ANNUAL REPORT 2000

PROJECT SB-02

ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

CIAT
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CONTENT

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 forages, 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. Transgenic 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 2000

CIAT

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

NARRATIVE SUMMARY	MEASURABLE INDICATORS (2000)	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 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

NARRATIVE SUMMARY	MEASURABLE INDICATORS (2000)	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
OUTPUT 1: Genomes characterized			
Activity 1.1 Molecular characterization of genetic diversity	• Characterization of core collections • Identification of sources of resistance to diseases • Genetic structure of wild and cultivated beans and cassava available Phylogeny trees based on ITS sequences.	• Report, articles, databases of molecular fingerprinting	• Availability of structured collections • Material supplied by GRU
Activity 1.2: Identification and mapping of useful genes and gene combinations	• Marker assisted scheme established for bean rice, and *Brachiaria* • Linkage detected between markers and important agronimical traits • *Framework* SSR and AFLP map for Brachiaria • QTL analysis of advanced backcross population in rice competed	• Draft articles, Annual report, publications	• Availability of mapping populations and phenotypic characterization
Activity 1.3: Development of molecular techniques for assessing genetic diversity and mapping useful genes	• Bean, Cassava and Brachiaria microsatellites developed • New technologies - SAGE and cDNA AFLP implemented • Resistances genes analogues identified, characterized and mapped in bean and cassava	• Sequences available • Report, draft articles	• Access to facilities in advanced labs,
OUTPUT 2: Genomes modified			
Activity 2.1 Transfer of novel genes and gene combinations by cellular/molecular techniques	• Artificial diet devolved and tested to rear C. clarkei • Generation of transgenic rice, beans and cassava plants • Transgenic rice resistant to RHBV • Transformation system for tomato and sugarcane established	• Transgenic plants in biosafety greenhouses • Report, draft articles	• Biosafety regulation approved • Biosafety greenhouse space available • Collaboration with NARS

NARRATIVE SUMMARY	MEASURABLE INDICATORS (2000)	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
Activity 2.2: Identification of points of genetic intervention and mechanism of plant stress.	• secondary metabolites and enzymes activity related to PPD screened • Representative of cassava genotypes screened for vitamin A and carotene content screened	• Report,	• Access to HPLC • Availability y of germplasm
Activity 2.3: development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base	• Propagation rates of cassava cultivars improved • Low cost cassava propagation method transferred to farmers association • Lines derived from anther culture distributed • RITA system to generate embryogenic callus of Brachiaria and rice established	• Farmers reports, level of adoption of technology	• Access to farmers association • Acess to RITA system
OUTPUT 3: Collaboration enhanced			
Activity 3.1 Organization of Networks, Workshops, training courses in biotechnology	• Contribution to training courses • Organization of network meeting for cassava molecular diversity • At least 10 trainees in service scheme • Participation of team members to international, regional conferences	• Reports	• Funding available
Activity 3.2 Data Bases and Genetic Stocks	• Floramap distributed • Databases for RAPD data established • Data base for gene constructs, plasmid, and vectors established	• Number of register users, report, access to databases, publications	• Continued core support
Activity 3.3 Publications, project concept notes, project proposals, biosafety, IPR	• At least 6 papers prepared • At least 6 concept notes/proposals prepared • Organize biosafety workshop; coordinate IP audit • Visit to private and public sectors	• Manuscripts, reports Concept notes Regulations updated, implemented	• Scientific dedicate time Donor support

PROJECT SB-2 HIGHLIGHTS 2000

Highlights for the period October 1999 - September 2000 are summarized within each of the project's three outputs:

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

1. The level of introgression in inter-specific (*P. vulgaris* x *P. acutifolius*) congruity-backcross lines was quantified using AFLP fingerprinting.
2. On-farm diversity of local common bean varieties in Nicaragua was characterized by microsatellites providing insights into traditional farmer managed local bean genotypes.
3. The ITS region was sequenced for seventy four accessions 60 *Phaseolus* taxa providing the background for the organization of the genus *Phaseolus*,
4. The genetic structure of cassava landraces from growing regions from Sub Saharan Africa and Latin America was assessed using a very large set of 92 SSR providing the basis for cassava improvement strategies using germplasm from the two continents.
5. Marker assisted selection was pursed this year for bean, rice and Brachiaria.
6. The mapping of resistance to *Thrips palmi*, bean angular leaf spot and anthracnose disease resistance genes were acheived.
7. The identification of a SSR and RFLP markers that flank at a distance of 10 cM and 6 cM respectively the single dominant gene controlling resistance to Cassava mosaic disease (CMD).
8. The QTLs analysis of early bulking in cassava resulted in the identification of QTLS associated with harvest index and dry foliage weight with strong influence on early bulking.
9. The QTL analysis of the advanced backcross rice 288 B2F2 families of BG 90-2 by *O rufipogon* was completed.
10. Collaboration with WARDA was pursued by sending 1283 segregating lines from *O. sativa* x *O. glaberrima* interspecific crosses devolved for the advanced backcross program.
11. Bean, Cassava and Brachiaria microsatellites were developed for mapping and fingerprinting.
12. A set of 768 bean cDNA clones were sequenced. This collection of new gene sequences for common bean represents as many sequences as are currently in the Genbank public database for all *Phaseolus* species together.
13. A framework molecular map for Brachiaria was developed using microsatellites and AFLP markers.
14. The Serial Analysis of Gene Expression (SAGE) method was implemented for cassava as part of a collaborative project with the Iwate Biotech Center in Japan.
15. Resistance gene analogs (RGAs) in cassava resulted in the characterization of 12 classes of RGS and one cDNA clones related to R gene.
16. Bean RGA were mapped on the Rils population of Dor 364 x G19833. Tight linkage with resistance genes was detected.

