polyacrylamide sequencing gels for 2 h at 100W, and DNA visualized by silver staining according to the Manufacturer's guide (Promega Inc., USA). SSR markers having a unique allele in either or both parents were analyzed in the entire F_1 progeny of 150 individuals and placed onto the existing map of cassava using the linkage analysis computer package MAPMAKER 2.0 (Lander et. al.1987), as described earlier (Fregene et. al. 1997).

Results and Discussion

SSR Markers

One hundred and sixty four, or 95%, of the 172 SSR containing clones for which primers were designed, were dinucleotide repeats while the balance were trinucleotide repeats save for one tetranucleotide repeat. Table 1 shows the breakdown of the clones into nucleotide repeat classes.

Table 1. Number, percentage and kind of SSR repeat sequences for which primers were designed

Type of SSR	Number	Percentage (%)	Type of SSR	Number	Percentage (%)
Enrichment A			**Enrichment B**		
GA/CT	12	34	GA/CT	80	58
CA/GT	5	14	CA/GT	30	22
(CA)(GA)	2	6	(CT)(CA)	15	11
ATT/TAA	5	14	(CA)(GA)	6	4
Others	11	31	Others	6	4
Total	35		**Total**	137	

SSR Parental Survey

All 172 primer pairs successfully amplified the corresponding SSR loci in the parents of the cassava mapping progeny; but with different $MgCl_2$ (1 and 1.5 mM) concentrations, and 2 annealing temperatures, 55 C and 45 C. One hundred and thirteen SSR loci, or 66% of all SSR markers tested in the parents, revealed a unique allele in at least one of the parents while 45 SSR markers, or 26% showed a unique allele for both parents.

Genome location of SSR markers

Thirty-six SSR loci from the 172 SSR markers analyzed to date have been placed on the male- and female-derived molecular genetic map (Fregene et al., 1997). The 36 SSR markers reveal a fairly even spread over the cassava genome – sixteen of the eighteen linkage groups have at least 1 SSR marker, with an exception of 9 SSR markers clustered on linkage groups C, D, and J.

Applications of the SSR markers

Aside from the on-going SSR mapping project, and the successful tagging of the ACMVD resistance locus (see section of this Annual Report on Molecular Mapping of Genes Conferring Resistance to the Cassava Mosaic Disease – CMD - in African Cassava Germplasm), the SSR markers are currently being used in the following ways in CIAT:

a) mapping of resistance to white fly in cassava; and
b) finger printing for CBB resistance.

At IITA, the markers are being used in mapping for resistance to ACMVD

Further activities

- Mapping of the identified 113 polymorphic SSR markers;
- Continued sequencing of the remaining over 900 positive clones;
- Searching for and sequencing SSR's in the 3' and 5' untranslated regions of expressed sequence tags (ESTs), expected from ongoing cassava EST projects; and
- Ultimately identifying at least 500 SSR markers and mapping at least 200 of them.

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Collaborators

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1.3.2 Identification and Characterization of microsatellites in *Phaseolus sp*

Background

Microsatellites or Simple Sequence Repeats (SSR) are short, 1-6 bp in length, tandemly repeated simple sequence of DNA (Asley & Dow, 1994). SSRs seem to be randomly dispersed in the genome (Weber JL, 1990). Dinucleotide repeats are reported to be the most abundant repeats in plants as well as in vertebrates: (GA)n repeats are predominant in plants and (AT)n repeats in animals (Morgante 1993). The number of repeats in SSRs or SSR Polymorphism is highly variable and can be detected by PCR using as primers conserved DNA sequences flanking the repeat motifs. Their characteristics (highly polymorphic at the interspecies level, PCR based co dominant markers, and amenability to semiautomated genotyping) make them highly suitable for genome analysis.

We have initiated this year a project to look at the abundance and characterization of AG/CT, CA/GT dinucleotide microsatellites in bean.

Methodology

A bean library was constructed in a collaboration with IACR in Bristol (England) following Edward's methodology (K.J. Edwards, et. at, 1996, Biotechniques 20:5) with some modifications. Bean DNA was digested using RsaI enzyme. Fractions were ligated to MluI adaptors using T4 DNA ligase. Enrichment for microsatellites was carried out using approximately 100 ng of the ligated, denatured DNA in 500 ul of hybridization buffer over night at 45°C. Bound DNA was then eluted in sterile distilled water by boiling for 5 min. 2.5 ul of the eluted DNA were amplified by PCR using 21-mer adaptor primer. Approximately, 5 ng of enriched DNA were taken directly from the final amplification, digested with 1 unit of MluI and ligated into a modified pUC19 vector

(pJV1) containing a BssHI site. Plasmids were transformed into DH5α and plated onto L-agar plates containing 100ug/ml ampicilin. Following incubation overnight at 37°C, colonies were transferred into microplates for long storage. Plasmids from individual colonies were prepared using the Promega Wizard kit and sequenced with CIAT ABI 377 sequencer using Dye Terminator Ready Sequencing Mix (Perkin-Elmer).

PCR amplification and evaluation of polymorphism: one hundred thirty six locus-specific pair or primers were designed using the PRIMER computer program version 3.0. These primers were tested for PCR amplification and polymorphism using DNA from the parents of mapping populations (DOR 364 * G 19833). The PCR reaction was carried out in a 25ul final volume containing 25 ng of genomic DNA, 0.25uM of each forward and reverse primers, 10mM Tris-HCL (pH 7.2), 50mM KCL, 1.5mM to 2.5mM MgCL2, 250mM of each dNTP and 1 Unit of Taq DNA Polymerase. Temperature cycling profile was: an initial denaturation step for 5 min at 94 C, followed by 30 cycles of denaturation at 94 C for 1 min, annealing since 48 C to 65 C for 2 min and primer extension at 72 C for 2 min. A final extension cycle of 5 min at 72 C was added. Between 2 and 3 ul of the PCR reaction was resolved on 6% polyacrylamide sequencing gels for 2 h at 100W, and DNA visualized by silver staining according to the Manufacturer's guide (Promega Inc., USA).

Results

Two hundred different microsatellites were identified in the bean library. We obteined clones with perfect sequences motif as well as imperfect sequences motif. Using CA and/or GA as motif for searching microsatellites in the library, we obteined several clones with tri or tetranucleotides (Table 1).

Table 1. Sequences motif obtenined using CA/GA microsatellites

Microsatellite Sequences	No. of Clones
CA>10	15
CA 10-20	7
CA>20	56
GA 10-20	2
GA>20	5
COMPOUND	25
OTHER MICROSATELLITES	14
REPITED	59
NO MICROSATELLITE	14
IMPERFEC	2
NEAR TO CLONING SITE	59

So far, primer pairs in the flanking regions of 136 microsatellite loci have been designed. As a first step to establish a set for high through put germplasm characterization selected primers were tested on accessions from the Mesoamerican and Andean genepool (Fig 1) and from different *Phaseolus* species (Fig 2). The level of polymorphism detected was more than adequate. The mapping of the primers pairs is underway using the cross of DOR 364 x G19833 (Fig 3).

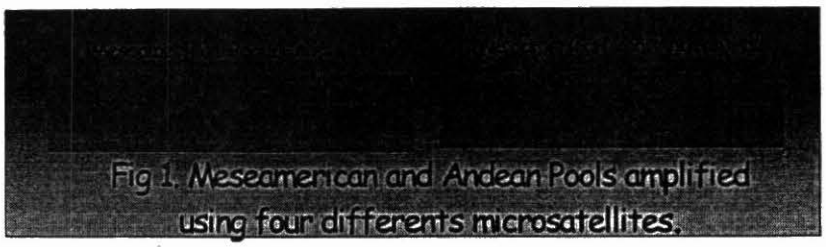


Fig 1. Meseamerican and Andean Pools amplified using four differents microsatellites.

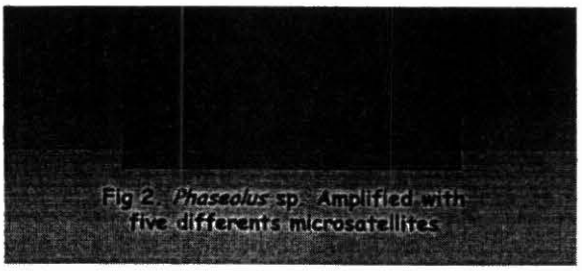
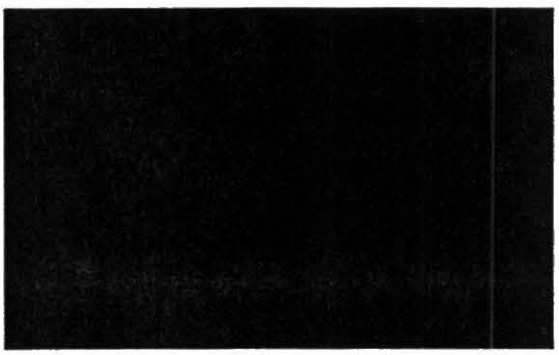


Fig 2. *Phaseolus* sp. Amplified with five differents microsatellites



On-going Activities

- Standardization of the PCR conditions for each primer in the parental DOR362 and G19833 and to check the polymorphism between them.
- Amplification in the segregant population the polymorphic primers and linkage analysis with the existing RFLP and RAPD bean map devolved at CIAT using the RIL from the cross DOR 364 x G19833
- Development of a set of 15 fluorescent labeled SSrs for high trough put germplasm fingerprinting.

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Collaborators

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1.3.3 Isolation and characterization of microsatellites in *Brachiaria* sp.

Background

Spittlebug species are considered as the main pests associated with *Brachiaria* in tropical Latin America. Spittlebug damage can result in complete loss of available forage. Chemical and biological control are used as a the main control method. *B. brizantha* cv. Marandu is known to be resistant against several species and genera of spittlebugs. (J.W. Miles, et. al., eds, 1996). Based on this positive attribute, Marnadu has been used in breeding programs to study the heretability of resistance to spittlebug in interespecific crosses.

We have initiated a project to assess the presence and abundance of microsatellites in *Brachiaria* and their potential as markers in mapping and tagging of spittlebug resistance and apomixis gene. As PCR-based markers, microsatellites will facilitate the application of markers in a breeding program. We also plan to design locus-specific primers and use them to analyze crosses between apomictic, sexual and spittlebug resistant parents to increase the saturation of the *Brachiaria*'s map (unpublished data). Such primers will

provide us with co-dominant, PCR-based and locus specific markers that could be used in gene tagging and marker assisted selection for Spittlebug resistance gene and apomixis gene.

Methodology

The enrichment methodology was carried out in collaboration with IACR-Long Ashton (England) and CIAT following the Edward's Methodology (K.J. Edwards, et,al, 1996) with some modifications.

Preparation of genomic DNA: Genomic DNA were digested with Rsa I. One microgram of a MluI adaptor (Consisting of a 21-mer: 5' CTCTTGCTTACGCGTGGACTA3' and a 25-mer: 5'TAGTCCACGCGTAAGCAAGAGCACA3') together with 10mM ATP were added to the digestion mixture along with 5 units of T4 DNA Ligase. The ligation is the allowed to proceed over night at 37°C. Finally 2.5 ul of digested/ligated DNA is amplified by PCR using the 21-mer adaptor primer in a 50 ul of final volume. The PCR product was cleaned using Phenol-Chloroform procedure and DNA was resuspended in 50 ul of water.

Characterization of enriched microsatellites: Enrichment for microsatellites was carried out using cleaned and denatured PCR in 500 ul of hybridization buffer containing 1 ug of the 21-mer oligonucleotide using a single hybond N+ filter with 10 μg of each oligonucleotide for 24 h at 45°C. DNA bound was the eluted by boiling for 5 min. Five microliters of the eluted DNA were amplified using a 21-mer oligonucleotide. Approximately 2.5 ug of enriched DNA were taken directly from the final amplification, digested with 1 unit of MluI and ligated into a modified pUC19 vector (pJV1) containing a BssHI site. Plasmids were transformed into DH5α (Life Technologies) and plated onto LB-agar plates containing 100ul/ml ampicilin. Recombinant colonies were transferred to a Hybond filter and hybridized with the oligonucleotide containing microsatellite motif radioactively labeled. Positive clones were sequenced using Dye Terminator Cycle Sequencing Kit with an ABI 377 sequencer (Perkin-Elmer).

PCR amplification and evaluation of polymorphism: sixteen locus-specific pair or primers were designed using the PRIMER computer program version 3.0. These primers were tested for PCR amplification and polymorphism using DNA from the parents of all two mapping populations (CIAT 606 * Clone 44-3 and CIAT 6294 * Clone 44-3). The PCR reaction was carried out in a 25ul final volume containing 25 ng of genomic DNA, 0.8uM of each forward and reverse primers, 10mM Tris-HCL (pH 7.2), 50mM KCL, 0.8 or 1 mM MgCL2, 200mM of each dNTP and 1 Unit of Taq DNA Polymerase. Temperature cycling profile was: an initial denaturation step for 5 min ar 94 C, followed by 30 cycles of denaturation at 94 C for 1 min, annealing at 68 C or 65 C for 2 min and primer extension at 72 C for 2 min. A final extension cycle of 5 min at 72 C was added. Between 2 and 3 ul of the PCR reaction was resolved on 6% polyacrylamide sequencing

gels for 2 h at 100W, and DNA visualized by silver staining according to the manufacturer's instruction (Promega Inc., USA).

Results

Two libraries were obtained using $(GA)_{20}$ and $(CA)_{20}$ oligonucleotides. Several clones from each library were sequenced and 30 different microsatellites were identified so far. Eleven of sixteen pair of primers were standardized to amplify in all three parental (apomictic parental - CIAT 606, sexual clone 44-3 and CIAT 6294) of two mapping populations. We obtained 10 polymorphic pair of primers when they were evaluated in all three parentals. The polymorphic amplification in the parentals using primers EGBRGT2 and EGBRCT5 can observed (fig 1). Each line is a different genotype and the number and size of each allele is different in each one. Some 300 clones containing a putative microsatellite motif have been identified. The sequencing of each clone is underway.

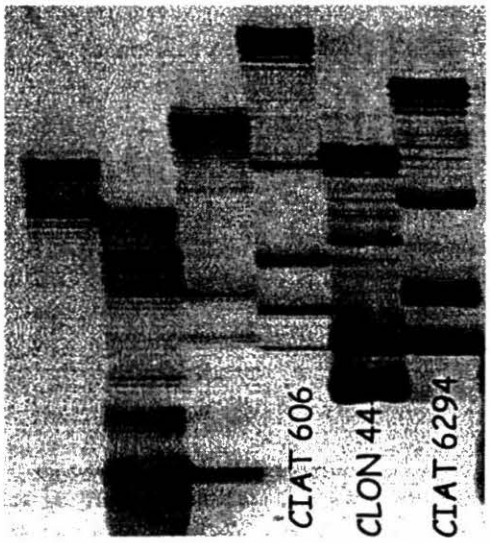

Fig. 1 Evaluación de parentales (sexual y apomíctico) utilizando dos pares de Microsatélites

On-Going Activities

- To continue sequencing available clones and design primers useful for mapping.
- Evaluation of all polymorphic primers in the F1 population.
- Construction of a molecular map in *Brachiaria* using microsatellites anchored with RFLP clones from other grasses.

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1.3.4 Characterization of *Xanthomonas axonopodis* pv. *manihotis* strains using AFLP, SAMPL and RAMPs.

Introduction

Xanthomonas axonopodis pv. *manihotis* (*Xam*) is the causal agent of cassava bacterial blight (CBB), a particularly and destructive disease of cassava in South America and Africa. Under favourable conditions yield losses can range between 12 and 100% (Lozano, 1986) and can be reduced by a combination of cultural practices and host resistance (Lozano 1986; Boher and Verdier 1994). The advent of molecular techniques for detection of genetic variability provides a useful tool to assess the genetic structure of microorganisms. AFLP is a recently developed technique for the fingerprinting of plant, bacterial, fungal and nematode genomes (Folkerstma *et al.*, 1996; O'Neill *et al.*, 1997; Vos *et al.*, 1995) and was recently used in studies for detecting genetic variation of *Xam* (Restrepo *et al.*, 1999). SAMPL and RAMPs were developed as new methods for characterizing genetic variation (Witsenboer *et al.*, 1997) and mapping of plants (Becker *et al.*, 1995) respectively. The objective of the present study is to assess the usefulness of the AFLP, SAMPL and RAMP techniques in characterizing the genetic diversity of *Xam* at the infrapathovar level. We aimed to establish the appropriate conditions for applying the SAMPL and RAMP techniques to the study of *Xam* populations.

Materials and Methods

Bacterial strains: Twelve Xam strains were used for this study that were cultured routinely at 30°C on LPG medium; colonies were used to start broth cultures. Cultures were grown at 30°C for 12 hours, and cells from 3 ml of broth were used for DNA extraction.The genomic DNA was extracted by the method of Boucher et al., (1985) and the conservation of the strains was done as previously reported.

Molecular markers: AFLP. A total of 500 ng of DNA was digested with two combinations of enzymes: *Eco*RI/*Mse*I and *Pst*I/*Mse*I and then ligated to the respective adapters. Ligation and PCR conditions were as previously described (Restrepo *et al.*, 1999). Amplified products were separated on a 6% denaturing polyacrylamide gel. Gels were stained using the silver staining technique. **SAMPL.** Template DNA for SAMPL analysis was prepared using the same amplification step as in the AFLP protocol, except for one of the primers used in the second amplification. This PCR primer, (*X*V) (5′-TTC GGC AAC GGC AGT GAC CAC C-3′), was developed based on the sequence of a 1.2-Kb *Xam* plasmid fragment. Cycling profile was as used for AFLP analysis. The AFLP primers were not selective (+0) and the enzyme combination used was *Eco*RI/*Pst*I. **RAMPs.** The DNA was digested with the enzyme combination *Eco*RI/*Pst*I and then ligated to the respective adapters as in the SAMPL protocol. However, one single amplification was performed, using one RAPD primer and a specific primer (*X*V). The RAPD primers were chosen from Kit F: F8, F11, F13 and F18. DNA was amplified at 94°C for 40s, 55°C for 35s and 72°C for 1 min. 20s; after that, a touch-down was performed with an initial annealing temperature of 53°C reduced by 2° to 40°C over 5 cycles. The rest of the thermal profile was performed at 94°C for 40s, 55°C for 35s and 72°C for 1 min. 20s over 25 cycles.

Results

The analysis of the AFLP results showed that banding patterns obtained by using restriction enzymes *Eco*RI/*Mse*I were more clear and easy to read, using T+T and T+A as selective bases on the primers. Fingerprints were more complex when G or C was the selective base on the primers. Banding patterns obtained by using the enzyme combination (*Pst*I/*Mse*I) were difficult to read due to the high number of bands. However, when C was the selective base for the *Pst*I primer and C and G for the *Mse*I primer, the banding patterns were clear and they showed the highest percentage of polymorphism.The two primer combinations used in SAMPL assays, (*Eco*RI(+0)/*X*V and *Pst*I(+0)/*X*V) showed a similar banding pattern, low number of total bands (17 bands) and low polymorphic bands when comparing with the AFLP technique. However, using the *Pst*I(+0)/*X*V combination more polymorphic bands were obtained (5/17) and the percentage of polymorphism (17.8%) was similar than the one obtained with the AFLP primer combination *Eco*RI+T/*Mse*I+T (17.5%).With the four combinations of RAMPs primers (F8/*X*V, F11/*X*V, F13/*X*V, F18/*X*V), we obtained a total of 23 bands. The number of polymorphisms obtained using the RAMP technique was low compared with the

AFLP technique. With the F13/*X*V and F18/*X*V combinations, the number of polymorphic bands (3/23 and 4/23, respectively) was high and the percentage of polymorphism (13% and 17.4%) was similar to the AFLP combinations EcoRI+T/MseI+T and EcoRI+T/MseI+A (11.4%).

Discussion

AFLP is an extremely useful and reliable technique for detecting polymorphism in bacterial populations and its reproducibility is very high ascertained the usefulness of this technique in assessing the genetic diversity of *Xam* at the infrapathovar level. Two novel techniques were used for the identification of polymorphisms in *Xam* strains: SAMPL and RAMPs. SAMPL analysis is a method of amplifying microsatellite loci using generic PCR primers, it uses one AFLP primer in combination with a primer complementary to microsatellite sequences, or an specific primer (Witsemboer *et al.,* 1997). The technique RAMPs combines most of the benefits of the microsatellite analysis to the universality of RAPD analysis, and it can use a specific primer too (Becker and Heun, 1995). Both techniques were standardized and applied to *Xam* genetic studies and it is the first report where the SAMPL technique was used for detecting genetic variation of microorganisms. SAMPL and RAMPs analysis extend AFLP technology to include amplification of a specific region of the *pth*B pathogenicity gene of *Xam*. The total number of bands obtained with SAMPL and RAMPs was lower than AFLP because these techniques use a specific primer. Furthermore, the RAPD primers used for RAMPs generate a low number of amplified bands. Based on this study, we suggest the use of a technique such as AFLP in assessing the genetic diversity of *Xam* and the use of SAMPL and RAMPs techniques to direct the search of pathogenicity factors. Cloning and characterization of these factors may elucidate the *Xam*-cassava interactions at molecular levels.

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OUTPUT 2: GENES AND GENE COMBINATIONS MADE AVAILABLE FOR BROADENING THE BASE OF MANDATED AND NON-MANDATED CROPS

ACTIVITY 2.1 Transfer of genes and gene combinations using cellular and molecular techniques

MAIN ACHIEVEMENTS

- As a component of a management strategy for the control of the cassava stemborer, gene constructs, harboring the *cry* 1A(b) gene, were made and regeneration-transformation was initiated with cassava var. Venezolana, prefered by farmers.

- *In vitro* multiplication of cassava is being implemented using two schemes. While one seeks to achieve highest rates of multiplication through bio-reactor systems, the other develops low cost, low input, artesanal tissue culture procedures.

- A practical cassava cryopreservation technique, using an encapsulation (artificial seed)- dehydration procedure resulted in 20% - 95% plant recovery rates from a sub-core cassava collection of 45 clonal accessions. This opens the way to a larger scale application of cryopreservation to cassava.

- QTLs from wild *Oryza* species associated with yield increases of 20-25% have been localized in the map. Similar approach has been initiated in common bean using Mexican and Colombian *Phaseolus* wild species. Molecular analysis shows introgression from wild accessions into backcross lines.

- While assessment of the RHBV-N transgenic resistant lines in underway, a range of mutated versions of an anti-pathogen protein gene is being tested in transgenic strategies against rice RHBV and *Rizoctonia*. Similar approach involves the RHBV non-structural protein. On the other hand, significant progress has been achieved in developing *Agrobacterium*-mediated transformation of *indica* and *japonica* rices.

- Extension of biotechnology tools, through collaborative arrangements, to non-mandated crops included (i) propagation of soursop (*Annona muricata*) and field testing of micrografted stocks in Colombia; (ii) genetic transformation of lulo (*Solanum quitoense*) and tree tomato (*Solanum betacearum*) towards transgenic resistance against nematode attack, a major pest in the Andean region; (iii) development of transgenic resistance to fruit worms and budworms of tomato, and (iv) transgenic resistance to the yellow leaf virus of sugarcane.

2.1.1 Cassava transformation for the integrated management of the cassava stem borer

Introduction

A technology of friable embryogenic callus (FEC system) in cassava became available in 1996 (Taylor et al. 1996). This technology has the potential for producing large amounts of transgenic cassava plants. In order to apply this technology to cassava cultivar MCol2215 and confer resistance to the stem borer (*Chilomina clarkei*) CIAT acquired the technology from ILTAB. We tested the regeneration potential (conversion of somatic embryos into plants) of the cell line TMS60444 (MNig11). Simultaneously we pursued the more traditional transformation system for cassava using young leaves or cotyledons from somatic embryos. The objective is to obtain transgenic cassava plants resistant to the stem borer using the plasmid pBIGCry (described in the Annual Report 1998, Project SB-02) which harbors the insect-resistance *cry*1A(b) gene derived from *Bacillus thuringiensis*.

Matherial and Methods. The plasmid pBIGCry was delivered into young, immature leaves through *Agrobacterium*-mediated transformation using the supervirulent strain C58C1. We also introduced the plasmid pBIGCry into cassava somatic embryos through biolistics and into tobacco plants via *Agrobacterium*.

To evaluate the effect of the CRY recombinant toxin (from the gene *cry*1A(b)) on the cassava stem borer *Chilomina clarkei*, we tested synthetic diets to rear *C.clarkei* larvae under laboratory conditions. We prepared four diets (Table 1 and 2) and evaluated the viability of larvae after feeding the diets (Table 3). Three individuals, as an average, were placed in small plastic dishes with 20-25 ml of the artificial diet. Each diet was evaluated five times. The experiments were carried out in darkness, at 25-30°C. Experiments lasted about 15 days Pupae were destroyed before adults appeared. The rate of food intake and the grade of development of larvae were indicators of the success of a diet.

Results and Discussion

The cell lines of TMS60444 acquired from ILTAB are maintained on solid or liquid medium. Although one of the lines has been maintained in vitro for over two years, it still produces embryos and plants at maximum frequencies of 10%. This percentage reflects the proportion of single somatic embryos that mature, germinate and convert to plants. Although it may seem to be low, given the potential of the system to produce plants, the effect of long exposure to tissue culture may influence the conversion ability. This effect has to be considered in the development of FEC for other cassava cultivars. We have already observed the production of FEC in MCol2215 on MS (Murashige and Skoog,

1963) and GD (Gresshof and Doy) medium, and we are currently testing the conditions necessary to increase the frequency of FEC and to propagate it.

Current experiments on transformation of MCol2215 with the *Agrobacterium* strain C58C1-pBIGCry showed that the T-DNA is efficiently transferred into young immature leaves, judged by transient expression of the reporter GUS gene. Approximately 10% of infected young leaves showed strong GUS expression (10 to 20 blue foci per leaf) after only 50 minutes of assaying. Scalpel wounding of explants was more effective than sonication-mediated wounding in inducing *Agrobacterium* multiple infections. Young leaves as well as whole embryos and cotyledons from somatic embryos infected with the bacteria are now under selection on embryo induction medium containing Paramomycin. Preliminary experiments using the particle gun showed that the plasmid pBIGCry can also be inserted and expressed in MCol2215 embryogenic tissues. On the other hand, we obtained six putatively transgenic tobacco plants transformed with C58C1-pBIGCry. All six plants express GUS and are currently growing in vitro on selection medium. They will be soon transferred to the greenhouse. These tobacco plants may be used to check if the *cry*1A(b) effectively kills *Chilomina* larvae by feeding with tobacco leaves.

Experiments showed that diets containing cassava flour (made out of dry cassava stems) were preferred by the larvae. Larvae did not feed well on diets containing maize flour (Table 3). The hardness grade of the diet was also important for its consumption. Larvae developed well on softer diets containing cassava flour and higher quantities of agar.

Table 1. Composition of diet to rear Helicoverpa larvae. Poitout & Bues (1970) modified by Betbeder -Matibet

Components	Quantity
Distilled water	500 ml
Sunflower Oil	0,5 ml
Agar-agar powder	8,12 g
Sorbic acid	0,75 g
Ascorbic acid	6,25 g
Cassava flour	71,25 g
Beer yeast	18,75 g
Wheat germ	18,75 g

Table 2. Composition of diet to rear Diatrea sacharalis. (Miskimen, G.W. 1965. And adapted to rear *C. clarkei* larvae

Components	Quantity
Cassava flour	450 g
Bacto-agar	3 g
Sorbi acid	2 g
Ascorbi acid	8 g
Distilled water	550 ml

Table 3. Evaluation of C. clarkei development upon feeding on artificial diets.		
Diet	**Diet intake**	**Larva Development**
Sunflower oil	None	Low
Maize oil	None	Low
Sunflower oil plus Cassava flour		Medium
Miskimen's diet plus Cassava flour	High	Medium

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2.1.2 Cassava micropropagation for rapid 'seed' production using temporary immersion bioreactors

Background

Demand for cassava planting material is high and conventional methods of propagation don't satisfy the needs (Buitrago, 1999). Recently a temporary immersion system has been described by Teisson and Alvard (1995) for plant propagation; this system, known as RITA, has been successfully used with banana (Alvard *et al* 1993), coffe (Berthouly 1991), rubber (Etienne *et al* 1993) and sugar cane (Lorenzo *et al* 1998).

Methodology

Cassava clone Venezolana (MCol 2215) was used to develop the technique because of large interest of farmers in the North Coast of Colombia. Plant-material of Venezolana was propagated by conventional solid medium 4E.

Two manifolds with capacity nodal cultures in of 24 RITA each one and with two different immersion periods (C1= 1min/6 hours and C2= 1min/4 hours) were conected. The system was compared to conventional cassava proliferation 4E medium (Roca, 1984) in different combinations of BAP or Kin (liquid media). The evaluation was done for 8 weeks and expressed in terms of number of new explants/initial single nodal culture.

Results and discussion

We observed that immersion period C1 increases propagation rate, showing that longer time between immersion cicles gave better results. In some media (BAP/Kin) hyperhydricity ocurred probably due to interaction of citokinins, concentration effects, and shorter immersion periods (C2). In some cases, shoots obtained in the RITA system showed ethiolation. Increases light intensity to the RITA system was implemented.

BAP-GA_3 medium was supplemented with asparagine and glutamine the propagation rate increased. Elimination of NAA from the propagation media reduced root induction and mechanical damages of tissue during propagation was reduced.

Table 1: Effect of medium composition and immersion period on propagation rate of Mcol 2215

Medium	Immersion period	Propagation rate(*)	
		Conventional solid phase	RITA system
4E	0	2-2.5	-
Liquid media (BAP/Kin)	C1	-	3-3.5
	C2	-	1.6-6.15
4E	C1	-	7.4
	C2	-	5.9

(*) Number of new explants (nodes) obtained per initial single node culture

Conclusion and future activities

- Propagation rate of MCol 2215 using the RITA system increased 7 times
- It is neccesary to adjust the immersion period and citokinin type and concentration in order to reduce hyperhidricity of tissues
- It is possible to increase the rate of shoot propagation by modifing media composition and growth conditions (light, temperature and photoperiod)
- We will introduce MCol 1505 (Verdecita) to the process.
- Tissue response should be monitored through during several cicles of RITA and plants performance in the field.

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2.1.3 Development of a low-cost, simplified, tissue culture system for cassava.

Background

Farmers from the Cauca region in Colombia have encountered limitations in the development of cassava-based systems, namely chronic problem of supply of planting material in adequate quantity of the desired varieties.

This coincides with a long-standing need perceived by CIAT to make tissue culture plant propagation an affordable technology for small farmers (Roca *et al*, 1991).

Methodology

To tackle the factors involved in cassava micropropagation we designed a pilot site outside the BRU laboratory. This site allow us to obtain information on the minimum microclimate standards needed for *in vitro* culture; moreover, the use of low cost equipment, and seeking alternative culture media using local available ingredients (fertilizers, rooting agents, gels, etc), were all included in the pilots study.

This work was implemented using MCol 1522 (Algodona), which is the prefered var. by Cauca farmers.

The work has been carried out in collaboration with an NGO from the area (FIDAR). FIDAR contributed with the participation of a farmer to join the tissue culture team at CIAT, and took the task to involve Cauca farmer associations into the project. The overall project received a small grant from the System Wide Program on Participatory Research.

Results and discussion

- Minimum microclimate conditions The pilot site adapted consisted of one small room, as tissue culture transfer area, one washing-preparing area, and a simple post-flask growing area.

  In the growing area, conditions were adjusted (temperature and light) using a plastic curtain which is opened according to sunlight availability.

- Low cost equipment. We replaced the pH meter, the scale, oven, pipettes, autoclave, glass ware by indicator paper, spoons, gas stove, syringes, pressure cooker and baby jars, respectively.

- Alternative culture media. Based on Roca (1984) that defined 4E medium as standard for cassava micropropagation, basal Murashige and Skook salts (1962), 2% sucrose, 1mg/l Thiamine, 100mg/l m-Inositol, 0.04 mg/l BAP, 0.05 mg/lGA_3, 0.02 mg/l NAA, 0,8% Agar pH 5.7-5.8), we defined some local available media ingredients such as all purpose fertilizer, table sugar, vitamin tablets with thiamin, jelly and running water.

  We considered several fertilizers some were discard because did not allow to fix the pH (®Cosmocel); others produced dirt probably due to plaster (15:15:15). We found that 10 ml of filtered 10:20:20 solution (1 teaspoon /80 ml water) was the best source of mineral salts.

  ®Hormonagro and ®Pro gibb were also tested as NAA and GA_3 sources, respectively. We observed that cassava tissue cultures could use higher doses of Progibb (0.05-0,5 mg/l) than Hormonagro (0.02 mg/l or less). We tested other supplements like malt extract, coconut water and peptone, but did not observe any effect besides improving callus induction and tissue necrosis.

Plants propagated under these conditions were rooted and transplanted to soil using a low-input post-flask management method. This consisted of a plastic bag containing humid soil; small holes were made on the plastic to allow for air exchange.

All transplanted cultures were healthy and become established with success.

- Participatory research. . Collaboration with a farmer (Carlos Hernandez) located by the Cauca community in CIAT and BRU group was organized throughout the experiment. The farmers was able to develop the abilities to prepare media, propagate cassava tissue and evaluate the experiments using simple, local methods.

Conclusion and future plans

- It is possible to replace some components of the 4E medium using local available ingredients, and the cassava cultures can be propagated under non control conditions,
- The success of low cost, simplified, tissue culture for cassava may depend as much on the interest of the farmers and the perceived benefits, as on their skills and the cost of the reagents and equipment,
- Adjusting relations among new sources of growth regulatos will increase propagation rate,
- This new protocol will be tested by farmers under their own local conditions in the Cauca area.
- A Farmer's Work Mannual will be made available with all information on the developed protocols to facilitate access of Cauca's communities to the low input technique.

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Proposal to the plant breeding small grants program. System-wide program on participatory research and gender analysis. Project: "Participatory development of low-cost simplified rustic tissue culture for cassava (micropropagación artesanal)"

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Collaborators

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2.1.4 Cryopreservation of cassava shoot tips using the encapsulation-dehydration technique.

Background

We have developed an encapsulation-dehydration technique as a means to cryopreserve cassava germplasm. This methodology was established by Palacio (1997) and adjusted by Rangel (1998). However, some clones showed a low response to the technique.

In 1999 through the adjustment of the bead quality and dehydration time it was possible to test a sub-core collection of 45 cassava clones.

Methodology

Adjustments were made in the quality of beads (3% alginate with 75mM CaCl2). In spite that 2-3% alginate with 50mM CaCl2 gave good response after freezing, the beads could not be established, to become deformed and shoots were lost under sucrose treatment. Dehydration time was increase to 24 hours. We tested 45 clones with this protocol.

Results and discussion.

Adjustment of beads, associated with dehydration time (24 hours), improved the response after freezing. Only 4.5% of the 45 clones tested showed low response (MNga 1 and MPar 110). Some shoots were small and grew slowly. Adjusting GA3 and light treatments increased total shoots per freezing experiment. We observed that clones from Brazil and Colombia (the most representative countries on the cassava collection) have a consistent response after freezing (46% and 49.9% on average, respectively). This is the first time that a larger group of clones were cryopreserved (Table 1).