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

1. A methodology for the reproducible genetic transformation of tepary bean was developed.
2. Production of Friable Embryogenic Callus (FEC) in MCol2215 (Venezolana) and regeneration of whole plants. Efficient expression, in FEC of Mcol2215, of constructs containing an insect resistant gene through *Agrobacterium*-mediated transformation.
3. Transgenic rice plants carrying the anti-fungal protein gene PAP were generated, plants with different levels of expression were selected, and will be evaluated for sheath blight resistance under greenhouse conditions.
4. CIAT obtained registration from the Colombian Biosafety Commission for the generation and importation of transgenic plants for germplasm development, as well as approval to conduct field trials with transgenic RHBV rice plants at CIAT experimental Station.
5. A total of 500 accessions (not from the core collection) from the cassava world germplasm bank held at CIAT were evaluated for vitamin C and carotene content. For vitamin C in the roots, there were at least 120 genotypes with concentrations higher than the highest value found in the core collection

6. Improvement of propagation rates of cassava cultivars Mcol 2215 and Mcol 1505 using the RITA system.
7. Sixty-six cassava accessions from the core collection cryopreserved and recovered after freezing in liquid nitrogen. Results were reproducible through time..
8. Validation of low-cost. cassava propagation methods with women (small farmers) from Santa Ana. Department of Cauca. Colombia
9. The automated temporary immersion system RITA has shown to be an efficient system for the expedite generation of large number of embryogenic callus of *Brachiaria* and rice.
10. A protocol for an increased rate of conversion from somatic embryos into plants suitable for genetic transformation was optimized for *Brachiaria*.

OUTPUT 3 Collaboration with public and private partners enhanced

1. CIAT obtained approval of a project from BMZ. Germany, to evaluate gene flow from beans and rice into weedy and wild species relatives.
2. During the period of Oct 1999-Sept 2000 a total of 59 researchers received training with Sb-2 staff.
3. The First Workshop on the Molecular Genetic Diversity of Cassava Network was organized at CIAT
4. A training course on bioinformatic was organized for the SB-02 assistants and post doc. FloraMap was officially released in 2000. Some 190 registered users from several countries have obtained a copy.
5. Two assistants from the genome lab received full fellowships to attend two advanced training courses on sequencing and bioinformatics at the Cold Spring harbor Lab US.
6. In the period of Oct 1999-Sept 2000, SB-02 projects members published 19 Scientific papers in referred journal and books, at least 10 abstracts and posters in conference proceedings and 3 theses. At the same time staff members gave keynotes presentations at international and regional meetings.
7. In the period of Oct 1999-Sept 2000. projects members increased the contacts with private sector at the regional and international level to establish collaborative projects on technology transfer and to obtain freedom to operate for key technologies. Same kind of activities were established with regional NGOs in order to transfer CIAT technologies.
8. In the same period, 8 proposals were approved and 5 more submitted

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

ACTIVITY 1.1 Characterization of genetic diversity

Main Achievements

- A core collection of *P. coccineus* and *P. polyanthus* was evaluated for BGMV, for web blight, and for a highly virulent isolate of the anthracnose pathogen, identifying resistance to all three pathogens. Molecular characterization using AFLP has been initiated.
- The relationship of phenotypic data of the *P.coccineus-P.polynathus* core on disease resistance and seed production revealed association with agroecological parameters and with ecological classes defined by different methods
- The level of introgression in inter-specific (*P. vulgaris* x *P. acutifolius*) congruity-backcross lines was quantified using AFLP fingerprinting. The overall amount of introgression was below the level expected suggesting a still genetic incompatibility after five cycles of congruity backcrossing
- On-farm diversity of local common bean varieties in Nicaragua was characterized by microsatellite providing insights into traditional farmer managed local bean genotypes.
- The ITS region was sequenced for seventy four accessions 60 *Phaseolus* taxa providing the background for the organization of the genus *Phaseolus*, the composition of secondary gene pools of the two major bean cultigens and species relationships in view of future interspecific hybridization
- The genetic structure of cassava landraces from growing regions from Sub Saharan Africa and Latin America was assessed using a very large set of 92 SSR developed at CIAT providing the basis for cassava improvement strategies using germplasm from the two continents.
- The diversity of *X. axonopodis* pv. *Manihotis* strain from Africa was completed using RFLP and AFLP fingerprinting. The results will be compared with the *Xam* database available at CIAT to gain insight into the origin and evolution of *Xam* strains in Africa.

1.1.1 Evaluation of the *P. coccineus* and *P. polyanthus* core collections for resistance to diseases

S. Beebe, P. Jones, C. Cajiao, M.C. Duque[1], C. Jara[2] , J. Beaver[3]
[1] SB-2 Project [2] IP-1 Project [3] University of Puerto Rico

Introduction

In a project financed by the Belgian government to explore the potential of *P. coccineus* (PC) and *P. polyanthus* (PP), a core collection was formed as reported in past years. The core was studied for several agronomic traits and for the relationship of these to the agroecological characteristics of the sites of origin of the accessions.