Table 1: Response of 45 sub-core cassava clones cryopreserved in liquid nitrogen using encapsulation-dehydration technique

Varieties	% response	
	Viability	Shoot formation
CG 1141-1	83.8	83.8
CM 4063-6	74	29.6
CM 523-7	72.1	53.8
CM 6082-1	90	50
M Bra 12	100	57.1
M Bra 69	86.2	23.7
M Bra 191	76.6	53.3
M Bra 325	90	60
M Bra 328	83.3	50
M Bra 337	56	50
M Bra 383	49.1	28.3
M Bra 474	-	-
M Bra 507	85.9	71
M Bra 514	53.6	26.6
M Bra 590	87.5	62.5
M Bra 632	80.5	64
M Bra 691	67.6	43.3
M Bra 698	63.3	20
M Bra 759	80	30
M Bra 769	83.3	43.3
M Bra 839	85.3	58.8
M Bra 881	92.8	32.14
M Bra 894	96.4	50
M Col 1468	35.7	28.5
M Col 1505	79.4	76.4
M Col 2016	70	20
M Col 22	95	95
M Col 32	33.3	23.3
M Col 40	-	-
M Col 511	93.7	56.2
M CR 113	60.7	42.8
M Cub 29	72.7	54.5
M Cub 39	-	-
M Cub 55	80	66.6
M Ecu 117	81.5	48.1
M Mal 2	71.1	55
M Mex 71	75	40.62
M Nga 1	45	15
M Nga 5	72.7	54.5
M Pan 51	100	84.8
M Par 110	46	0
M Par 71	96	96
M PTR 19	77.5	21.8
M Ven 232	94.4	50

Conclusions and future activities.

- 45 cassava clones were cryopreserved and shoots were recovered after freezing in liquid nitrogen
- 42 cassava clones showed recovery rates up to 20%
- It is possible to increase shoot recovery rate up to 5-10% by modification on growth conditions and recovery media.
- The number of cassava clones for cryopreservation will be increased to 105

Reference.

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Collaborators

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SB-2 Project

2.1.5 Use of wild beans for the identification of QTLs for yield improvement

Rationale

Wild germplasm is now recognized as an important source of genes for yield and other agronomic and quality traits. The advanced backcross scheme for incorporating useful alleles from unadapted wild relatives into the genome of a crop species (Tanksley and Nelson, 1996) has been shown to be a promising technique for the improvement of inbred crops. Last year we reported on progress in the development of populations to introgress genes from wild to cultivated bean. The populations were prepared by backcrossing cultivated x wild bean hybrids twice to the cultivated parent and developing lines by a modified backcross inbred method. This year we have the first yield data from those populations.

Materials and Methods

Four populations of crosses with wild beans have been tested so far. Two populations were derived from an Andean recurrent parent (the large-seeded rojo bolon variety 'Cerinza Radical') and two from a Mesoamerican recurrent parent (the small black-seeded DOR390). The population were all tested in the BC_2F_5 generation over at least 2 locations each with from 2-6 replications.

The first of the Andean populations, was derived from the cross of Cerinza x G24390, a wild bean from Nayarit, Mexico. This population was field tested in two seasons in Popayan and once in the dry season in Palmira. An ascoquita epidemic severely affected the second season's planting in Popayan and was not harvested. This same population was also sent to Puerto Rico and Panama for evaluation, and data is already available for Puerto Rico. A second Andean population, was derived from the cross of Cerinza x G24404, a Colombian wild bean. This population was also tested in two seasons in Popayan and withstood the ascoquita epidemic better than the first popuation, giving low but informative yields.

Meanwhile, the first of the Mesoamerican populations, resulting from the cross of DOR 390 x G24390, was planted in one yield trial in Santander de Quilichao. A second Mesoamerican population was derived from the cross of DOR 390 x G24423, another wild bean from Colombia. This population was yield tested in two environments, Santander de Quilichao and Palmira. The wild progenitors for this work were selected on the basis of AFLP analysis that suggested that all these wild accessions were relatively distant genetically from cultivated beans.

For each of the four populations, a basic set of 100 lines was selected and used in yield trials which were planted in a 10 x 10 lattice design. Four replications were used in the dry season in Palmira, two in Puerto Rico and three in all the other locations. Another set of extra additional lines was selected for each of the populations. These lines were planted in unreplicated trials for observation and yield estimates. However, the additional lines of the Cerinza x G24390 population were also planted in a replicated yield trial, during the dry season in Palmira. This trial consisted of two 10 x 10 lattices with four replications. All together a total of 300 lines were tested for this population. Large numbers are important for statistical accuracy of the QTL analysis with the backcross inbred population structure. In addition to collecting yield data, the days to flowering, days to maturity and 100 seed weight were determined.

In the laboratory, we are beginning to conduct the marker analysis on the populations. In a preliminary study, DNA from the lines of the Cerinza x G24390 population was extracted and has been analyzed with 10 RAPD primers, that were selected because they produce bands that have been mapped and characterized in terms of prevalence in the core collection. Both the basic and additional sets of lines of this population were analyzed to maximize the population size.

Results

The results from the testing of the Andean populations shows that yields were significantly correlated over seasons in the same location. In Popayan, a proportion of the introgression lines significantly out performed the recurrent parent by as much as 400 kg/ha. The results were not significant in Puerto Rico because of high coefficient of variation, however there were lines there that also outyielded the recurrent parent by 400 kg/ha. Some of the lines that significantly out-performed Cerinza in terms of yield, had

smaller seed size than Cerinza, but a few were of a similar size and appearance as Cerinza. While Cerinza has a medium red, square shaped grain, some of the introgressions from the wild beans produced seeds that were darker red, purple, black or red mottled and rounded. The plant type, days to flowering and days to maturity of the introgression lines were very similar to the recurrent parent. Very few of the lines were indeterminate or late-flowering in those populations.

In the case of the Mesoamerican populations, although the derived lines were significantly different in yield in both populations, neither population produced lines that were statistically superior to DOR 390. The best line in each case produced about 300 kg more than DOR 390, which was not a significant advantage. This is not a surprise since introgression from the wild bean normally reduces yield, and it was anticipated at the outset that it might be necessary to recur to molecular markers to recognize positive QTL and to separate their effects from the overriding negative effects of wild beans.

The differences observed between the advantage of the wild crosses in Mesoamerican and Andean genepools was unexpected. It is possible that the Andean lines benefited more from the incorporation of wild germplasm because of the more limited diversity of the Andean gene pool when compared to the Mesoamerican gene pool. It is also possible that introgrssions from wild beans has occurred in the past more frequently with the Mesomarican gene pool, thus already capturing the advantageous alleles that the wild accession can provide. The same may not have occurred with Andean beans. It would be interesting to quantify the potential and likelihood of introgressions acruing from wild beans into cultivated beans, by evaluating where the range of wild and cultivated beans tends to overlap the most.

According to results with RAPD markers it appears that introgressions from the wild accessions were successfully incorporated into the backcross lines. Polymorphism between the wild and cultivated beans appeared to be high and evidence of outcrossing was not observed. Overall, the amount of introgression observed was in agreement with the ratio expected for a second backcross generation. Segregation distortion, which is the lower than expected occurrence of a given allele, was observed for some loci, represented by individual RAPD bands, more than others. This probably reflects the linkage of these loci to undesirable traits that were eliminated during the by selection of lines that were more like the recurrent type. Distortion segregation was greater for the 100 selected lines than for the additional lines, indicating that great selection pressure was applied during the development of these lines. For the upcoming year we will be using single-copy markers, such as the microsatellites and SCARs to supplement the results from the RAPDs and to provide a framework map on which to analyze the quantitative data. QTL analysis will be conducted with qGENE and QTL cartographer software. The molecular analysis will be extended to the additional Andean crosses and the Mesoamerican crosses as well in the near future.

Conclusions

While it is too early to draw final conclusions, it can be stated that additional variability can be incorporated into cultivated beans from wide crosses with wild accessions. We hope to identify the genomic location of the yield genes that have been introgressed into the recurrent parent background by conducting a QTL analysis combining the filed and laboratory data. Molecular markers will be a necessary element to follow the yield genes in these crosses. The identification of important yield genes in this project should lead to further more detailed studies. Marker assisted selection will be useful to transfer the yield genes into other genetic backgrounds. We are already making the crosses to recover useful QTLs from this initial study into other Andean seed-types (red mottled and red kidney). Fine-mapping around the QTLs can be used to determine whether the important loci are single genes or blocks of several genes together.

Meanwhile, we are developing other populations derived from wild beans from Argentina, Mexico and N. Peru. Four other populations of Cerinza and DOR390 crossed with wild parents from Argentina and Mexico are being advanced to the BC_2F_3 generation and might also be used for the QTL analysis. The first populations will be tested in a preliminary replicated yield trial this season, while the the others will be evaluated to identify promising lines. Early generation testing is possible with the advanced backcross family structure since selection occurs among F_1 plants and return to commercial type is rapid. The BC_2F_1's of the final population, Cerinza x Peruvian wild accession, were selected this June in Popayan and will be advanced.

Collaborators

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2.1.6 Genes from wild rice contribute to yield increase in cultivated rice

Introduction

Twenty one wild species and two cultivated species (*O.sativa* and *O. glaberrima*) represent ample 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. However, limited use of this variability has taken place. Barriers still exist in effectively utilizing genes from wild species and molecular mapping techniques are needed to readily detect these new alleles in segregating populations.

CIAT started in 1994 a collaborative project aimed at characterizing and utilizing wild rice species for the improvement of cultivated rice. We here report on progress made in the identification of QTLs associated with yield increase in *O. rufipogon* and *O. barthii*.

Plant materials and field trials

The 300 BC_2F_2 families derived from the cross BG90-2/ *O. rufipogon*, and the 326 BC_3F_2 families from the cross Lemont / *O.barthii* were planted in replicated yield trials in CIAT- Palmira.

Transplanting (20x30) was done and a completely randomized design with two reps., 2 row-plot, 5 m. long was used. Data on 12 agronomic data, including plot yield/family were taken on 10 randomly selected plants/plot. Based on yield potential and good agronomic traits, 38 BC_2F_2 families from the cross BG90-2 / *O. rufipogon* were selected and further evaluated for grain yield as BC_2F_3 families; a completely randomized design with four reps, 4 row-plot, and 5 m. long was used.

Materials and Methods

Two improved rice cultivars (BG90-2 and Lemont) were crossed *to O. rufipogon* and *O. barthii*, respectively. Few plants (2-3) in each of the wild species were hybridized to several plants of each of the improved cultivars recurrent parents). Single crosses were obtained and grown in the greenhouse at CIAT in 1994. Three F_1 hybrid plants were backcrossed to the improved cultivar (Fig 1 and 2); 153-198 BC_1F_1 seeds were obtained per cross combination. The resulting BC_1F_1 plants were transplanted (30x50 cm) and evaluated based on phenotype; negative phenotypic selection for undesirable agronomic traits (spreading plant type, excessive shattering, long awns, dark-color grains, high sterility, etc.) was used to narrow the selection down to the best (30-50) individuals. Each selected BC_1 individual was back crossed again to the recurrent parent and approx. 30 BC_2F_1 seed were sown in wooden trays in the screenhouse and later on transplanted (30x40 cm) under irrigated conditions. A negative phenotypic selection was applied again and best individuals per cross were selected and harvested individually to generate BC_2F_2 seed; 300 BC_2F_1 plants were selected in the BG90-2/ *O. rufipogon* cross for field testing, whilst high sterility was found in the Lemont / *O. barthii* cross. Therefore, another BC to Lemont was done and 326 BC_3F_1 plants were selected for field testing as BC_3F_2 families.

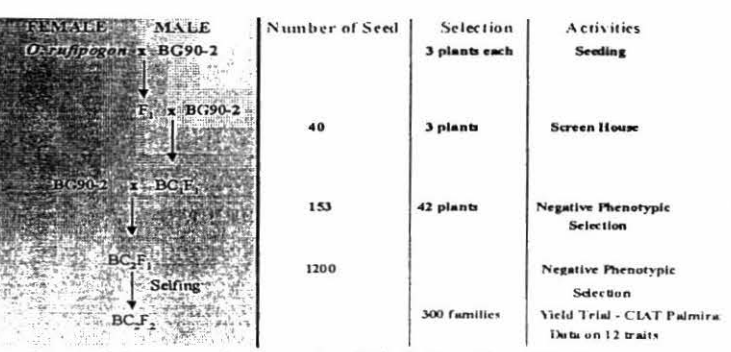

Fig. 1. Scheme used to develop BC_2F_2 families with *O. rufipogon*

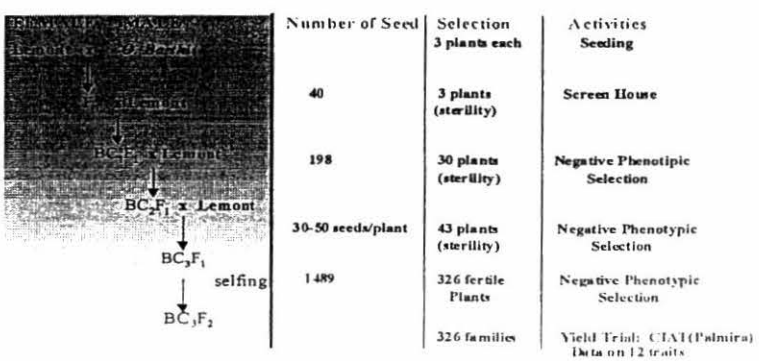

Fig. 2. Scheme used to develop BC_3F_2 families with *O. barthii*

Molecular Characterization

DNA of young leaves from the parentales genotypes, the BC_3F_2 families was extract by the Dellaporta Method (McCouch et al. 1988) modified for PCR assay by CIAT Biotechnology Research Unit (unpublished data). Parental surveys filters containing *O. rufipogon, O. barthii*, BG90-2, and Lemont were prepared using five restriction enzymes (EcoRI, EcoRV, HindIII, XbaI and DraI). Aproximately 140 markers from the rice molecular framework linkage map were selected at 10-20 cM intervals throughout the genome. A set of 78 mapped rice microsatellite markers, developed at Cornell University, was also used to complement the RFLPs in QTL analysis.

Results and Discussion

The distribution of grain yield (kg/ha) of 300 BC_2F_2 families (BG90-2/*O. rufipogon*) derived from plot yields of 40 plants (20 plants/row x 2 row) averaged over two replications.

Transgressive segregation can be observed, with several lines (11%) having between 5 and 25% higher yield than the recurrent parent BG90-2. Transgressive segregation for other yield components was also observed. Grain yield data taken on 38 BC_2F_3 families (Table 1a) confirmed results obtained in the BC_2F_2 generation.

Based on the 88 RFLP and 39 microsatellites from the RF- Cornell framework map screened on 300 BC_2F_2 families, putative linkages were identified with yield, and yield

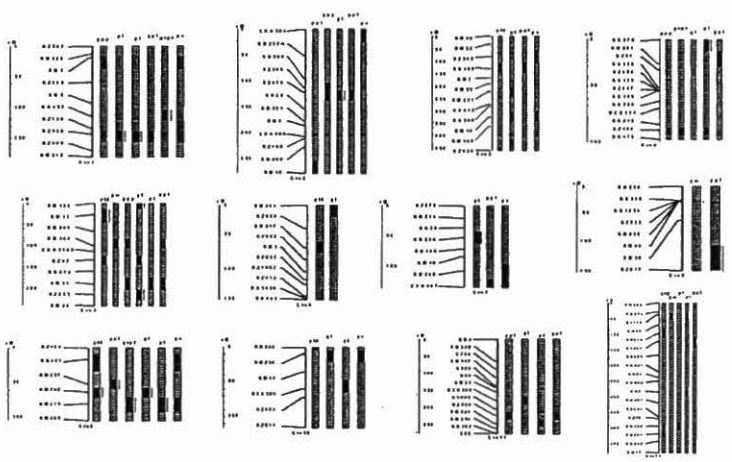

Figure 3a. y 3b. Molecular mapping and QTL alleles for yield and yield components (BG90-2 x *O. rufipogon*).

components from replicated data available for the whole mapping population. Results using Qgene macintosh software, version 2.30_AA (Nelson 1997) indicate associations between markers and yield on chromosomes 3, 5, 6, 9, 10 and 12. Results obtained in

chromosomes 5 and 12 showed similar associations to Xiao *et al.* 1996 and 1998 results (Fig. 3a and 3b). No linkage for yield was detected on chromosome 1 as reported by J. Xiao and S. McCouch. Other associations were also identified for yield and the various yield components (Fig. 3a and 3b). These data from different groups working with diverse recurrent parents suggest that DNA introgressed from *O. rufipogon* can contribute positively to yield in elite rice cultivars.

The distribution of grain yield (kg/ha.) of 326 BC_3F_2 families (Lemont / *O. barthii*) derived from plot yields of 40 plants (20 plants/row x 2 row) averaged over two replications. Transgressive segregation can be observed, with several lines having up to 30% higher yield than Lemont (Table 1b). Based on the 54 microsatellites from RF-Cornell framework map screened on 326 BC_3F_2 families derived from the cross Lemont / *O. barthii* putative linkages were identified. Preliminary results using one way anova and t-test indicate association between markers and yield on chromosome 2 similar to Xiao et al. and on chromosome 7.

Grain Yield (Kg/ha) of some BC_2F_3 families from BG90-2/*O. rufipogon* cross at CIAT. 1997

Line/pedigree	Yield	(%) of BG90-2
CT13941-11-M	7880	21
CT13958-12-M	7746	19
CT131946-1-M	7535	16
CT13976-7-M	7519	16
Ct13946-26-M	7359	13
BG90-2	6496	0
O. rufipogon	4998	-23

* Minimum signif difference. Dunnett's = 1582

Grain Yield (Kg/ha) of some BC_3F_2 families from Lemont/*O. Barthii* cross at CIAT. 1997.

Line/pedigree	Yield	(%) de rendimiento
CT14949-28	6193.5	136.05
CT14937-24	5899.24	129.59
CT14964-3	5629.59	123.66
CT14938-5	5620.73	123.47
CT14937-10	5526.44	121.4
CT14955-15	5514.25	121.13
CT14966-9	5513.34	121.11
CT14937-5	5498.15	120.78
CT14946-69	5496.81	120.75
CT14937-26	5450.14	119.72
Lemont	4552.39	100
O. barthii	1065.72	23.41

Table 1a and 1b. Some families of the backcross populations in this study that have a higher yield than recurrent parent.

On-Going Activities

1. Complete the characterization of agronomic and molecular data, and QTL analysis to determine the number of QTLs associated with yield increase across environments for Lemont x *O. barthii* cross.
2. Determination of contribution for positive alleles of each of the parents.
3. Development of NILs based on QTL analysis carrying specific QTLs for use in breeding programs
4. Start agronomic and molecular characterization of several other populations involving crosses with *O. glaberrima* to determine of QTLs for yield increase.

Acknowledgments

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Collaborators

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

2.1.7 Foreign genes as novel sources of resistance: Resistance of rice to rice hoja blanca virus (RHBV) and *Rhizoctonia solani.*

Background

Last year, results from research conducted by our research group suggested that the resistance conferred by the N transgene towards RHBV disease is expressed independently of the genotype background. The N transgene could be used to complement the natural resistance source to the virus, when crossing selected transgenic lines with diverse genotypes carrying the breeding resistance gene(s) since it is expressed on plants at 10-day-old. Significant progress has been attained, however, immunity at this early age has not been recovered yet in the hybrids containing the RHBV-N

transgene and the breeding resistance source. The resistance conferred by the N gene is characterized by a significant delay in the progression and severity of disease respect to inoculated non-transgenic controls. Besides RHBV, *Rhizoctonia solani* (sheath blight) is already causing important rice yield losses in the Southern cone of South America and increaseing spreads had been reported in Colombia, Mexico and Venezuela. All rice varieties are susceptible and there is not known source of stable genetic resistance in rice. Biological control of this disease has not been successful either. At present, the control depends on heavy use of fungicides (Dr. Fernando Correa, CIAT Rice Pathologist, Cali, Colombia, personal communication). Therefore, incorporation of resistance for this disease by genetic engineering is an attractive approach. Work conducted by another principal investigators in this project (Nilgun Tumer , Rutgers University, USA) showed that a pokeweed antiviral protein (PAP), a 29-kDa protein isolated from *Phytolacca americana*, has a ribosome-inactivating ability and a potent antiviral activity against many plant and animal viruses, including HIV (Tumer et al., 1997). Interesting enough, mutated versions of PAP gene also confer resistance to fungal infection (Zoubenko et al., 1997). Homozygous progeny of transgenic tobacco plants expressing these PAP genes displayed resistance to the fungal pathogen *Rhizoctonia solani*. These results suggest the possibility of designing molecular strategies for incorporating dual antiviral and fungal resistance by introgressing mutant PAP gene(s) in transgenic rice plants. Here we report the progress made during the seven months when this project was initiated.

Materials and Methods

Indica rice varieties Cica 8 (control for transformation efficiency), Palmar, Cimarrón, and PNA004 will be used as targets. Palmar and Cimarrón shows high and moderate tolerance to sheath blight, and PNA004 carries the Colombian 1 source of resistance to RHBV. *Agrobacterium* mediated transformation of these varieties is being optimized (see work on this annual report). To generate new point mutations in the PAP gene, a quick change site directed mutagenesis kit from Startagene was used. Genes constructs carrying various mutant versions of the PAP gene, or the RHBV NS4 (non-structural RHBV RNA4) gene in sense or antisense directions are being placed in vectors driven by 35S CaMV promoter or maize ubiquitin promoter, and using hygromicin resistance as gene selection.

Results

Eight new PAP mutations were generated directed to change aminoacid composition in the PAP protein (Table 1). These new mutated genes were placed into yeast vectors, and transformed into yeast to check for no toxicity. The non-toxic mutated genes will be transformed into tobacco first to check the gene expression before using them for rice trasnformation.

Table 1.- New mutations generated in PAP gene.

	Mutation	AA Change	Name in Plant Vector	Name in Yeast Vector
1.	PAPI Del	I4M	NT296	NT299
2.	PAPI Del	T18M	NT298	NT300
3.	PAPI Del	I13M	NT317	NT311
4.	PAPI Del	V8M	NT319	NT312
5.	PAPI Del	Y16M	NT	NT
6.	PAPI Point	Y16A	NT	NT
7.	PAPI Point	Y16S	NT	NT
8.	PAPI Point	Y16Phe	NT	NT

Two mutated versions of PAP (I and II) already tested for no toxicity in turfgrass (another monocot species) will be used as the first approach to transform rice (Table 2). These genes driven by the ubiquitin promoter were placed in the plasmid vectors pWBVec8, pWB10a, and pBGXiHGFP kindly supplied by Dr. Peter Waterhouse (CSIRO, Australia). These plasmid had been used succesfully by Waterhouse to trasnform rice via Agrobacterium. They contain a hpt gene with a CAT-1 intron for increased expression of hygromycin resistance and selection in rice, a gus-intron-gene, or a gfp (green fish fluorescent) gene, respectively, to aid the recovery of transgenic plants.

The RHBV NS4 gene in sense and anti-sense orientation driven by the 35S CaMV promoter were placed into the plasmid pCAMBIA 1301 carrying the gus-intron and hygromycin resistance gene (Table 3). The NS4 gene in both directions driven by the unbiquitin promoter is ready to be moved into vectors carrying the hygromycin-cat 1 intron gene from Watehouse laboratory.

Table 2.- Description of PAP constructs for Plant Transformation generated.

	GEN	Promoter	Vector/orientation	Other genes	Transf	Name
1	PAPId	Maize Ubiq.	NT168	Amp	B	NT178
2	PAPId	Maize Ubiq.	NT294	Spec,GFP,Hygi	A	NT301
3	PAPId	Maize Ubiq.	PWBVec10a	Spec,GUS,Hygi	A	NT303
4	PAPId	Maize Ubiq.	PWBVec8	Spec,Hygi	A	NT306
5	PAPII	Maize Ubiq.	NT168	Amp	B	RTT126
6	PAPII	Maize Ubiq.	NT294	Spec,GFP,Hygi	A	NT302
7	PAPII	Maize Ubiq.	PWBVec10a	Spec,GUS,Hygi	A	NT304
8	PAPII	Maize Ubiq.	PWBVec8	Spec,Hygi	A	NT305

Table 3.-Description of RHBV-NS4 constructs generated in CIAT

	GEN	Promoter	Vector/orientation	Other genes	Name
1	NS4	35S	PC1300/sense	Hyg,Kan	pIC001
2	NS4	35S	PC1300/asense	Hyg,Kan	pIC003
3	NS4	35S	PC1301/sense	Hyg,Kan,GUSi	pIC002
4	NS4	35S	PC1301/asense	Hyg,Kan,GUSi	pIC004
5	NS4	Maize Ubiq.	NT168/sense	Amp	pIC005
6	NS4	Maize Ubiq.	NT168/asense	Amp	pIC006
7	NS4	Maize Ubiq.	PWBVec8/sense	Spec,Hygi	pIC007
8	NS4	Maize Ubiq.	PWBVec8/asense	Spec,Hygi	pIC008
9	NS4	35S	PWBVec8/sense	Spec,Hygi	pIC009
10	NS4	35S	PWBVec8/asense	Spec,Hygi	pIC010

Future Plans

- Optimize the genetic transformation mediated by *Agrobacterium* of the genotypes selected using the gene constructs carrying the gus-intron gene.
- Generate transgenic rice carrying the different versions of PAP or NS4.
- Evaluate the gene integration by Southern, and gene expression by Northern and Western of the transgenic plants evaluated.

- Characterized transgenic plants for stable expression and inheritance of the transgenes.
- Study the level and stability of the RHBV resistance confer by PAP alone or in combination with the RHBV-viral trasngenes and/or the Colombia 1 breeding resistance source.
- Evaluate spectrum of *Rhizoctonia* resistance encoded by PAP gene.

Collaborators

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2.1.8 *Agrobacterium* mediated genetic transformation of *indica* and *japonica* rice

Background

Last year, we reported the preliminary results on the optimization of Agrobacterium mediated transformation of local rice varieties. It included *indica* rices (CICA 8, IR-72, INIAP 12) adapted to irrigated (flooded) conditions, and *japonica* rices (CT 6241, CT 10069, and Lastisday-Fofifa) adapted to acid soils (savanna) and highland (hillside) environments. Protocols established for rice were reviewed (Hiei *et al.*, 1994; Aldemita,1996; and Toki , 1997) and the necessary modifications for optimization using the selected breeding genotypes were introduced. The studies conducted suggested that the selection of the explant source and preculture conditions of the target tissue are major key factors for *Agrobacterium* mediated transformation of rice. Embryogenic scutellum derived callus showed higher transient GUS expression than immature embryos. Higher number of hygromycin resistant (Hyg^r) callus was recovered by preculturing the callus three days before co-cultivation with the Agrobacterium. Between 60% and 100% of these Hyg^r callus showed stable GUS expression (GUS^+) 90 days after infection. Following we report the efficiency on plant regeneration after selection on 50 mg/l hygromycin, the analysis on Southern blot of the T0 plants, and the inheritance of GUS^+

and Hyg^r in T1 plants. Furthermore, preliminary results on three other indica genotypes (Palmar, Cimarrón, PNA004) using the established protocol are also described.

Materials and Methods

The three protocols developed for rice (Hiei *et al.*, 1994 and 1997; Aldemita,1996; and Toki , 1997) were tested. Preliminary results showed differences between the three protocols established for rice transformation. Following are described the modifications introduced into Aldemita and Hodges (1996)'s protocol, which gave the highest response for the genotypes tested so far. Instead of using N6 as recommended by Aldemita and Hodges (1996), scutellum derived callus was induced and sub-cultured for 0 to 3 days on NBA medium (Li te al., 1993) containing proline and NAA prior the co-cultivation (preculture) with the bacteria. The the callus was co-cultivated with *A.tumefaciens* strain LBA4404 (pTOK233) or AGL1 (pCAMBIA 1301) in NBA-AS medium containing 100μM acetosyringone for 3 or 5-6 days. Casamino acids and kinetin were omitted in the co-cultivation and selection media, and 20μl 100μM acetosyringone were added 2 hours prior the co-cultivation and onto the co-cultivated callus to reactivate further the *vir* genes. After co-cultivation, the agro-infected callus were washed with N6 salts (Chu et al., 1975) containing carbenicillin (250 mg/L), cefotaxime (100 mg/L), and hygromycin (50 mg/L) to kill the bacteria. The callus were then transferred onto the selection medium A [NBA containing carbenicillin (250 mg/L), cefotaxime (100 mg/L), and hygromycin (30 mg/L)] for three weeks. The healthy looking callus were sub-cultivated onto medium B [NBA containing carbenicillin (250 mg/L), cefotaxime (100 mg/L), and hygromycin (50 mg/L)] for other three weeks. Following the transgenic calli were first transferred onto a proliferation medium (LS with 0.5 mg/l 2,4-D and 50 mg/L hygromycin) for 3 weeks and then to a regeneration medium (MS with NAA 1 mg/l, kinetin 4 mg/l).

Results and Discussion

A total of 306 Hyg^r callus derived from co-cultivation with LBA 4404 (pTOK233) were transferred onto regeneration medium containing 50 mg/l hygromycin. Between 20% to 68% of the callus regenerated plants. Total of 86 T0 plants were transferred to the greenhouse, and 80% to 96% of these plants were GUS^+. Southern blot and PCR analysis confirmed the integration of both GUS^+ and Hyg^r genes in 50% to 100% of the regenerated plants. These results suggest a transformation efficiency of 7% to 18%. T1 seeds from plants showing integration of both transgenes were harvested and analyzed for germination on medium containing 50 mg/l hygromycin and GUS expression. For Cica 8 (indica), 73% of the T1 lines derived from independent T0 transgenic lines inherited GUS expression; whereas for CT 6241 and CT10069 (japonica), 100% of the T1 lines were GUS^+ (Table 1 and 2). Segregation for GUS expression at the T1 generation indicated a Mendelian segregation of 3:1 or 1:0 ratio for GUS^+:GUS^- suggesting the presence of one (3:1) or more active GUS locus. Similar results were obtained for the inheritance of the Hyg^r gene indicating either a 3:1 (eight T1 lines) or 1:0 ratios (four T1 lines).

Table 1.- Gus expression in T1 generation derived from Cica 8 plants transformed with LBA 4404 (pTOK233).

T0 line	Seeds	Seedlings	GUS$^+$	GUS$^-$
5	25	25	25	0
7	25	25	25	0
12	27	25	25	0
18	25	25	0	25
21	28	23	23	0
22	28	25	25	0
25	26	25	25	0
26	28	26	26	0
27	28	27	0	27
28	27	27	0	27
30	28	27	27	0
34	28	27	26	1
38	28	27	27	0
40	27	26	0	26
41	27	26	26	0
Cica-8 control	26	26	0	26

Two lines showed a significant lower number of Hyg^r plants than expected suggesting a 1:1 ratio (Table 3). Of the 14 T1 lines analyzed showing resistance to hygromycin, 21% did not co-segregated for GUS expression, indicating gene silencing or sorting out of the GUS gene throughout meiosis. Preliminary results on transient gus expression on recalcitrant rice genotypes, such the indica varieties Palmar, Cimarón, and PNA004, using the *Agrobacterium* strain AGL1 (pCAMBIA 1301) suggest that the protocol establish for LBA 4404(pTOK233) can be used with minor modifications. These include increasing the days of agroinfection from 3 to 6 days. Selection of these callus on hygromycin containing medium is in progress.

Table 2.- Gus expression in T1 generation derived from CT 6241 and CT 10069 plants transformed with LBA 4404 (pTOK233).

Genotype	T0 line	GUS$^+$	GUS$^-$
CT10069	3	25	0
	5	21	4
	6	25	0
	7	20	5
	8	23	0
	9	24	0
	11	25	0
	12	24	5
	13	24	5
	14	23	2
	15	21	4
	17	24	1
	18	24	1

Genotype	T0 line	GUS$^+$	GUS$^-$
	1	20	5
	5	19	6
	6	16	7
	11	23	2
	15	15	10
CT6241	16	17	8
	17	23	2
	18	19	6
	19	19	6
	20	25	0
	21	20	5
	22	21	4
	24	24	1
	25	17	8

Table 3.- Co-segregation for hygromycin resistance and GUS expression in T1 generation derived from Cica 8 plants transformed with LBA 4404 (pTOK233).

T0 line	Seeds	Seedlings Hyg^r : Hyg^s	Seedlings Hyg^r tested for Gus expression	GUS$^+$	GUS$^-$
5	25	23:2	20	17	3
7	24	23:1	6	6	0
12	26	21:5	6	6	0
18	22	16:6	6	0	6
21	25	22:3	6	6	0
22	24	21:3	6	6	0
25	22	18:4	6	6	0
26	27	16:11	6	6	0
27	22	16:6	6	0	6
28	23	17:6	6	1	5
30	23	17:6	6	6	0
34	24	19:5	6	6	0
40	26	14:12	6	0	6
41	25	18:7	6	6	0
Cica-8	22	0:22	0	0	0

Future Plans

- Complete phenotypic and genetic inheritance analysis of T1 lines.
- Comparison of transformation efficiency between LBA 4404 and AGL1 strains.
- Comparison of efficiency between *Agrobacterium* mediated transformation and particle bombardment.
- Use of *Agrobacterium* transformation to incoprorate NS4 and PAP genes for RHBV and *Rhizoctonia* resistance in indica rice.

Collaborators

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2.1.9 *In vitro* clonal propagation method of soursop (*Annona muricata* l.)

Introduction

In 1998 we developed a new micrografting method for micropropagation of soursop using elongated axillary shoots of the clone "*Elita*" (Rios Castaño et al., 1996) as scions and *in vitro* germinated "*Elita*" plantlets as rootstocks (Royero et al. 1998).

This year the method was improved (method B) to reduce the time required for production of micrografted plants.

Growth evaluation of cultivated micrografted plants and the standardization of agronomic practices began at farms located on different microenvironments.
New commercial clones and related species of *A. muricata* were selected to begin the application of the micrografting method.

Results and Discussion

Table 1. Production of micrografted, disease-free soursop plants

Steps	Average success and time required	
	Method A	**Method B**
Mother plants selection and preparation in the screen-house (trimming, fungicide and antibiotics sprying, hormone treatment); selection of best stems for *in vitro* planting.	8 stems / mother plant [60 days]	
Stem planting *in vitro* and elongation of axillary buds.	47 % of stems produced optimum scions [30 days]	100 % [45 days]
Axillary bud multiplication rate.	**256 / year**	**16380 / year**
Rootstock production from sexual seed (seed sterilization, *in vitro* germination and growth)	81 % of seeds produced optimum rootstocks [45 days]	83 % [45 days]
In vitro micrografting.	61 % succesful micrografts [45 days]	73 % [45 days]
Hardening in greenhouse conditions.	66 % of micrografts developed in the greenhouse. [180 days]	90 % [180 days]
Total.	**360 days**	**315 days**

In the method B we used an *in vitro* stock of micrografted "*Elita*" plants as source of axillary buds. The use of sterile micrografted plants to obtain buds allow the elimination of the establishment of mother plants (first step in method A). Growth factors in rootstock of micrografted plants stimulate the elongation of axillary buds more efficiently and contribute to the improvement of axillary bud multiplication rate that is theoretically of 4^7 per year.

The *in vitro* development of 3 or 4 complete leaves of micrografted plants is an advantage to a successful establishment in the green-house.

The micrografting method will be applied to new selected clones and *A. muricata* related species (Table 2) to evaluate the method and to look for new scion/rootstock combinations.

Sets of forty Elita/Elita micrografted plants, hardened in greenhouse during six months, were transplanted to different places (Table 3) for field testing. During the first two years, the growth of micrografted plants will be evaluated. Every six months data of stem perimeter, treetop perimeter and anthracnose presence will be recorded. In Venecia III micrografted plants v.s grafted plants will be compared in a split plot design.

Table 2. Clones and *Annona* species selected for micrografting.