Materials and methods

Last year we noted a very high level of resistance in P.coccineus/polyanthus to isolates of anthracnose (*Colletotrichum lindemutheanum* or CL) that were obtained locally in Popayán. These isolates had been characterized as Andean or Mesoamerican type in relation to bean differential varieties, but in fact they were Colombian in origin. This raised the question whether isolates from Central America or Mexico (region in which coccineus/polyanthus evolved as species) might in fact present more virulence than Colombian isolates. Thus, we wished to confirm this result of broad resistance with a truly Mesoamerican isolate of CL. We selected an isolate CL–77 from Costa Rica which had previously proven to be among the most virulent of Mesoamerican CL isolates, overcoming the resistance of G2333 which possesses three resistance genes. An evaluation of the core was carried out in the greenhouse under controlled conditions at CIAT headquarters.

The core collection was also shared with colleagues outside of CIAT. Reaction to BGYMV was evaluated in Puerto Rico in 1999 by Dr. James Beaver using inoculation with white flies in the greenhouse. Dr Beaver also evaluated resistance to web blight (*Thanatephoris cucumeris*) in the field and greenhouse. Web blight is one of the primary limitations on the cultivation of bean in the lowland wet tropics, and prevents the planting of bean in the coastal areas of Central America in Guatemala and El Salvador. Production in these regions would obviate the need for continuous deforestation on fragile hillsides. Resistance to web blight has been an elusive objective for breeders, and while differences in degrees of susceptibility exist, no *P. vulgaris* is capable of withstanding a severe attack of web blight. Thus, resistance to web blight is a logical objective for looking at the potential of interspecific crosses.

Results

Exactly the same result was found with the Costa Rican isolate of the anthracnose pathogen as with the Colombian isolates: coccineus/polyanthus accessions were almost uniformly resistant to the Costa Rican isolate. Thus, the broad resistance of coccineus/polyanthus seems to hold even to virulent Mesoamerican isolates. While this ought to be confirmed further, it appears that the distance among Phaseolus species is more important in limiting pathogenesis than is the fact that the pathogen evolved in the same geographical region and supposedly could have been exposed to the coccineus/polyanthus host over millions of years.

In the evaluation of BGYMV, six accessions were evaluated as resistant, all of which are coccineus from Mexico or Guatemala. Another 10 presented no chlorosis, which is only one of several manifestations of symptoms of the virus, and all 10 were coccineus, mostly from Mexico and Guatemala. Thus, this evaluation has opened the possibility that more accessions of coccineus are resistant to BGMV, in addition to G35171 and G35172 that were reported previously. It is noteworthy that all resistant accessions are coccineus and none are polyanthus. In our own experience with greenhouse inoculations of polyanthus, we have seen that it is especially susceptible. This points up an important difference between the two species that was not evident previously.

In the evaluation of the core for reaction to web blight, several materials presented a high level of resistance, in particular G35006 and G35513. Additional seed was supplied to Dr Beaver for a confirmation trial and the resistance was confirmed. Thus, for the first time bean breeders access to high levels of resistance for web blight. These sources are now being investigated in more detail to determine if they hold against all isolates of the pathogen.

Conclusions

The foregoing results and those of past years invite reflections on the host-pathogen relations of coccineus/polyanthus, and certain tentative conclusions:

- PC and PP have resistance genes that are absent in PV. This was recognized previously, especially in the case of ascochyta blight, and to some degree for BGMV and Bean Stem Maggot. Now this conclusion can be extended to web blight and perhaps to Angular Leaf Spot and/or anthracnose.

- The resistance of PC and PP is almost universal in the case of diseases that present clear-cut physiological races with common bean, ie, anthracnose and ALS, both of which are found in the ecological regions where PC and PP evolved. The resistance of PC/PP appears to hold up to those races that evolved within the same region in which the two species evolved, thus proximity of the races did not appear to be conducive to breakdown of resistance. Evidence with wild accessions suggest that this resistance is ancestral.

- *Resistance* is also observed but at lower frequency for diseases that did not evolve in the ecological region of PC and PP. Web blight is more commonly found in the hot, wet tropics, and BGMV is also limited to the warm tropics.

- Although the two species are closely related, in the case of disease resistance there are some similarities and some differences between the species. PP is uniformly resistant to ascochyta and PC presents ample variability. Both species appear to be largely resistant to anthracnose and ALS, and variable for web blight resistance. Up to now, resistance to BGMV only has been found in PC.

This perspective on the host-pathogen relationships of these two species has been made possible due the formation and use of a core. The core permits confidence in drawing generalized conclusions about a species that otherwise would be limited to one or a few accessions.

1.1.2 Relationship of phenotypic data of the coccineus/polyanthus core with ecological origin

S. Beebe, P. Jones, C. Cajiao, M.C. Duque[1], C. Jara [2], J. Beaver [3]
1 SB-2 Project, 2 IP-1 Project, 3 University of Puerto Rico

Introduction

Since the selection of the core collection was based on agro-ecological variability among the sites of origin of the collections, we wished to determine if any of the traits that were evaluated were in fact associated with the agro-ecological parameters. In other words, were these parameters in fact useful criteria to recover genetic diversity in the selection of the core?