Material	Origin	Characteristics
Clones		
Costa Rica	Costa Rica	- high productivity (80 – 100 kg/tree/year) - adapted to Huila soils. - delicious fruit, ^{0} brix : 15-18 - big fruit (4 - 5 kg), without spicules
Rojas	Campo Alegre (Huila)	- high productivity (80 – 100 kg/tree/year) - delicious fruit, ^{0} brix : 15-18 - big fruit (4 - 5 kg), short spicules
San Francisco	Yaguará (Huila)	- high productivity (>100 kg/tree/year) - delicious fruit, ^{0} brix : 15-18
Rootstock		
Rojas	Campo Alegre (Huila)	- adapted to Huila soils.
Anón amazónico	Vaupes	- rustic, adapted to amazonian soils
A. montana	Chocó	- rustic, adapted to Chocó soils - possible resistance against anthracnose
A. cherimola	Anzoátegui (Tolima)	- adapted to high lands (>1200 msnm.)

Table 3. Field evaluation of soursop micrografted plants in different microenvironments.

Date of sowing	Place	Genotype (scions/rootstock)
January, 99	Empresa Comunitaria San Francisco, Yaguará (Huila)	*Elita / Elita*
January, 99	Hacienda El Castillo, Santa Elena (centro del Valle)	*Elita / Elita*
March, 99	Hacienda La Esneda, Santa Elena (centro del Valle)	*Elita / Elita*
April, 99	Hacienda Cerritos, Yaguará (Huila)	*Elita / Elita*
April, 99	Montebello, Nilo (Cundinamarca)	*Elita / Elita*
August, 99	Hacienda Venecia III, Caicedonia (norte del Valle)	*Elita / Elita*
September, 99	Hacienda Normandía, Toro (norte del Valle)	*Elita / Elita*; *Elita / Rojas*

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2.1.10 Genetic transformation of lulo and tree tomato

The tropical fruits are very important for the international market; lulo (*Solanum quitoense*) and tree tomato or tamarillo (*Solanum betacearum*) have been recently identified as the best for its flavor and aroma qualities. A major limitantions is the attack of nematodes (*Meloidogine* spp.), that makes it necessary to develop genetic transformation techniques. The use of the *MI* –1.2 gen (Milligan *et al.* 1998) was selected to obtain resistance by means of genetic engineering.

Materials and Methods

Transformation

A medium was developed to be used in the transformation of leaf pieces of lulo and tree tomato. After 15 days of *in vitro* germination, shoots from both fruits are subjected to ultrasonic treatment to wound the tissues. After infection with *Agrobacterium*, tissues are left for three days at 22°C and 12 photoperiod hours. Then the tissue is cleaned with liquid media containing 40 mg/L of Vancomicine and 200 mg/L of Cefotaxime. In this medium they are left for 2 days, and finally subcultured for regeneration in solid medium supplemented with selected antibiotics.

Lulo o naranjilla (*Solanum quitoense*) and tree tomato (Cifomandra betacea) are important commercial fruit crops in Colombia although they are very susceptible to nematodes. To produce varieties resistant to rook knot nematodes we acquired the gene Mi-1.2 (plasmid pC201 from the Department of Nematology of the University of California, Davis). The Mi-1.2 gene was originally introudced into cultivated tomato from Lycopersicon peruvianum to confer resistance to three of the most damaging species of root knot nematodes (Meloidognyne spp; Milligan et al. 1998). The Mi-1.2 gene has baout 9 kb of size and does not have regulator sequences. Our objective is to make genetic constructs containing the Mi-1.2 (Figure 1) and insert them into binary vectors for Agrobatericum-mediated transformation of commercial cultivars of lulo and tree tomato.

Results

The plasmid pTRAMi was constructed to place regulatory sequences at both ends of the *Mi*-1.2 gene (Figure 1). pTRAMi has approximate 12 kb and contains an ampicillin resistance gene. The *Bam*H I fragment of *Mi*-1.2 is in front of an enhanced 35S promoter and followed by the transcription termination signal from a tumor morphology large gene (*tml*) from *A. tumefaciens*. Currently we have six clones with the *Mi*-1.2 gene plus regulatory sequences. We will confirm which clone has the correct orientation to introduce it into the binary plasmid pCAMBIA 2301 to place it into *A. tumefaciens* strains for plant transformation. The new binary vector will contain slectable (*npt*II for antibiotic resistance) and scorable (*gus*-intron for color detection) genes.

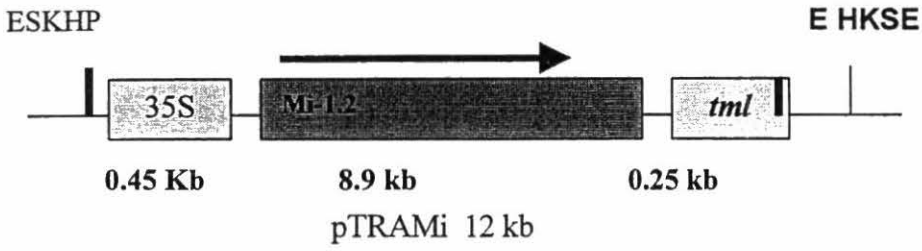

Figure 1. Physical organization of pTRAMi plasmid. pTRAMi contains the *Mi*-1.2 coding sequence between the 35S promoter of CaMV and the transcription termination signal of the morphology large gene (*tml*) from *A. tumefaciens*.

Preliminary results using the methodology already described with the LBA4404 and pTOK233 strains showed a trnsitory high expression for both fruit plants. At this time, lulo and tamarillo explants are placed in a selected medium.

Regeneration

At first we intended to obtain regeneration through organogenesis, since it is an efficient system for *Solanaceae,* especially tobacco and tomato (HENDRIX *et al.*, 1987). A preliminary work was started in July 1999, where different growth media and different hormonal concentrations were used (Table 1). Cotiledoneous leafes differentiation was obtained. These results open the way to obtain organogenic shoots in both fruits. Somatic embriogenesis is also tested using different auxin (2,4-D) concentrations.

Table 1. Trials using different media for organogenesis in lulo and tree tomato.

CULTURE MEDIUM	NAA (g/L)	BAP (g/L)	KIN (µM)	IAA (µM)
MS 104[1]	0.1	1	-	-
B5[2]	0.2	0.3	-	-
	0.2	1	-	-
	0.2	3	-	-
B5		1	-	-
		3	-	-
		9	-	-
MS[3]	-	-	23	0.06
	-	-	46	0.06
	-	-	93	0.06
	-	-	116	0.06
MS	-	-	23	0.57
	-	-	46	0.57
	-	-	93	0.57
	-	-	116	0.57

[1]MS104: Salts MS, vitamins B5, sucrose 3%, agar 5.8 g/L, pH: 5.8
[2]B5: Salts MS, vitamins B5, sacarose 2%, hydrolizate caseine 200 g/L, agar/gelrite (3:1), pH 5.8
[3]MS: Salts MS, Agar (7g/L), sucrose 3%, vitamins B5, pH: 5.8

Future Activities

With the established transformation and regeneration methodology, we will conduct trials with *A. tumefaciens*: AT650 and the pC*Mi* plasmid. This is a construction already used in the Biotechnology Unit - CIAT (MANCILLA, 1999).

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2.1.11 Genetic transformation of tomato variety UNAPAL Arreboles for resistance to fruitworm (*Neoleucinodes elegantalis*) and budworm (*Scrobipalpuloides absoluta*).

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 atractive to tomato growers such as fruit firmness and good adaptability specially to the Valle del Cauca region. But this variety is susceptible to the two major limitations to tomato in this region: the fruitworm (*Neoleucinodes elegantalis*) which damage the fruit even at early stages of development, and the budworm (*Scrobipalpuloides absoluta*), which eats the tomato buds and young leaves. It had been dificult to breed tomato resistant to these two pests 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 interspecific breeding program has not been successful (Lourencao et al., 1985). The main objective of this work is to transform the tomato variety Arreboles with the Bt gene cryIA(b), which had been used susscessfully to obtain resistance against Lepidoptera pests in various economical important crops (i.e. maize, cotton).

Last year we reported 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).

Materials and Methods

Two *Agrobacterium* mediated transformation protocols developed and widely used for tomato (McCormick et al., 1986; Fillatti et al., 1987) will be evaluated using the variety Arreboles. Agrobacterium strains LBA4404 and AGL1 containing the pBIGCry construct (Lida Mancilla, CIAT) will be used. This plasmid contains the cry1a,b gene driven by the 35S CaMV promoter, the nptII gene for kanamycin resistance as selection markers, and the gus-intron as reporter gen.

Results

The review of key factors affecting the efficiency of *Agrobacterium* mediated genetic transformation of tomato Arreboles is in progress.

Collaborators

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2.1.12 Resistance to sugar cane yellow leaf virus (ScYLV): Genetic transformation as an alternative aid to sugar cane breeding.

Background

Yellow leaf syndrome disease caused by ScYLV was introduced in Colombia in1998 through the Brazilian variety SP 71-6163 (Victoria et al., 1999). The main source of the disease is the use of vegetative seed from clonal propagated infected plants, and transmission by the aphid vector *Melanaphis sacchari,* widely present in the sugar cane region of Colombia. The disease is associated with reduction in sucrose content and crop yield, although plants may be symptom-less. In Brazil, the variety SP 71-6163 showed yield losses of 60% to 80%. The molecular characterization and cloning of ScYLV was performed at Texas A&M (Mirkov et al., personal communication). Several constructs were generated, and one gene version containing part of the coat protein gene encoded high levels of protection in transformed sugar cane USA varieties. The main objective of this project is to transform sugar cane with the truncated version of ScYLV coat protein gene via biolistics.

Materials and Methods

Initial attempts to transform sugar cane will be conducted using the PDS-1000 He particle accelerator device. A construct (pScYLV) containing the truncated version of the ScYLV coat protein gene, driven by the ubiquitin promoter, and the nptII gene for genetycin resistance will be used. The protocol optimized at CIAT for rice transformation will be tested and modifications will be introduced accordingly. In this regard, preliminary experiments were conducted using plasmids pGV1040, pCAMBIA 1201, and pCAMBIA 1301 all carrying the gus gene driven by the 35S CaMV promoter, to optimize bombardment conditions evaluating GUS transient expression. Simultaneously, callus induction and plant regeneration of the varieties CC 87-434, CC 85-63, CC 85-92, CC 85-96 and CC 84-75 will be tested to select the most responsive genotype and use as target. Initially, callus will be induced in the dark from apical meristems of *in vitro* plantlets cultured on MS salts supplemented with 3 mg/l 2,4-D, 100 mg/l inositol, 18% coconut water, 3% sucrose, and 0.2% gelrite. Modifications for callus induction and plant regeneration will be introduced as needed.

Results

Preliminary studies indicated that the routine protocol used for clonal propagation of sugar in CENICAÑA, although efficient for the generation of plants, it is not appropriate for the maintenance of embryogenic callus needed for bombardment, since plant differentiation occurs as soon as callus induction is obtained. A modification of the standard protocol was introduced by culturing the induced callus under dim light rather than direct light. This change in light culture conditions restrained callus differentiation by 85%, allowing the maintenance of embryogenic callus at a optimal for bombardment.

Callus cultured under dim light conditions, showed about 80% of plant regeneration when transferred to direct light. These results suggest that changes of light conditions at early stages of callus induction, does not have deleterious effects on the plant differentiation process.

Initially, embryogenic callus were bombarded using plasmids pGV1040, pCAMBIA 1201, and pCAMBIA 1301. Following a two step-wise bombardment under 1,100 psi and at 6 cm target distance, followed by 1,300 psi at 9 cm indicated that between 20% and 49% of callus showed GUS transient expression 48 hours after bombardment (Table 1). Optimization of the bombardment and selection protocol is in progress.

Table 1.- GUS transient expression of embryogenic callus derived from sugar cane apical meristems.

Plasmid	Experiment	Callus No.	No. GUS$^+$	% GUS$^+$	Mean (Sd error)
pVG1040	1	32	19	59,38	49.4 (10.1)
	2	28	11	39,29	
p1301	1	9	0	0	23.3 (23.3)
	2	9	0	0	
	3	10	7	70	
p1201	1	35	20	57,14	23.4 (7.4)
	2	33	0	0	
	3	55	6	10,9	
	4	45	30	66,66	
	6	16	0	0	
	7	17	3	17,65	
	8	14	3	21,43	
	9	14	4	28,57	
	10	15	5	33,33	
	11	16	0	0	

Future Plans

- Optimization of bombarment conditions will be performed using the plasmid pAct1-D, containing the GUS gene under the control of the actin 1 promoter-1 intron from rice. This promoter, likewise the ubiquitin promoter of maize, show higher level of expression on monocots than the 35S CaMV promoter. This gene sequence will allow to optimize bombarment conditions closer for the use of the ubiquitin promoter.
- Lethal and sub-lethal concentrations of genetycin will be determine to establish the optimal protocol for selection of stable transformants at the embryogenic callus, plant regeneration, and plant rooting stages.
- Once the optimal conditions for bombardement and genetycin selection are establish, intitially tissue will be co-bombard with pScYLV and pAtc 1-D plasmids to aid the selection at the callus level up to regeneration. Later, the pScYLV plasmid will be used alone after the conditions to select tissues with genetycin and high level of stable GUS expression are optimized.

- Putative transgenic plants will be evaluated for stable expression and inheritance of ScYLV coat protein gene, and selected plants will be screen for resistance to yellow leaf syndrome disease under biosafety greenhouse conditions.

Collaborators

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ACTIVITY 2.2 Identification of points of genetic intervention and mechanisms of plant-stress interactions

MAIN ACHIEVEMENTS

- A set of secondary metabolites arising during the process of cassava root physiological deterioration, have been identified in a collaborative research underway.
- Evaluation of the cassava core collection for Vit. C and carotene content was completed. Leaves contain 10 times more Vit. C and 100 times more carotene than roots. Carotene showed higher stability than Vit. C after processing the roots through bioling and solar drying.
- The cassava bacterial blight pathogen (*Xam*) pth B protein was cloned, as a first step toward elucidating its interaction with genetic resistance. The interaction of the pth B gene with host plant resistance genes involved in the recognition of the protein was also studied.

2.2.1 Identifying target points for the control of post-harvest physiological deterioration in cassava.

Introduction

The major constraint of cassava production is the rapid post-harvest deterioration starting within 48 hours after harvest rendering the root unpalatable and unmarketable (Beeching *et al* 1995). The post-harvest deterioration process in cassava occurs in two stages: primary or physiological post-harvest deterioration (PPD) and secondary or microbial deterioration (Rickard 1982).

PPD is characterised by vascular streaking, a blue-black discoloration of the xylem parenchyma, rapidly followed by a general discoloration of the storage parenchyma. Prior to the general discoloration of the storage parenchyma an intense UV fluorescence in the storage tissue is observed.

PPD in cassava shows many parallels with the wound responses of other plant systems. Defensive wound responses include the activation of lytic enzymes as chitinases and ß-1,3 glucanases, protease inhibitor proteins and hydroxyproline-rich glycoproteins (Bowles 1990). The induction of the phenylpropanoid pathway plays a determinant role in PPD because it involves several aspects of the wound response, such as the production

of flavonoid pigments, allelochemicals, antioxidants, phytoalexins, signaling molecules and polymers (lignin and suberin) (Wenham 1995, Beeching 1998). Phenylalanine ammonia-lyase (PAL) is the key entry enzyme to the phenylpropanoid pathway (secondary metabolism). PAL increases in activity during PPD (Rickard 1982) leading to the accumulation of phenolic and polyphenolic compounds in cassava roots. Some of these compounds fluoresce under UV light or are pigmented, so they can be involved in PPD symptoms. Other enzymes are also induced during cassava PPD, including peroxidases and polyphenol oxidases (Plumbey *et al* 1981 and Kato *et al* 1991). These enzymes oxidise phenolic products of the phenylpropanoid pathway resulting in tissue browning. The oxidation of phenolic compounds can result in the formation of quinones that can be toxic to micro-organisms or can polymerise to produce brown precipitates (Beeching *et al* 1998).

The aim of this project is the identification of biochemical markers for post harvest physiological deterioration, studying the occurrence of secondary metabolites produced by the activation of the phenylpropanoid pathway and the activity of enzymes that may play a determinant role during PPD process. The identification of these PPD markers will generate the context and tools necessary for the improvement of cassava germplasm with respect to PPD by means of breeding and genetic modification. The finding of biochemical PPD markers will also help with the development of screening methods for use in germplasm evaluation of breeding programs.

Methodology

The strategy is based on the evaluation of cassava cultivars with different reactions to PPD. After we determine the key biochemical tests, they will be used to screen the progeny of a cross between to cultivars with different susceptibility to PPD, which is being used at CIAT to construct the genetic map of cassava.

Plant Material

Roots from plants at 11 months of development and different responses to PPD are being used for the first phase of the project. CM2177-2 and MCOL22 as high response, MNGA2 and MVEN77 as medium response and MBRA12 and MPER183 as low response.

Post-Harvest deterioration induction

Once the roots are harvested the proximal and distal ends are cut and the distal end wrapped with cling film in order to induce deterioration. The roots are stored at environmental conditions, following a time course of seven days.

Secondary metabolites analysis

Ethanolic extraction. The roots were cut into small pieces, after transferring them into ethanol (EtOH). The tissue was homogenized using an ultra turrax. The extract was incubated at room temperature, then filtered and finally concentrated by evaporation.

Determination of the "Total Phenol Content". For the determination of the "total phenol content" of the extracts a modified Folin-Ciocalteu method is used as described by Cliffe et al 1994. Defined solutions of gallic acid were used as a standard and the total amount of phenolics in the cassava root extracts was expressed in gallic-acid-equivalents (GAE).

Chromatographic Analysis. Thin layer chromatography (TLC), high performance TLC (HPTLC) and high performance liquid chromatography (HPLC) are used for qualitative and quantitative determination of secondary metabolites.

Enzyme Activities. Enzymes to be studied are Phenylalanine ammonia-lyase (PAL), Polyphenol oxydase (PPO), Peroxidase (POX), Catalase (CAT), chitinase and ß-1,3 glucanase.

Results

The first phase of the work was concentrated in optimizing various techniques for conditions at CIAT. Previous research carried out at Bath University was corroborated by the experiments on freshly harvested field grown material.

The TLC analysis indicated no significant differences in the compound patterns during the time course. Analysis of root extracts by means of HPLC resulted in the detection of four coumarins (scopoletin, scopolin, esculetin and esculin), three catechins (epicatechin gallate, (-)-epicatechin and (+)-catechin) and one flavonid (rutin). Most of these compounds correspond to those described by Tanaka *et al,* 1983. HPLC chromatograms at the beginning and end of the time course have significant differences, as the increase of peak areas and the occurance of new peaks in the area corresponding to non-polar compounds. Depending on their day of initial detection, during the storage time course, these compounds may be related with vascular streaking. The other compounds of the root extracts are still unknown.

Total phenol content determination showed no large differences between cultivars.

Regarding to enzyme activity analysis several methodologies either previously described for cassava or other plant systems, were tested in order to develop protocols suitable for cassava root tissue in terms of efficiency, reproducibility and low cost.

On going activities

Experiments and future research include the identification of these unknown secondary metabolites in cooperation with Bath University; conclusion of the enzyme activity assays and quantification of secondary metabolites.

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2.2.2 Exploring the genetic potential to improve micronutrient content of cassava

Introduction

Improving the efficiency with which cassava acquires micronutrients and accumulates them in the roots and leaves can have an enormous potential not only in terms of human nutrition, but also in terms of crop production. We have greater expectation that new cultivars with higher contents of micronutrients will also have an agronomic advantage, which will ensure they are competitive in the market place. We have done some work during 1998 exploring the genetic diversity of the CIAT cassava core collection (630 genotypes) with respect to vitamin C. During 1999 the screening of the collection with respect to carotenes content was completed. The nutritional value also depends on the availability of nutrients to the human body once the product is processed and consumed.

The overall objective of this project is to improve the nutritional status of people living in marginal environments of the tropics, by selecting and promoting cassava genotypes with high and good bio-availability of micronutrients and vitamins.

Materials and Methods

Carotene concentration measurements: the extraction procedure outlined by Safo-Katanga et al. (1984) was adjusted. The protocols for the extraction from cassava leaves and roots tissue is described in the past annual report (1998).

Ascorbic acid concentration measurements: the protocol for the determination of ascorbic acid by Fung and Luk (1985) was adjusted for cassava leaf and roots taking as base the procedure outlined in the past annual report (1998).

Results

Analysis of Vitamin C and Carotene content: evaluation of the core collection (601 accesions, that constitute the core collection of the cassava world germplasm bank collection held at CIAT), for vitamin C and carotene in both roots and leaves has been completed. This screening include accesions from CIAT Elite Clones (33), Argentina (7), Brazil (98), Colombia (124), Cuba (18) , Ecuador (26), Guatemala (12), Malasya (14), Mexico (17), Paraguay (33), Perú (68)y Venezuela (52) among others. However, because of lack of adequate quality or quantity of sample tissue from some genotypes, some data are missing.

Sampling study: Sampling variation was measured to elucidate the plant variation (within the same genotype); root to root variation (within the same plant); and the variation due to the experimental measuring procedure. Results suggest that a homogenized sample of 2-3 roots (ideally from 2-3 different plants) would be adequate to

represent the genotypes. Stability of vitamin C and carotene was not highly dependent on sampling.

Ascorbic acid on roots and leaves: ascorbic acid concentration in leaf tissue ranged from 1.68 to 419.25 mg/100g FW; Concentration of ascorbic acid in the roots ranged from less than 1.00 to 39.52 mg/100 g of fresh roots (Table 1). Both distributions showed a strong skewness, with values concentrating on the left, and long right tails. It is obvious that ascorbic acid concentrates on the leaves, rather than in the roots. Its mean concentration on leaves was more than 12 times larger than the mean concentration on the roots. There was no correlation between the ascorbic acid concentration on leaves and roots (ρ = 0.045).

Carotene in roots and leaves: carotene concentration on leaves ranged from 23.28 to 86.22 mg/100 g FW. As in the case of vitamin C, carotene distribution was also skewed to the left, but to a lesser degree. Carotene concentration on roots showed a strongly skewed distribution to the left, ranging from 0.102 to 10.40,mg/100 g FW (table 2). Following the same trend observed with ascorbic acid, carotene concentrated 100 times more on leaves than in roots, illustrating once again the excellent nutritive value of cassava leaves. There was no correlation between carotene concentration on leaves and roots.

Stability of vitamin content after different processing procedures: Carotene content was considerably more stable than ascorbic acid after the different processing procedures evaluated. Boiling was the procedure that allowed maintaning a higher proportion of the original vitamin content (53.2 % of the carotene original and 14.4% of ascorbic acid), whereas solar drying resulted in the highest losses (40% of the carotene and 0% of the ascorbic acid original).

Table 1. Ascorbic acid concentration in leaves and roots of 551 and 530 cassava accesions, respectively.

Data from leaves		Data from roots	
Range (mg / 100 g FW)	Frequency	Range (mg / 100 g FW)	Frequency
0.0 - 14.9	47	0.00 - 1.49	26
15.0 - 29.9	43	1.50 - 2.99	49
30.0 - 44.9	40	3.00 - 4.49	38
45.0 - 59.9	30	4.50 - 5.99	56
60.0 - 74.9	37	6.00 - 7.49	71
75.0 - 89.9	41	7.50 - 8.99	54
90.0 - 104.9	34	9.00 - 10.49	49
105.0 - 119.9	38	10.50 - 11.99	43
120.0 - 134.9	32	12.00 - 13.49	30
135.0 - 149.9	27	13.50 - 14.99	25
150.0 - 164.9	35	15.00 - 16.49	22
165.0 - 179.9	32	16.50 - 17.99	16
180.0 - 194.9	18	18.00 - 19.49	11
195.0 - 209.9	26	19.50 - 20.99	6
210.0 - 224.9	23	21.00 - 22.49	12

Data from leaves		Data from roots	
Range (mg / 100 g FW)	Frequency	Range (mg / 100 g FW)	Frequency
225.0 - 239.9	16	22.50 - 23.99	3
240.0 - 254.9	16	24.00 - 24.49	2
255.0 - 269.9	8	25.50 - 26.99	5
270.0 - 284.9	5	27.00 - 28.49	2
285.0 - 299.9	4	28.50 - 29.99	3
300.0 - 314.9	3	30.00 - 31.49	2
315.0 - 329.9	5	31.50 - 32.99	0
330.0 - 344.9	1	33.00 - 34.49	3
345.0 - 359.9	0	34.50 - 35.99	0
360.0 - 374.9	1	36.00 - 37.49	1
375.0 - 389.9	1	37.50 - 38.99	1
390.0 - 404.9	4	39.00 - 40.49	0
405.0 - 419.9	1	40.50 - 41.99	0
Minimun[§]	0	Minimun[§]	0
Maximun	419.25	Maximun	37.52
Total	568	Total	530
Median	109.30	Median	8.09
Skewness[†]	0.69	Skewness[†]	1.28
Mean	120.16	Mean	9.48
St.Dev.	84.14	St.Dev.	6.50

[§] Measurement below detection threshold of equipment.

[†] Skewness test ranges from negative values (left tales); to 0.0 (perfect symmetry); to positive values (right tales). Larger magnitudes imply larger asymmetry.

Future plans

- To complete the evaluation of carotene and ascorbic acid contents in the cassava collection held at Ciat (5400 genotipes).

- The short post-harvest storage life of cassava is a characteristic that limits the marketability of the root and necessitates either consumption or processing shortly after harvesting. An important component of the post-harvest physiological deterioration (PPD) responses are the oxidative processes. Ascorbic acid and carotene are known to have antioxidant properties. Therefore, PPD will be measured in a wide sample of genotypes (4000) to evaluate the potential correlation between these two vitamins and PPD.

Table 2. Carotene concentration in leaves and roots of 551 and 530 cassava accesions, respectively.

Data from leaves		Data from roots	
Range (mg / 100 g FW)	**Frequency**	**Range (mg / 100 g FW)**	**Frequency**
≤ 27.25	2	0.100-0.135	14
27.26-29.00	1	0.136-0.170	106
29.01-30.75	2	0.171-0.205	249
30.76-32.50	6	0.206-0.240	44
32.51-34.25	9	0.241-0.275	5
34.26-36.00	17	0.276-0.310	15
36.01-37.75	15	0.311-0.345	9
37.76-39.50	26	0.346-0.380	10
39.51-41.25	33	0.381-0.415	10
41.26-43.00	40	0.416-0.450	9
43.01-44.75	41	0.451-0.485	3
44.76-46.50	49	0.486-0.520	5
46.51-48.25	45	0.521-0.555	0
48.26-50.00	43	0.556-0.590	7
50.01-51.75	41	0.591-0.625	3
51.76-53.50	37	0.626-0.660	2
53.51-55.25	31	0.661-0.695	2
55.26-57.00	33	0.596-0.730	3
57.01-58.75	15	0.731-0.765	1
58.76-60.50	13	0.766-0.800	1
60.51-62.25	14	0.801-0.835	0
62.26-64.00	8	0.836-0.870	0
64.01-65.75	7	0.871-0.905	1
65.76-67.50	2	0.906-0.940	0
67.51-69.25	3	0.941-0.975	1
69.26-71.00	4	0.976-1.010	1
71.01-72.75	4	1.011-1.045	2
≥ 72.76	3	1.044-1.080	1
Minimun	23.28	Minimun	0.102
Maximun	86.22	Maximun	1.040
Total	544	Total	504
Median	47.72	Median	0.185
Skewness†	0.48	Skewness†	3.26
Mean	48.26	Mean	0.232
St.Dev.	8.61	St.Dev.	0.137

† Skewness test ranges from negative values (left tales); to 0.0 (perfect symmetry); to positive values (right tales). Larger magnitudes imply larger asymmetry.

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1. SB-02, Project; 2. IP-3 Project

2.2.3 Role of the PthB protein of *Xanthomonas axonopodis* pv. *manihotis* in host plant interaction

Introdution

Generally, phytopathogenic bacteria have a limited host range; often to member of single plant species or genus. Microbial genes involved in plant-microbe interactions may be functionally classified into four broad categories: those involved in parasitism, pathogenicity, host range (pthB gene), and avirulence (1). Avirulence genes present in the pathogen are negative factors because they restrict the host range and allow the pathogen to infect its host. The products of these avirulence genes interact with matching resistance genes products in the host to trigger a cascade of responses in the plant. The major plant defense mechanisms include the production of anti-microbial compounds and enzymes, cell-wall fortification, production of reactive oxygen species, and the hypersensitive reaction (HR), characterized by the rapid appearing of necrosis at the site of infection and the accumulation of phytoalexins (2), (3).

Recently, a gene family in *Xanthomonas* was identified (*avrBs3*); it is constituted by some avirulence and pathogenicity genes. The pthB gene of *Xanthomonas axonopodis* pv. *manihotis* belongs to this family (4). It is possible that the repetitive domain of Pth or Avr proteins may also function as a ligand binding site, specifically interacting with the leucine-rich repeats encoded by the corresponding plant resistance genes (7).

The purpose of this study, is to identify the role of the PthB protein of *Xanthomonas axonopodis* pv. *manihotis* and its activation domain (AD) in the interaction with cassava (*Manihot esculenta* Crantz).

Materials and Methods

PCR primers. Primers XVV and XV were used for the amplification of the activation domain of the protein PthB . Primer XVV was designed based on the restriction site *Xho*I found in the sequence of the *pth*B gene of *Xanthomonas axonopodis* pv. *manihotis*.

These primers directed the amplification of a 1.4 Kb DNA fragment (4548-5899 bp). The amplification product was subjected to electrophoresis in agarose gel and then cleaned with the GENOMED kit.

Bacterial strains and plasmids. *Escherichia coli* strain DH5α, plasmids pGEMT and pGEX series $A_3$15 were used to make the constructions. *E. coli* strains transformed were grown in Luria-Bertani (LB) plus ampicilin (100 mg/liter) medium at 37 °C.

Recombinant DNA techniques. Plasmids were isolated from *E. coli* cells by alkaline lysis and purified by polietilenglicol (6). Restriction enzyme digestion with *Xho*I and *Not*I (GIBCO BRL) was made to verify the position of the insert and to liberate it from pGEMT. Then, the fragment was inserted in the pGEXA3-15(see annual report 1998) that had previously been digested with the same enzymes described above.

Expression of the *pth*B gene and detection of the PthB-AD/GST protein. Transformed *E. coli* strains were grown in LB plus ampicilin medium with IPTG (1.0 mM) to induce the expression of the proteins PthB/GST and PthB-AD/GST, the bacteria was grown until they got a O.D600 of 0.6. Bacterial suspensions were run in SDS-PAGE and stained with coomasie blue R-250 to detect the proteins.

Results and Discussion

The cloning of the *pth*B-AD fragment in pGEX series 5X-3 was successful; only two clones had the fragment properly orientated for the expression when evaluated with *Xho*I and *Not*I enzymes. Induced proteins were separated by SDS-PAGE. Comassie blue-stained profiles obtained after electrophoresis of extract of transformant *E. coli* cells, strain DH5α displayed a pattern of eighteen bands with molecular weights between 29 and 170 Kda, where a band of 163.7 KDa belongs to the protein fusion PthB-AD/GST as expected. The control samples were: Proteins PthB/GST induced (showing a 117 KDa band), PthB/GST non-induced (without this band).

Ongoing Work

- Purification of the PthB-AD/GST protein
- Quantification of the protein
- Evaluation of the protein activity in susceptible cassava plants

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2.2.4 Assessing the two hybrid system for studying the interaction between cassava R genes and the *pth*B protein of *Xanthomonas axonopodis* pv. *manihotis*.

Introduction

Plants are in close contact with a vast number of bacterial species, but infection of the plant by bacteria only occurs in certain specific cases. The host range of a bacterial plant pathogen is generally limited to certain species (or cultivars) of plants. In the case of Gram-negative plant pathogenic bacteria, the development of an interaction is often associated with the elicitation of a hypersensivite response (HR) characterized by a rapid collapse of the plant cells and the necrosis at the site of infection (1). In many plant-pathogen interactions, defense response are activated upon recognition of a pathogen carrying a specific avirulence (avr) gene by a plant host containg a corresponding resistence (R) gene (2). Most of the R genes cloned to date confer disease resistance in a gene for gene manner by recognition of specific avirulent strains of the corresponding pathogen. These R genes encode components of signalling pathways that ultimately would lead to defense response. These contain imperfect leucine-rich repeats wich are thought to be involved in protein-protein interaction (3).

The purpose of this study is to characterize cDNA containing R protein, involved in the recognition of the PTHB protein of ***X. axonopodis pv. manihotis*** (4) by using the trap/two hybrid system. The method is based on the properties of the yeast LEXA protein, which consists of separable domains responsible for DNA-binding and

transcriptional activation (6). The two system used in our work involve plasmids encoding two hybrid proteins. One consisting of the LEXA DNA-binding domain fused to protein *pth*B (bait), that is constructed and introduced into yeast and the second one the cDNA library that act as the "prey". The interaction between *pth*B and R proteins active the transcription of a reporter gene containing a biding site for LEXA.

Materials and Methods

Yeast and bacterial strains and plasmids. Strain used was *S. Cereviseae* EGY 48 (MAT A URA3 LEU2::pLEXAopG-LEU2) and *Escherichia coli* DH5α. Plasmids used were PlexA, pGAD, pJK101 and pSH 18-34. Strain yeast growed in YEPD medium (10g yeast extract, 20g peptone , 20g glucose, 20g agar, Difco) and transformants growed in CM (selective minimal medium with glucosa whitout histidine and uracil) Transformant strains *E. Coli* DH5α-with plasmid pLEXA and pGAD were grown in Luria Bertani (LB) with ampicillin 50ng/ml.

Transformation of yeast. Yeast strain EGY48 was transformed by the high-efficient method. β-Galactosidase activity was assayed on plates containing X-Gal (40mg) and 100ml of 1M potassium phosphato (7)..

Construction of a cDNA library. For the "prey library, we prepared cDNA from mRNA isolated from the cassava resistant variety, MNGA 19, that was inoculated with a *Xam* strain.

Results and Discussion

The entire open reading frame, ORF1, of *pth*B gene was cloned to the C-terminus of the DNA-binding domain of LEXA in the "bait" plasmid pLEXA, this clon was sequenced to verify the right sense of the pthB protein, and was transformed into yeast strain EGY 48 containing two reporter genes(lexAop-LEU2 and lexAop-lacZ). The plasmid pLEXA-*pth*B did not activate transcription of the two reporter genes in the activation assay, and a repression assay indicated that the LEXA-pthB fusion protein entered the yeast nucleus.

Ongoing Work

- Construction of Yeast genomic Libraries.
- Screening Activation Domain Libraries. Yeast strain EGY48 transformed with pthB will be transformed simultaneously with of the libraries of genomic DNA fragments.

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Collaborators

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CIAT-IRD

ACTIVITY 2.3 Development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base.

MAIN ACHIVEMENTS

- In the effort to develop efficient transformation for common bean, a massive wounding procedure, based on sonication, was implemented for *Agrobacterium* inoculation. On the other hand, the higher competence of tepary bean to *Agrobacteriuim*-mediated transformation is transfered to common bean through interspecific hybridization.
- Transformation technique of *Brachiaria*, mediated by *Agrobacterium* progressed significantly

2.3.1 Identifying the constraints-limiting the genetic transformation of common bean, and production of *Agrobacterium* competent common bean genotypes through interspecific hybridization with the tepary bean

Introduction

Although transgenic plants of the common bean have been produced in repeated occasions through particle bombardment (Russell et al., 1993; Aragao et al., 1996), there is worldwide no transgenic common bean plant with agronomic important traits in the market. The reason for this may be: i. transformation methodologies available are still too inefficient, and/or ii. The stable expression of the transgenes has not been as desired.

Often problems in gene expression and gene silencing have been encountered after using direct transformation methodologies (biolistic, electroporation, etc.), and these are possibly caused by plasmid deletions, or multiple plasmid insertion. Contrary to this, genetic transformation through *Agrobacterium* offers the possibility to control the length of the transferred DNA fragment, and the number of copies inserted in the plant genome. This results in a better expression of the transgenes.