Materials and Methods

The agroecological parameters that served to select the core were tested individually as factors in an ANOVA, and appear in Table 1 under the heading "Agro-ecological parameters": soil, temperature (represented as length of growth cycle, long cycles being typical of cool temperatures, etc.), rainfall and daylength at flowering. The effect of species is included, as are the interaction terms of species x ecological parameters, to test if the genetic resources of the two species demonstrate the same variability in relation to ecological parameters.

The same agro-ecological parameters described in the previous paragraph served to define classes. Potentially a total of 54 classes could exist (3 levels of soil x 3 of temperature x 3 of rainfall x 2 of daylength). A total of 27 such classes were represented in the core but some included only one accession, thus 21 classes were included in the ANOVA. This classification is identified in Table 1 as "Agro-ecological classes". Since

the parameters used to define the classes were the same as in the previous analysis, this analysis is in a sense duplicative, but is more comparable to the Holdridge classification (see following paragraph).

A third option that was not employed to select the core but is included here for comparison is the Holdridge system of classifying native vegetation and therefore environments. It was a simple task to identify the Holdridge equivalents of the sites of origin, to apply the ANOVA to accessions as grouped by Holdridge classes.

Another criterion that was used to select the core was based on a multivariate analysis of climatic data (average temperature, day-night temperature difference, and rainfall), and thus was an alternative to the former system of classifying the environments where accessions originated. Since this was based on a cluster analysis of quantitative climate data at the sites of origin of each accession, this analysis was performed species by species, and the test of this classification is done within species. In Table 1 this analysis is identified as "Climate clusters".

Five data that were taken on the core collection were analyzed by the foregoing classification systems: reaction to ALS-Andean races and ALS-Mesoamerican races; reaction to ascochyta; reaction to web blight; and total number of seeds produced. Data on anthracnose were not included due to minimal variability in the resistance reaction. Data on BGMV resistance were taken as resistant or susceptible and thus were not subject to ANOVA.

Results

The effect of species was evident in the reaction to ascochyta (as expected), to ALS-And, and to ALS-Meso, and was independent of the classification system, as it should have been. In the analysis of the effect of individual ecological parameters, significant effects were noted for temperature (on ALS-And and only marginally for ALS-Meso and seed production), and for rainfall (on ascochyta resistance, especially in coccineus in which accessions from wetter climates presented better resistance). But the most important effects were those of daylength at flowering (ALS-And, seed production and marginally web blight).

The interaction terms of species x ecological parameters were significant for: species x soil (seed production); species x temperature (ALS-And); species x day length (ALS-And and marginally for web blight); and only marginally for species x rainfall (seed production). But what was important in this regard is that the interaction terms were significant in all cases due to polyanthus and not coccineus. That is, the variability in ecological parameters was associated with variability within polyanthus but not within coccineus. Does polyanthus present adaptation to more specific ecological niches than coccineus?
With regard to the methodology used to select the core, all ecological parameters used were associated with genetic diversity in one or more traits, for one or both species.

Thus, the use of ecological criteria probably served to recover genetic diversity in these species. This validates the method and commends it for use in other species.

When ANOVA was applied to the agroecological classes (as opposed to the individual ecological parameters that defined the classes), relatively few significant effects were noted. This was probably due in large part to having lost statistical power, since many classes were represented by only 2 or 3 accessions which functioned as repetitions in the analysis. The analysis by Holdridge classes was statistically firmer in terms of more uniform representation of classes. However, the two systems recognized very similar patterns of diversity in the data taken on accessions, and in spite of statistical difficulties, the agro-ecological classes recognized slightly more differences although at marginal significance levels. Thus, the agroecological classes are at least as good as and possibly better than the Holdridge classes for revealing genetic diversity.

The analysis by climatic clusters was different than the other analyses since it was performed only within species. This was because the clusters were derived from the data on each species individually. Surprisingly, the climatic clusters did not have any significant effect at all on the structure of data taken on coccineus, but this system was the most successful in distinguishing variability within polyanthus! Significant effects were noted for ALS-And (*), for web blight (the only effect significant at p=0.05 for this variable), and for seed production (**). In relation to seed production, this system of classification recognized three environments, accessions from which were very poorly adapted in Popayán. Ironically, two of these environments were in Colombia and one in Peru (and Mexico). Environments that produced accessions of PP with good production formed two clusters, both centered in Guatemala.

The cluster of polyanthus that presented the best reaction to web blight was centered on Chiapas (Mexico) and Totonicapan (western Guatemala), as well as individual accessions from Cajamarca (Peru), Venezuela and Veracruz (Mexico). Another 44 accessions from this climate cluster in Mexico and Guatemala exist that could be assayed for resistance to web blight.

Although the effects of the climatic clusters on coccineus were not significant statistically, patterns could be discerned in seed production by clusters. Again, the two poorest clusters were dominated by Colombian environments, while the two environmental clusters that produced best were centered on Oaxaca and surrounding states of Mexico.