However in several labs in the world, the genetic transformation of common bean through *Agrobacterium* has been attempted but yet not achieved. In the Genus *Phaseolus* only a wild genotype of tepary bean has been transformed through this way (Dillen et al. 1997).

Our objective is to develop genetic transformation methodology principally through *Agrobacterium*, which could be used for introducing and expressing efficiently and routinely genes of agronomic importance into common bean.

Methodology

Meristematic calli of the genotype Bayo Madero has been induced as reported before (Mejía-Jiménez et al. 1998).

To develop common bean genotypes with higher competence for *Agrobacterium* transformation and meristematic callus induction, an interspecific hybridization program was started, which involve the best tepary bean and common bean genotypes identified for *Agrobacterium* competence and m-callus induction. Also the wild genotype of tepary bean NI576, which was transformed by Dillen et al. (1997), has been included. As facilitators of the crosses, Congruity Hybrids between the common and the tepary bean, developed previously (Mejía Jiménez et al., 1995), have been used (Table 1).

Inoculation, and coculture conditions are being optimized with the hypervirulent *Agrobacterium* strain LBA4404 pTOK233 (Hiei et al. 1994). Also hybrid populations developed are being screened for transformation competence and m-callus induction using this strain.

Single stranded DNA plasmids of pCambia 1304 carrying the codogenic (SS GUS+) and the none codogenic (SS GUS-) sequences of the GUS genes were produced by cloning the whole plasmid, into the pBluescript SK + or - plasmid, a M13 derived plasmid which produces single stranded DNA molecules.

Results

A rapid and a efficient methodology for massive wounding and inoculation with *Agrobacterium*

Using a modified sonication-assisted *Agrobacterium* transformation methodology (SAAT; Trick and Finer, 1997) large amounts of bean tissue can now be rapidly and efficiently wounded and inoculated with *Agrobacterium* for transformation.

Tepary bean (*P. acutifolius*) shows much higher competence to *Agrobacterium* transformation than common bean

Although we have used the most virulent *Agrobacterium* strains available, the vir-Genes inducing cocultivation conditions, different regenerable tissues (m-calli or cotyledonary nodes) and very efficient wounding methods in our genetic transformation studies of *Phaseolus* beans, we have always observed very few or no transient expression of the GUS gene in common bean tissues. However in tepary bean, a much higher competence for *Agrobacterium* transformation has been detected (Table 1).

Table 1. Effect of the *Agrobacterium* strain and common or tepary bean genotype in the transient GUS expression. Sonified m-calli were cocultured for 3 days with hypervirulent *Agrobacterium* strains (OD 0.5-0.8) in MI medium (NaH_2PO_4 0.24 g/l, MES 3.9 g/l, Glucose 5 g/l, AB Salts, Acetosiringone 100 µM, pH 5.7; Gelvin, 1997) at 22°C

	LBA4404 ptok233	EHA101 pGINRT	EHA101 pIBGUS	C58C1 pBIGcry	AT650 pSGH
P. vulgaris					
Bayo Madero	++	-	-	-	-
A295	-	-	-		
C20	-	-			
BAT477	-				
ICA Pijao	-		-		
P. acutifolius					
G40001	+++				
G40013	+++				
G40022	+++				
G40025	+++	+	+		
G40043	+++				
G40065	+++			++	
N. tabacum (leafs)	+++	+++	+++	+++	+++

+++ More than 16 GUS expressing foci per 100 mg transformed m-calli
++ Between 6 and 15
+ Between 1 and 5 expressing
- No expression .
-

This may be one reason, besides its recalcitrance to plant regeneration protocols, why world wide transformation of common bean with *Agrobacterium* has not achieved success. The results presented above encouraged us to develop common bean lines with improved competence for *Agrobacterium* transformation, through interspecific hybridization with tepary bean. In a previous work we were able to overcome interspecific incompatibility barriers between these two species (Mejía Jiménez et al., 1994), and to transfer from tepary bean high levels of resistance to Bacterial Blight (Singh and Muñoz, 1999).

Development of common bean cultivars with high competence to *Agrobacterium* transformation

Dillen et al. (1997) succeeded the transformation with *Agrobacterium* of the wild genotype of tepary bean NI576, and proposed the use of this genotype for transferring transgenes to common bean through interspecific hybridization. However, many years would be needed for introgression of one transgene to an agronomic important common bean genotype following this strategy. In contrast we are attempting to develop common bean lines with higher competence to *Agrobacterium* transformation through interspecific hybridization with tepary bean (Table 2).

Table 2. Strategy for the introgression of factors involved in tissue culture response (m-callus formation) and *Agrobacterium* transformation competence from selected genotypes of tepary bean to a common bean genetic background.

	x	G40065 G40022 G40025 NI576 ↓		
CBC5 hybrids*		CBC6-F_1** (self sterile plants)	x Bayo Madero ↓	
			CBC7-F_1(Partial fertile plants) ↓ screening F_2 ↓ screening F_3 etc.	x Bayo Madero (Fertile plants) ↓ F_1 ↓ screening F_2 etc.

*Congruity Backcross Hybrids 5 (Mejía-Jiménez, et al. 1994), developed by alternate backcrossing of F_1 hybrids between common and tepary bean, with genotypes of both species. This are being used as facilitator of the crosses

** Hybrids already obtained

Eighty-five CBC6-F_1 hybrids, which involve all the four-selected tepary bean genotypes, have been obtained. These have shown to be self-sterile as expected and are being backcrossed with the genotype Bayo Madero, to recover fertile hybrid plants. Fertile hybrid populations will be soon screened for *Agrobacterium* competence and meristematic callus induction capabilities.

Does exist in mersistematic calli of *Phaseolus* beans and tobacco defense mechanisms against stable integration of foreign genes introduced through particle bombardment or *Agrobacterium*?

In the last few years we have attempted to transform meristematic calli of common and tepary bean through particle bombardment and *Agrobacterium* coculture without success. Only transient but no stable expression of the marker genes was obtained. Particle bombardment experiments of tobacco and common bean meristematic calli with single stranded DNA plasmids, performed during 1998, gave us the explanation of what is occurring.

Single stranded DNA plasmids, which carry the non-codogenic GUS sequences (SS GUS DNA– Plasmids) are useful tools to detect if insertion of foreign DNA is occurring after particle bombardment of a plant tissue. The GUS Gene should only be expressed in the plant cell, after its DNA is integrated in the plant genome and the opposite GUS codogenic strand is synthesized. Since no expression of this gene was obtained after particle bombardment of the bean and tobacco meristematic calli, we can conclude that no integration occurs in this tissue.

Thus meristematic calli of tobacco and common bean posses an unknown mechanism (DNA-degradation?) which impede stable foreign DNA integration in the plant genome.

In tobacco meristematic calli this mechanism is so strong, that also the transient GUS-Expression is inhibited. However this defense mechanism is not present in other meristematic tissues of the common bean plant, since we have been able to recover stable transformed (GUS expressing) bean tissue after particle bombardment and *Agrobacterium* inoculation of cotyledonary nodes (Table 3).

Table 3. Effect of the tissue type and plasmid configuration on the transient and stable GUS expression.

GENOTYPE AND TISSUE	PARTICLE BOMBARDMENT MEDIATED TRANSFORMATION						*AGROBACTERIUM* MEDIATED TRANSFORMATION	
	Transient GUS-Expression (two days after Bombardment)			Stable GUS-Expression (1 month after bombardment			Transient GUS-Expression (three days after inoculation)	Stable GUS-Expression (1 month after inoculation
	Double Stranded Circular	Single Stranded Circular +	Single Stranded Circular-	Double Stranded Circular	Single Stranded Circular +	Single Stranded Circular-		
Bayo Madero (Common bean)								
Meristematic Calli	+	+	-	-	-	-	+	-
Cotyledonary nodes	+			+		*	+	+
Tobacco								
Leafs	+			+			+	+
Meristematic calli	-	-	-	-	-	-	-	-

- Planned

Genetic transformation of tepary bean cotyledonary nodes and recovery of hygromycin resistant GUS+ meristematic calli.

After inoculation of mature seed tissue of the cultivated tepary bean genotype G40065 with the *Agrobacterium* strain LBA4004 pTOK233 three putatively transformed, hygromycin resistant and GUS expressing meristematic calli have been obtained. These results confirm the hypothesis, that cotyledonary nodes of tepary bean are competent for *Agrobacterium* transformation. It will take about two months to regenerate plants from these and confirm its transformed nature.

Conclusions

Meristematic calli of common and tepary bean are useful in genetic transformation, because they allow to apply efficiently in vitro selection to eliminate non transformed, and recover stable transformed regenerable, tissue. But for stable transformation the meristematic cells present in mature or immature seeds, should be transformed before meristematic callus induction.

Our results show also that is unlikely that transgenic plants of the agronomic important common bean genotypes can be obtained through inoculation of meristematic cells, since

these genotypes show very low or no competence for *Agrobacterium* transformation, even with the most virulent *Agrobacterium* strains.

Developing highly competent common bean genotypes for *Agrobacterium* transformation which form meristematic calli of good quality, through interspecific hybridization with tepary bean, seems to be the way of choice to develop efficient genetic transformation methodologies in this recalcitrant legume crop.

Future Plans

Cotyledonary nodes of the genotype Bayo Madero will be bombarded with SS GUS-plasmids in order to test the integration hypothesis.

The methodology of genetic transformation of cultivated *Agrobacterium*-competent tepary bean genotypes will be further optimized and transformations efficiencies evaluated.

Screening of hybrid populations for *Agrobacterium* competence and meristematic callus induction will continue. From this screening, recombinant lines highly competent for *Agrobacterium* transformation are expected to be obtained. These lines can be used to introgress transgenes to common bean genotypes of agronomic importance.

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Collaborators:

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2.3.2 *Brachiaria* genetic transformation mediated by *Agrobacterium tumesfaciens.*

Backgorund

Brachiaria species are important components of the pastures grown in the tropical lowlands of America, Asia, Africa, and Australia. *B. decumbens* cv *Basilisk* is one of the most extensively cultivated species, which has adaptation to acid soils, rapid growth, good soil coverage, and high nutrional value as a feed pasture. This species however, is highly susceptible to spittle bug (homoptera). Resistance to this pest is present in ***B. brizantha*** which does not outcross with *B. decumbens. B. ruziziensis* is used as a bridge between *decumbens* and ***brizantha*** species, thus recurrent backcross is needed to recover the agronomic characteristics from *decumbens*. Plant genetic transformation offers an expedite alternative to transfer genes between unrelated species. A protocol for genetic transformation of *Brachiaria* will be particularly useful to introduce resistance gene(s) for this homoptera pest, and to improve further the quality traits associated with the nutritional value of the pasture.

Earlier work at CIAT's included the establishment of tissue culture methods for plant regeneration (Lenis, 1993), and genetic transformation by direct methods using particle bombardment (Galindo, 1997) of *Brachiaria* species. Last year we reported the progress made on the improvement and adaptation of callus culture and plant regeneration for genetic transformation of *Brachiaria*. Following is described the progress made towards the establishment of a protocol for a reproducible and efficient method for *Agrobacterium* mediated transformation of *Brachiaria* species.

Materials and Methods

Mature embryos and embryogenic scutellum derived callus of *B. decumbens* cv Basilisk accession 606 are used as target explants. The bacteria strains LBA 4404 carrying the hypervirulent plasmid *p*TOK233 (50.35 Kb, kindly provided by Dr. Toshihiko Komari,

Japan Tobacco Inc., Japan) and the hypervirulent strain AGL1 carrying the binary vector pCAMBIA 1301 (provided by Dr. Richard Jefferson, Australia) 1301 are being tested. The *p*TOK 233 plasmid is a cointegrated system with three chimeric genes for expression in plants, including hygromycin resistance (*hph)*, *gus-intron* (*uid-intron*) and neomycin resistance (*npt II)* genes driven by the 35S promoter. The pCAMBIA1301 contains the hygromycin resistance (*hph)*, and *gus-intron* (*uid-intron*) genes. Protocols developed for Agro-transformation of various monocots species are being tested and modifications include accordinghly (Aldemita et al., 1996; Dong et al., 1996; escudero et al., 1996; Hei et al., 1994; Ishida et al., 1996; Rashid et al., 1996; Tingay et al., 1997).

Results

Preliminary results indicate a higher response to *Agroinfection* when using the hypervirulent strain AGL1. Transient GUS expression was noted on mature embryos preculture 4 days in callus induction medium prior the infection as well as in embryogenic callus. GUS expression is mainly localized at the active cell division region between the scutellum and callus . Treatment with the surfactant pluronic F-68 increased the percentage of tissue with transient GUS expression, but tissues tend to brown and die with this treatment. The phenolization caused by pluronic acid was significantly reduced by including ascorbic acid in the callus induction medium. A possitive correlation between GUS expression and days of co-cultivation was found, suggesting a need of at least 4 days of coculture. Jointly with this work, a study had been initiated to establish the optimal conditions for the bioassay of two insecticidal proteins (GNA, and *Mi* 1,2) against spittle bug (*Aeneolamia varia*), the major pest of *Brachiaria* sps. At present, developments towards the establishement of the artificial diet for this homoptera is in progress.

Future Plans

- To define optimal explant and age of explant for agroinfection.
- To establish the optimal period (#days) of cocultivation with *Agrobacterium.*
- To establish conditions to delay differentiation and maintain callus under embryogenic stage for a longer period of time in order to ease the production of appropriate explants for transformation.
- To optimize bioassays conditions to test the effect of insecticidal proteins on the growth of spittle bug.

Collaborators

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OUTPUT 3. COLLABORATION WITH PUBLIC AND PRIVATE SECTOR PARTNERS ENHANCED

MAIN ACHIEVEMENTS

- In the area of biosafety - public awareness, Project SB-02 members delivered a two-day workshop for the press, at CIAT, addressed to present/discuss fundamentals of gene technology and the major issues highlighlited by the press about transgenic crops. On the other hand, with support from the Rockefeller Foundation an international workshop was organized to discuss issues involved in the release of transgenic RHBV-resistant rice lines; representatives from 10 LAC countries joined U.S. and European and CIAT scientists.

- Two training courses wre organized for LAC in 1999, one on biodiversity assessment using molecular markers'the other on molecular plant breeding.

- A patent on plant regeneration and transformation of *Brachiaria* was filed jointly with Embrapa, Brazil. Another patent on soursop micrografting is in preparation with the Corp. Biotec, Colombia.

- In the period Oct 1998-Sept 1999, SB-02 project members published 15 scientific papers in Refereed Journals, and 17 abstracts and posters in Conference Proceedings.

- In 1998-99, SB-2 project members increased activities toward developing collaborative biotechnology linkages with the private sector. Genes, selection markers and collaboration in biotech training have been the major activities.

- In same period, 15 Concept Notes and Proposals have been prepared and/or followed up. Out of these 10 have been approved for funding.

- In the period Oct. 1998-Sept 1999, 10 funding organizations from developed and developing countries have contributed to project SB-02 research activities.

ACTIVITY 3.1 New Collaborative Arrangements and Organization of Workshops and Training Courses

3.1.1 Collaboration with Private Sector

In 1998-99 the following activities were carried out through collaborative arrangements with private sector partners:

- A Bt gene (Cry 1 Ab), acquired jointly with other CG Centers fromJapanese Co 'Plantek', is being used for cassava transformation against the cassava stem borer, a devasting pest in the Colombian North Coast.

- A mannose isomerase gene (pSG-mann 1), acquired from Sandoz Co. through collaboration with ISAAA, will be used to develop a non-deleterious, biosafety friendly, marker gene for cassava transformation.

- Plants have been made to test transgenic maize lines for resistance to the stem borer in collaboration with Novartis-Colombia.

- Collaboration with Novartis-Colombia for partial support to a training course on Molecular Breeding was arranged.

- Generation of transgenic rice with pest resistance, using proprietary genes, in collaboration with Rutgers University, the IDEA (Venezuela) and with support from Empresas Polar, Venezuela

- CIAT, jointly with EMBRAPA, has filed a patent on plant regeneration and genetic transformation of *Brachiaria* (registration in Colombia and Brazil)

- CIAT jointly with Corp. BIOTEC is preparing the patenting of micropropagation of *Anona muricata* through micrografting.

3.1.2 Biosafety Workshop

With support from the Rockefeller Foundation, an international workshop was organized at CIAT, focused on specific issues related to the release of RHBV transgenic resistant rice lines. Key scientists with experience in releasing virus resistant transgenic plants, rice breeders and biosafety officers from USA, Europe and 10 Latin American and Caribbean countries will attend the workshop. Major issues to be discussed, and recommendations to be drawn on, will include: transgene flow to red rice and wild *Oryza* spp, issues of viral encapsidation and alergenicity, risk assessment procedures and policies for releasing transgenic rice in tropical environments. Workshop proceeding will be published in english and spanish.

3.1.3 Genes and the Media Workshop.

For the first time CIAT organized a two-day workshop aimed at presenting to major Colombian newspaper, tv and radio, the main elements DNA - Gene - Gene manipulation technologies and discuss the mejor contemporary issues of public concern relating to transgenic plants and derived foods e.g. gene regulation and the so called 'terminator' technology, geneflow and the case of the 'Monarc' buterflies, and the potato-lectin case. The outcome of the workshop was to initiate a better understanding of bio-technology potential and biosafety considerations from the part of the media.

3.1.4 Training Courses

- A course on Molecular Assessment of Biodiversity and Diversity analysis through Parsimny methodologies was conducted in CIAT in collaboration with the A.von Humboldt Institute, and the Smithonian Institute, Washington D.C. Participants from Colombia, Costa Rica, Ecuador and Central America attended the Course.

- A course on Molecular Plant Breeding, with focus on rice, was conducted in CIAT with the support from the organization of American States (OEA), ICETEC-Colombia and Novartis-Colombia. A total of 21 professionals from 10 LA countries attended the course. Main topics included: aplications of anther culture and genetic transformation in rice breeding, applications of molecular markers and marker-assited selection in rice breeing, use of DNA fingerprinting in the control of rice blast and plant host-pathogen interaction.