Both the climatic system of classifying environments and the classification by individual environmental parameters suggest the same conclusion: that *P. polyanthus* presents more specific adaptation to ecological niches than does *P. coccineus*. This is a surprise, since one would expect more inherent genetic variability within coccineus, for several reasons. First, the wild ancestor of coccineus is distributed over a much larger area than wild polyanthus, at least based on collections made in this century. (This does not rule out a wider distribution of wild polyanthus in the past.) Wild polyanthus has been found only in Guatemala, while wild coccineus is found from central Mexico south and east as far as

Honduras. This same geographic range is associated with wide genetic diversity in *P. vulgaris*. Thus, one would expect that as a species, *P. coccineus* should present broad variability, and wider than *P. polyanthus*. Secondly, and of lesser weight, is the wider morphological variability of domesticated coccineus compared to domesticated polyanthus. Coccineus presents seed colors including white, black, purple, and cream, while polyanthus is largely cream colored. Similarly, coccineus presents both climbing and bush (type 3 and even type 1) habits, while polyanthus is limited to climbing types. These raise questions about visible vs inherent variability that may be elucidated by DNA analysis of the core collection.

Conclusions

The agroecological parameters were in fact associated with phenotypic data of resistance to several diseases and seed production, thus the parameters had served to capture genetic variability. Classification of accessions by environmental parameters suggest that *P. polyanthus* presents more specific adaptation to ecological niches than does *P. coccineus*.

Table 1: Test of mean squares for factors used to classify the accessions in the core collection, classing accessions by different ecological parameters and systems of grouping environments.

Source of variation	df	ALS-And	ALS-Meso	Ascochyta	Web blight	Seed production
Agro-ecological parameters						
Species	1	*	***	***		
Soil	2					
Temperature	2	*	(.06)			(.10)
Rainfall	2			**		
Day length at flowering	1	**			(.06)	**
Species x Soil	2					**
Species x Temperature	2	*				
Species x Rainfall	1					(.06)
Species x Day length	1	**			(.10)	
Agro-ecological classes						
Species	1	***	***	***		
Class	20	*				(.08)
Species x Class	16	**			(.06)	(.06)
Holdridge classes						
Species	1	***	***	***		
Class	11	*				
Species x Class	10	**				
Climatic clusters						
Clusters of *P. coccineus*	14					
Clusters of *P. polyanthus*	13	*			*	**

1.1.3 Level of introgression in inter-specific (*P. vulgaris* x *P. acutifolius*) congruity-backcross lines.

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[1] IP-1 Project

Introduction

Congruity backcrossing (CBC) was shown by Mejía-Jiménez et al. (1994) to be a useful technique for increasing the success of introgressing tepary bean (*Phaseolus acutifolius*) genes into a common bean (*P. vulgaris*) background. This technique uses multiple back-crosses done alternately between both species which allows the recovery of mature back-cross F1 hybrid plants. Congruity backcrossing was postulated by the authors to reduce inter-specific hybridization barriers such as hybrid sterility, genotype incompatibility, embryo abortion often found in crosses between *P. vulgaris* and *P. acutifolius*. In their original study, Mejía-Jiménez et al. (1994) used seed protein (phaseolin) and morphological markers to verify introgression among the congruity backcross lines generated by a cross between the Tepary accession G4001and the common bean variety, ICA Pijao.

In this study, our primary objective was to quantify the level of introgression using random DNA markers generated by AFLP analysis on a group of congruity backcross lines derived from the study described above. Over 420 congruity backcross introgression lines were initially tested in the field from which a smaller group was selected based on their tolerance to drought conditions. AFLP markers have been applied before to study wild species of *Phaseolus* (Tohme et al., 1996) and lima bean, *P. lunatus*, accessions and their close relatives (Caicedo et al., 1999)

Materials and Methods

A total of 61 genotypes were used in the experiments. The first part of the experiment included four lines that were derived from a single backcross of the inter-specific F1 hybrid to the common bean parent (CBC_1F_5), and 35 lines that were derived from five alternating congruity backcrosses (CBC_5F_{10}). Among the latter,18 lines were drought tolerant and 17 were drought susceptible (Table 1). The parents of the introgression lines, G40001 and ICA Pijao, were used as controls to confirm the identity and source of the DNA polymorphisms.

In a second part of the experiment the level of introgression in lines derived from standard recurrent backcrossing and selection was compared with the level of introgression found in the lines developed by congruity backcrossing. Fourteen common bean advanced lines (from the XAN and VAX series) and varieties ('Tara' and 'Jules') derived from tepary beans by standard recurrent backcrossing and selection were included in the experiment. Many of these genotypes were originally developed for resistance to common bacterial blight, a trait that was identified in several tepary bean accessions, such as G40020 and PI207262 (= G1320), which were included in this study.

Three other tepary beans, G40035, G40036, G40006, were selected for this study to represent other accessions of *P. acutifolius* and to confirm the identity of AFLP bands introgressed from tepary parents into the common bean advanced lines and varieties.

Total genomic DNA was extracted from 2 g of fresh leaf tissue. Amplicon-template preparation, pre-amplification, and selective amplification were as described for the protocol of the Gibco BRL AFLP analysis system I kit for small genomes. An initial parental screening was conducted with 24 primer combinations based on the *Eco*RI (E) - *Mse*I (M) adapters and using primers with 3 selective nucleotides each. The most primer polymorphic combinations were chosen for the two parents, G40001 and ICA Pijao. This initial screening included bulked DNA of 10 drought tolerant and 10 drought-susceptible lines. The full set of introgression lines was then analyzed with the most four polymorphic AFLP combinations given in Table 2. Fragments were sized by comparison to a 50bp ladder molecular weight size standard. All the polymorphic AFLP bands between 100 and 350 bp were scored for presence or absence among the lines. Genetic similarities between genotypes was determined with the Dice coefficient using the software packages SAS (SAS Institute, 1989) and NTSYS 2.02 (Rohlf, 1993).

Results

In the twenty-four AFLP combinations that were tested on the parents and bulks from the congruent backcross set, from 73% to 95% of bands were polymorphic. Some combinations produced generally larger or smaller bands and were discarded. Four AFLP combinations had high polymorphism rates, clear amplification profiles and well-distributed ranges in PCR product sizes. These four combinations generated 207 bands including monomorphic and polymorphic bands across the 41 congruent backcross individuals and their two parents. On average each primer combination produced 51.8 bands, however some primer combinations produced more bands than others (Table 2). Each band was scored as a possible introgression event if it was present in one of the parents and in several of the introgression lines. Markers showing predominantly the tepary allele in the congruent backcross lines were not considered. Non-parental bands (bands present in the introgression lines but absent in either parent) occurred on average 2.25 times per AFLP combination. The remaining genotypes, including the common bean varieties and advanced lines and the tepary accessions, were evaluated for the same bands that were present in the introgression lines and their parents. Analysis of the genotypes with the four different primer combinations produced genetic similarity matrices that were significantly correlated.

A multiple correspondance analysis was done for the datasets from each AFLP combination for the complete set of 61 genotypes. The results were similar for each combination, therefore only one analysis is presented here (Figure 1). Three major clusters were observed. 1) The most distinct cluster consisted of the four tepary bean accessions; 2) a second cluster contained all of the common bean varieties or advanced lines without tepary bean parentage (ICA Pijao, DOR476, BAT41). This cluster also contained previously developed genotypes with tepary beans in their pedigrees (Tara, Jules, MAR1, SEL1309 as well as all the VAX and XAN lines), all of the $CBC_1F_{3:5}$

introgression lines, and a group of 9 $CBC_5F_{6:10}$ introgression lines. 3) The remaining cluster was closely related to the other common beans but consisted solely of $CBC_5F_{6:10}$ introgression lines. Drought tolerant introgression lines were found in both of the common bean clusters.

Congruity backcross lines derived from 5 cycles of inter-specific hybridization ($CBC_5F_{6:10}$) showed 6.0% introgression on average while the lines derived from a single backcross (CBC_1F_5) showed only 3.6% introgression on average (Table 1). The levels of introgression in both sets of lines were far below those expected.

Discussion

Compared to a single recurrent backcross, the process of congruity backcrossing helped increase the level of introgression from *P. acutifolius* into *P. vulgaris*. However, the overall amount of introgression was still below the level expected, indicating that there must be genetic incompatibilities in the inter-specific crosses that are still not resolved even with five cycles of congruity backcrossing. Introgression was seen to be even lower in cultivated varieties and advanced lines previously generated from inter-specific hybridization with tepary beans that did not use congruent backcrossing. In addition, many of the initial common bean genotypes containing tepary genes have now been used in multiple generations of crosses and recurrent selection.

The lower than expected introgression rate has serious implications for the transfer of quantitative traits from tepary bean to common bean. While a simple trait such as common bacterial blight resistance has been transferred successfully from tepary bean to common bean advanced lines and varieties, it may be more difficult to transfer complex traits which would rely on the simultaneous introgression of the right combination of tepary genes. Despite this concern it was noted that drought tolerance was not confined to the lines with the most introgression suggesting that a few well-placed loci may be having a greater influence on this trait than previously thought - and that these can be successfully transferred even when introgression levels are low. It remains to be confirmed whether any particular marker is associated with drought tolerances.

Future plans

In the future, the genotypic data presented here will be correlated with phenotypic data for the same introgression lines tested in replicated field experiments under drought and non-stress conditions. We also plan to determine the genetic diversity in a larger range of *P. acutifolius* accession using AFLP markers so as to identify how genotypes used in inter-specific hybridization are related to other accessions within the species, especially since tepary bean diversity may have arisen from more than one domestication event.

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Table 1. Level of introgression in congruity backcross lines and their parents

species/generation	genotype - selection history	No. of fam	% bands introgressed			Expected introgression
			min	max	avg	
P. vulgaris parent	ICA Pijao (parent)		--	--	0.0	na
P. acutifolius parent	G40001(male parent)		--	--	100.0	na
inter-specific CBC_1	ICA Pijao x (ICA Pijao x G40001)F1	4	2.5	4.9	3.6	25.0
inter-specific CBC_5	ICA Pijao x (CBC_4)	35	1.2	11.0	6.0	32.8

Table 2. Number of bands that were monomorphic, non-parental or polymorphic (introgressed or non-introgressed) in four AFLP combinations tested on the congruent backcross individuals and parents, ICA Pijao (*Phaseolus vulgaris*) and G40001 (*P. acutifolius*).

					polymorphic			
	combination	total	Mono-morphic	Non-parental	Introgressed	%	Non-introgressed	%
1	E-ACC M-CTA	48	7	1	10	25.0	30	75.0
2	E-ACA M-CAT	62	10	5	23	48.9	24	51.1
3	E-AAG M-CTC	34	9	0	11	47.8	12	52.2
4	E-AAG M-CTT	63	10	3	16	32.0	34	68.0
	total	207	36	9	60	37.5	100	62.5