ACTIVITY 3.2 ASSEMBLING DATA BASES, GENETIC STOCKS, MAPS, PROBES AND RELATED INFORMATION, PATENTING AND IPR

- A large number of plasmids, bacterial strains, and *Agrobacterium* vector strains have been assembled in the BRU as a result of exchange, donation, transfer and acquisition activities with a range of institutions world wide. As a first step in the development of a reliable storage-retrieval-consultation system, a data base system will be developed for this purpose, in collaboration with CIAT biometricians.

3.2.1 Assembling of data bases, genetic stocks, etc.

- Plasmid vectors pWBVec8, pWB10a, and pBGXiHGFP were kindly supplied by Dr. Peter Waterhouse (CSIRO, Australia) under restricted use BMTA. These plasmids contain a hpt gene with a CAT-1 intron for increased expression of hygromycin resistance, a gus-intron-gene, or a gfp (green fish fluorescent) gene, respectively. Responsible Z. Lentini.

- Plasmid vector NT168 kindly supply by Dr. Nilgun Tumer (Rutgers University, USA) under restricted use BMTA containing the maize ubiquitin promoter. Responsible Z. Lentini.

- Eight different constructs carrying two versions of PAP gene driven by the maize ubiquitin promoter, and hygromycin resistance as selection gene, and some with gus-intron gene. Responsible Z. Lentini.

- Ten different constructs carrying the RHBV NS$ gene in sense and anti-sense directions driven by the maize ubiquitin promoter or 35S CaMV promoter, and hygromycin resistance as selection gene, and some with gus-intron gene. Responsible Z. Lentini.

3.2.2 Genetic stocks

- Pokeweed antiviral protein (PAP), isolated from *Phytolacca americana*, with a ribosome-inactivating ability, potent antiviral and anti-fungal activities, kindly supply by Dr. Nilgun Tumer (Rutgers University, USA) under restricted use BMTA. Responsible Z. Lentini.

- F1 and F2 plants from crosses between Cica 8 transgenic plants carrying the RHBV-N gene and indica varieties Oryzica 1, Iniap 12 and Fedearroz 50.

3.2.3 Patents

Filed: Genetic transformation, Embryogenic Callus Induction and Whole Plant Regeneration for *Brachiaria* Species. Inventors: Z. Lentini (CIAT), V. Carneiro (CENARGEN, Brazil), L. Galindo (CIAT), and S. Lenis (CENARGEN). Depósito Instituto Nacional de Propriedade Industrial-INPI, Brazilia, Brazil. July 28, 1999.

In preparation: Soursop (*Annona muricata*) micrografting technique in collaboration with the Corporación Biotec, Colombia.

ACTIVITY 3.3 PUBLICATIONS AND PROJECT PROPOSALS

3.3.1 Publications by SB-02 Project Staff in the period October 1998-Sept 1999.

3.3.1.1 Refereed Publications

- Caicedo, A.L., E. Gaitán, M.C.Duque, O. Toro, D.G. Debouck and J. Tohme. 1999. Amplified fragment lengh polymorphism (AFLPs) analysis of *Phaseolus lunatus* L. and related wild species of South America. Crop Science. *In press*.

- Sanchez G, S Restrepo, M C Duque, M Fregene, M Bonierbale , and Ver dier. 1999. AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB). Genome, 42:163-172

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- Chavarriaga-Aguirre P., Maya M.M., Tohme J., Duque M.C., Iglesias C.,nierbale M.W., Kresovich S. and Kochert G. (1999) Using microsatellites, isozymes and AFLPs to evaluate genetic diversity and redundancy in the cassava core collection and to asses the usefulness of DNA-based markers to maintain germplasm collections. Molecular Breeding 5:263-273

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- Fregene, M.A. Bernal, A. Dixon, A. Roca, W. and Tohme, J. (1998). AFLP analysis of African cassava (Manihot esculenta crantz) germplasm resistant to the cassava mosaic disease (CMD). Theor and Appl Gent. *In press*

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- Lentini Z., L. Calvert, I. Lozano, E. Tabares, L-F Fory, Jerson Dominguez, and Maritza Cuervo 1999. Expression and inheritance of resistance to rice hoja blanca virus in transgenic rice mediated by the viral nucleoprotein. Molecular Breeding (submitted).

3.3.1.2 Other Publications

(a) Conference Proceedings and Book Abstracts

- Lentini, Z.1999. Vision of Public Organization on Development and Use of Biotechnology . XLV Annual Meeting PCCMCA. Guatemala City, Guatemala. April 12-15, 1999. Proceedings. Invited Speaker.

- Lentini Z. 1999. Plant Biotechnology at CIAT: Powerful Tools for the Characterization, Conservation, and Broadening the Genetic Base of Breeding Germplasm. ICGEB China Workshop on Plant Biotechnology. Beijing, China. May 19-22, 1999. Proceedings. Invited Speaker.

- Lentini, Z. 1999. Transgenic Plants in Plant Breeding for Agroecosystems in Latin America. First Biosafety Meeting. Tegucigalpa, Honduras, June 29-30, 1999. Proceedings. Invited Speaker.

- Lentini, Z. 1999. Biosafety for the Cultivation of Genetically Modified Plants. First Biosafety Meeting. Tegucigalpa, Honduras, June 29-30, 1999. Proceedings. Invited Speaker.

- Lentini, Z. 1999. Molecular Markers and Genetic Transformation for Broadening the Genetic Base of Rice. First Brazilian Rice Meeting. Pelotas, Brazil, August 2-6, 1999. Proceedings. Invited Speaker.

- Lentini Z., L. Calvert, I. Lozano, E. Tabares, C. Gamboa, L-F Fory, B-C Ramirez. 1999. Resistance to RHBV by the Nucleocapsid Gene in Transgenic Indica Rice and Co-Expreession with Oryza Resistance Genes. International Program on Rice Biotechnology. September 20-24, 1999. Phuket, Thailand. Proceedings. Speaker.

- Steve Beebe, Alma Viviana González, Judith Rengifo, Matthew Blair, 1999. The Inheritance of Iron and Zinc Content in Common *Bean (Phaseolus vulgaris L.)*.Abstract 9th . Gathlinburg Symposium Plants, Nutrition and Human Health,

- Jorge, V., M. Fregene, M. Bonierbale, J. Tohme, V. Verdier. 1999. Molecular genetic mapping of resistance to *Xanthomonas axonopodis* pv. *manihotis* in cassava. P 150 In Proc. Plant Genome VI, January, , San Diego California, (Abst.P283)

- Jorge, V., M. Fregene, P. Zuluaga, J. Tohme, V. Verdier. 1999. Genetic analysis of quantitative disease resistance in cassava. 9th International congress of Molecular Plant-Microbe Interactions. Amsterdam. July 25-30.

- Restrepo S., C. M. Vélez, V. Verdier. 1999. Role of the host in causing differentiation among *Xanthomonas axonopodis* pv. *manihotis* strains. 9th International congress of Molecular Plant-Microbe Interactions. Amsterdam. July 25-30.

- Chavarriaga P., Mancilla L., Segovia V. and Roca WM. Developing cassava transformation strategies against stem borers. Abstracts Fourth International Scientific Meeting of the Cassava Biotechnology Network (CBN), Salvador, Bahia, Brazil, November 3-7 (1998) pp. 29.

- Roa AC., Maya MM., Chavarriaga P., Duque MC., Mesa E., Bonierbale MW., Tohme J., Kochert G. and Iglesias C. In search of the closest relatives of cassava: A morphological and molecular approach. Abstracts Fourth International Scientific Meeting of the Cassava Biotechnology Network (CBN), Salvador, Bahia, Brazil, November 3-7 (1998) pp. 18.

- Fregene M., Angel F., Suarez MC., Gutierrez J., Chavarriaga P., Roca WM., Bonierbale M. and Tohme J. Genome mapping in cassava improvement: challenges, achievements and opportunities. Abstracts Fourth International Scientific Meeting of the Cassava Biotechnology Network (CBN), Salvador, Bahia, Brazil, November 3-7 (1998) pp. 24 .

- Chavarriags P., Maya MM., Duque MC., Iglesias C., Fregene M., Tohme J., Bonierbale M., Kresovich S. and Kochert G. Microsatellites in cassava: discovery, inheritance, variability and uses for germplasm characterization. Abstracts Fourth International Scientific Meeting of the Cassava Biotechnology Network (CBN), Salvador, Bahia, Brazil, November 3-7 (1998) pp. 23.

- Roa AC., Chavarriaga P., Duque MC., Bonierbale MW., Thome J., Kochert G. and Iglesias C. Microsatellites as a tool for assessing genetic diversity in Manihot species. Abstracts Fourth International Scientific Meeting of the Cassava Biotechnology Network (CBN), Salvador, Bahia, Brazil, November 3-7 (1998) pp. 17.

- Bellotti, A.C. B. Arias, V., C. Iglesias and E. Barrera, 1998. Host plant resistance to whiteflies in cassava. Poster presented at Entomology Society of America. Las Vegas New. USA No. 1998

- Fregene, M. Okogbening, E. Duque M.C., Roca, W. and Tohme, J. 1999. QTL mapping of earlines and root quality traits conference January 1999, San Diego, California (Poster Presentation)

(b) Degree Theses

- Silvia Restrepo. 1999. Population structure of xanthomonas axonopodis pv. manihotis in Colombia. PhD thesis 150p, University Paris VI.

- Carolina Gonzales Aplicacion de tecnicas moleculares para la busqueda de marcadores asociados a patogenicidad en *Xanthomonas axonopodis* pv. *manihotis* . Universidad de Los Andes, 110p, Sept 99

- Paola Zuluaga Amplificacion y caracterizacion de analogos a genes de resistencia en *manihot esculenta* Crantz utilizando la técnica de cebadores degenerados. Universidad de Los Andes, Sept 99, 77p.

- Ospina, B.L. Smith and A.C. Bellotti. 1999. Adapting participatory research methods for developing integrated crop management for cassava-based system, Northeast Brazil. In: Systems and Farmer Participatory Research. Sam Fujisaka, Ed. CIAT pp 61-75.

3.3.2 Project Proposals and Concept Notes

- Gene flow and biosafety. Proposal to BMZ. Approved. To begin in year 2000

- High quality 'seed' multiplication for cassava farmers in the Colombian North Coast. Proposal to CEGA-DGIS, Colombia. Approved for funding by mid 1999.

- Transgenic resistance to the cassava stem borer. Proposal to CEGA-DGIS, Colombia. Approved for funding by mid 1999.

- Artesanal, low input, cassasva micropropagation system. Proposal to the System wide Program on Participatory Research. Approved for funding begining 1999.

- Analysis of cassava bacterial blight pathogen in Colombia. Proposal to Colciencias-Colombia. Approved for funding begining 1999.

- Soursop (*Annonas muricata*) micrografting. Proposal to Pronata of Agriculture, Colombia, in collaboration with Corp. Biotec. Approved to begin year 2000

- Mapping ACMD resistance genes in collaboration with IITA. Project extension proposal to the Rockefeller Foundation (RF). Approved for funding in late 1999.

- Training of African scientists in applications of the cassava molecular map/genomics. Proposal to the RF. Approved for funding begining 2000.

- Extension of rice biotechnology research at CIAT, with support from the Rockefeller Foundation, for 3 year starting year 2000

- Ensuring Stable and Durable Resistance of Rice to Pathogens and Pests: Rice hoja blanca virus (RHBV), and *Rhizoctonia solani*. Funded. Donor: Centro Tecnológico Polar. Responsible: Z.Lentini (CIAT), Nilgun Tumer (Rutgers University, USA), María Angélica Santana (IDEA, Venezuela).

- Genetic Engineer in the Development of Crops less Dependant on Agrochemicals : Resistant Rice to Insect Pests. Funded. Donor: Accepted. Responsible: Z. Lentini (CIAT), Anan Mercedes Espinoza (Univerisdad de Costa Rica), Merardo Pujol

(CIGB, Cuba), Marcelo Gravina de Moraes (UFRG, Brazil), Lidia Fiuza (UNISINOS, Brazil), Emmanuel Guiderdoni (CIRAD, Franncia), Blanca San Segundo (CSIC, España), and Salomé Pratt (CSIC, España).

- Soursop (*Annonas muricata*) transgenic resistance to anthracnose Concept Note to Ministry of Agriculture, Colombia

- Exploring Biodiversity: Amphibian's as Novel Sources of Antimicrobial Proteins for Genetically Engineered Resistance in Transgenic Plants. Submitted. Donor: European Community. Responsible: Z. Lentini (CIAT); Guido Jack (Max Planck Institute, Germany); Alejandro Mentaberry (INGEBI, Argentina).

- Enabling genomic tools for an orphan crops: Developing and exploiting Expressed Sequence Tags for cassava Starch and bacterial blight resistance. Submitted to the Agropolis, Platform Biotechnology.

- Integrated managment of cowpea diseases submitted to the INCO -DC call (European community).

- Disease control and clonal multiplication of oil palm in Colombia. Concept Note to USDA/USAID.

- Transgenic resistance to papaya ring spot virus. Concept Note to Ministry of Agriculture, Colombia

ACTIVITY 3.4 Donors contributing to Project SB-02 in the period Oct. 1998-Sept. 1999

1. The Rockefeller Foundation.

- Rice Biotechnology: (i) mapping rice blast resistance genes; (ii) analysis of rice blast pathogen structure, (iii) transgenic resistance to RHBV.

- Cassava Biotechnology: (i) generation and mapping of SSR; (ii) identification and mapping of ACMD resistance genes; (iii) identification and mapping of CBB resistance and earliness genes.

- Workshop on Biosafety: field release of transgenic RHBV - resistant rice lines.

2. USAID

- Utilization of *Oryza* wild species in rice breeding

- Cassava disaster relief in Ecuador: planting material, training

3. **CEGA-DGIS, Colombia**

   - Development of bio-reactor (RITA) system for rapid in vitro clonal multiplication of cassava. Collaboration with CORPOICA.

   - Transgenic resistance to cassava stem borer. Collaboration with CORPOICA.

4. **DfID, U.K.**

   - Genes involved in cassava PPD. Collaboration with university of Bath.

   - Biochemical mechanism of PPD. Collaboration with Univ. of Bath

   - Transgenic cassava. Collaboration with Univ. of Bath

5. **SDC, Switzerland**

   - Generation of SSR markers for the cassava molecular map.

6. **COLCIENCIAS**

   - Analysis of diversity and resistance to CBB pathogen (*Xam*)

7. **A. von Humboldt Institute**

   - Training Course on Molecular assessment of biodiversity

8. **Fundación Polar, Venezuela**

   - Transgenic resistance to rice RHBV and Rhizoctonia.

9. **Corporación BIOTEC**

- Soursop micrografting

10. **Organización de Estados Americanos (OEA) e ICETEX**

    - Training Course on Molecular Breeding

11. **Novartis - Colombia**

    - Training Course on Molecular Breeding

PROJECT SB-02 STAFF (1999)

Name	Discipline	Area	Dedication %
J. Tohme	Genetics	(Molec. Markers)	1.0
S. Beebe	Breeding	(Breeding)	0.3
M. Blair	Genetics	(Mol. Markers)	0.6
A. Bellotti	Entomology	(IPM)	0.2
Z. Lentini	Cell Biology/genetics	Tissue culture/genetic transformation	0.8
M. Fregene	Molec. Genetics	(Molecular markers)	0.6
A. Mejia	Biology	(Tissue culture.transformation)	0.2
I. Sanchez	Genetics	(Diversity-CORPOICA)	1.0
V. Verdier	Molec. Pathology	Microbial diversity	1.0
D. Debouck	Botany	(Genetic Resource)	0.2
W. Roca	Physiology	(Tissue culture/transformation)	0.8
Chikelu Mba	Breeding	(Molec. Marker)	1.0
P. Chavarriaga	Molec. Biology	(Transformation)	1.0

Genome Modification

L.F. Galindo	Young Research- COLCIENCIAS
F. Giraldo	Assistant
L.I. Mancilla	Research Assistant
J.J. Ladino	Assistant
R. Escobar	Research Assistant
E. Tabares	Research Assistant
L. Duque	Student Univ. Nacional
C. Flores	Student Doctorado Univ. . Nacional
H. Ramírez	Student Doctorado Univ.. Nacional
Pablo Herrera	Téchnician
M. Valenciano	Téchnician

Genome Diversity

G. Gallego	Coordinador Investigación
A. Almeida	Research Assistant
M.C. Suarez	Research Assistant
E.Gaitan	Research Assistant
E. Barrera	Research Assistant
J.P. Gutierrez	Research Assistant
J. Lopez	Research Assistant
A. Bohorquez	Research Assistant
J. Vargas	Research Assistant
D.F. Cortés	Student
N. Reyes	Technician

Plant-Stress interactions

A.L. Chaves	Associated Research
G. Patiño	Student
A. Hernández	Student
J.M. Bedoya	Student
Monica Chavez	Research Assistant
Andrea P. Zuluaga	Student
Marcela Santaella	Student
Carolina Gonzalez	Student
Claudia M. Velez	Visiting Researcher
Sandra Ojeda	Visiting Researcher
Veronique Jorge	Visiting Researcher
Silvia Restrepo	Visiting Researcher

Administrative

O.L. Cruz	Bilingual Secretary
C.S. Zuñiga	Bilingual Secretary

A.V. Humboldt

J.D. Palacio	Visiting Researcher
J.F.Fernández	Visiting Researcher
Camilo Florez	Visiting Researcher
Eulalia Banguera	Visiting Researcher
Carol Rosero	Visiting Researcher
Pilar Corredor	Visiting Researcher

SINCHI

F. Rodriguez	Visiting Researcher
M. Rodriguez	Visiting Researcher

Corporación BIOTEC

N. Royero	Visiting Researcher
Gladis Perdomo	Visiting Researcher

CORPOICA

I. Sanchez	Visiting Researcher
V. Segovia	Visiting Researcher

CENICAÑA

Paola Rangel	Visiting Researcher