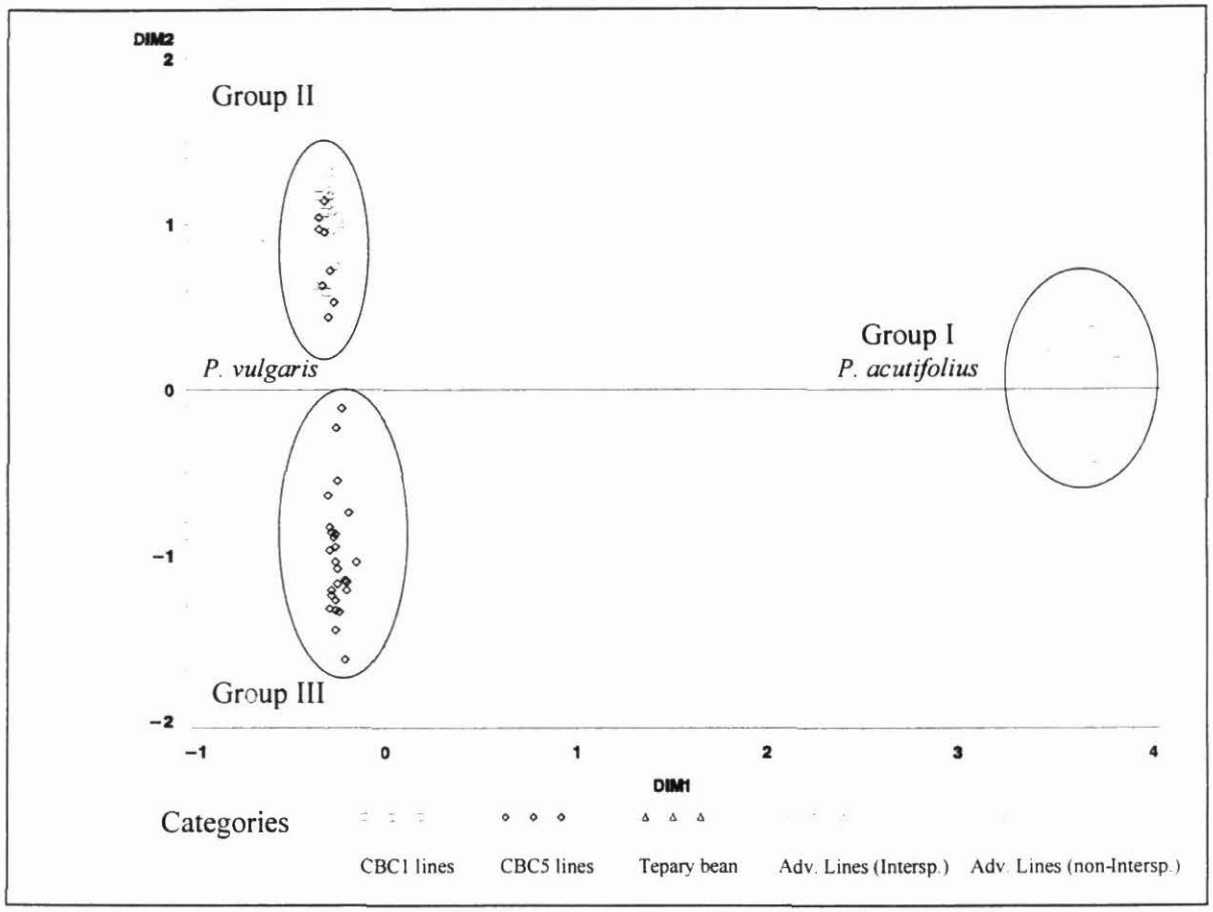

Figure 1. Multiple correspondance analysis showing the association between 61 genotypes of *Phaseolus* (5 Tepary beans, *Phaseolus acutifolius* and 21 common bean, *P. vulgaris*, accession and 35 interspecific hybrids from congruity backcross breeding).

1.1.4 Genetic diversity of microsatellites in common bean

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Introduction

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

Phaseolus microsatellite markers for their allelic variability on a panel of 18 common bean genotypes representing diverse germplasm, both cultivated and wild, Mesoamerican and Andean, within the species.

Materials and Methods

The genotypes consisted in 18 common bean genotypes, including five Andeans, ten Mesoamericans, two wild Andeans, and one wild Mesoamerican (Table 1), which are the parents of 11 mapping populations being studied at CIAT for the inheritance of micronutrient content, abiotic stress tolerance, disease and insect resistance. These included eight intra-genepool (three Andean x Andean and five Mesoamerican x Mesoamerican) and three inter-genepool populations (Table 2). The genotypes were evaluated with a total of 70 microsatellite markers (of which 34 were derived from genomic libraries and 36 were derived from cDNA or gene sequences). The markers were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The PCR products were resolved on silver-stained polyacrylamide gels and microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

Results and Discussion

The average polymorphism rate between the parents of the inter-genepool crosses (61.1%) was higher than that of the intra-genepool crosses (30.0% for Mesoamerican x Mesoamerican and 39.5% for Andean x Andean) (Table 2). Genomic microsatellites detected more polymorphism than cDNA microsatellites in the intra-genepool crosses but were about equally effective in uncovering polymorphism in the inter-genepool crosses. Overall the average polymorphism rate for cDNA microsatellites was 36% versus 46% for the genomic microsatellites.

Significantly fewer average alleles per locus were found for microsatellites from genes (2.9 alleles) than for microsatellites from non-coding sequences (5.1 alleles) (unpaired t-test: P= 0.03). The gene-derived microsatellites frequently were bi-allelic and distinguished the difference between Andean and Mesoamerican genepools. Meanwhile the genomic microsatellites detected more alleles and were thus able to resolve some within-genepool variation.

The polymorphism information content (PIC) of the gene-derived microsatellites was lower (0.401) than for the genomic microsatellites (0.520). The PIC values were positively correlated with the number of alleles produced at the locus, which were likewise lower on average for the gene-derived microsatellites (2.9) than for the genomic microsatellites (5.0). Null alleles were uncommon in both types of microsatellites. The allele size range was generally a good predictor of the number of alleles present for a locus. The allele range was 50% wider for the genomic microsatellites (29.6 bp) compared to the gene-derived microsatellites (19.3 bp). However there were several individual microsatellites with large size ranges but few alleles.

The differences in allelic variability observed at specific bean microsatellite loci are probably due to the differences in the mutation rate inherent for each locus. Microsatellites mutate when they add or subtract a small number of perfect repeats or undergo changes in the flanking regions of the SSR. These changes can occur due to polymerase slippage, unequal crossing-over and/or insertion-deletion events. Although microsatellites are believed to have some of the highest mutation rates observed at any type of molecular loci, some microsatellites will evidently be more stable than others. In this study as in others before, microsatellite variability seems to be influenced by the structure, motif, SSR length and genomic context of the locus.

The more polymorphic genomic microsatellites may well become the mainstay of mapping studies since they will be useful even in narrow intra-genepool crosses. They will also be very useful for to analyzing recent changes in population structure and selection history in closely-related germplasm from a given area or from a specific commercial class. Meanwhile the more conserved and stable cDNA-derived microsatellites may find their greatest utility in mapping in wide inter-genepool or inter-specific crosses and in the phylogenetic analysis of the genus *Phaseolus*.

Future Activities

We plan to continue testing all new *Phaseolus* microsatellites on the existing panel and if the need arises, will create another panel of varieties to survey for polymorphisms in the parents of additional populations. In the future it will be very useful to genotype many of the common parents and genetic sources used at CIAT, as this will allow us to implement whole-genome marker assisted selection that is specific to the genetic crosses made in our bean breeding program.

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

	Variety	Genepool	Purpose	Origin
1	G19833	Andean	Disease resistance, Abiotic stess	Peru
2	G21078	Andean	Micronutrient	Argentina
3	G21242	Andean	Micronutrient	Colombia
4	G21657	Andean	Micronutrient	Bulgaria
5	RAD-CER	Andean	Cultivar	Colombia
6	BAT477	Mesoamerican	Abiotic stess	CIAT line
7	BAT881	Mesoamerican	Abiotic stess	CIAT line
8	DOR364	Mesoamerican	Disease resistance, Cultivar	CIAT line
9	DOR390	Mesoamerican	Cultivar	CIAT line
10	G4825	Mesoamerican	Micronutrient	Brazil
11	G11360	Mesoamerican	Micronutrient	Mexico
12	G11350	Mesoamerican	Micronutrient	Mexico
13	G14519	Mesoamerican	Micronutrient	USA
14	G21212	Mesoamerican	Abiotic stess	Colombia
15	G3513	Mesoamerican	Abiotic stess	Mexico
16	G24404	Wild - Andean	Exotic germplasm	Colombia
17	G19892	Wild - Andean	Exotic germplasm	Argentina
18	G24390	Wild - Mesoamerican	Exotic germplasm	Mexico

Table 2. Polymorphism rate among 11 parent combinations for 70 microsatellite loci (36 cDNA and 34 genomic).

Population	Cross	Polymorphism Rate		
		cDNA derived	Genomic	Total
BAT 477 x DOR 364	M x M	24.2	38.9	31.9
BAT 881 x G 21212	M x M	31.3	32.4	31.9
DOR 364 x G 3513	M x M	18.2	35.1	27.1
G 11360 x G 11350	M x M	33.3	44.1	39.3
G 14519 x G 4825	M x M	21.6	17.2	19.7
G 21657 x G 21078	A x A	13.0	50.0	34.0
G 21078 x G 21242	A x A	33.3	56.7	45.0
DOR 364 x G 19833	A x M	59.4	47.4	67.1
G 24404 x RAD-CER	Wild A x Cult A	52.9	62.5	59.1
RAD-CER x G 24390	Wild M x Cult A	45.2	68.6	57.6
DOR 390 x G 19892	Wild A x Cult M	63.6	53.1	58.5

1.1.5 Characterization of on-farm diversity of local common bean varieties in Nicaragua

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Introduction

Common bean (*Phaseolus vulgaris* L.) is a self-pollinated crop with a very low rate of out-crossing. It is a common assumption that in such a crop, modern varieties grown on medium to large farms are maintained as pure lines while traditional landrace varieties grown on small farms are maintained as populations of mixed genotypes. In this study we attempt to describe the amount of genetic variation at the molecular level that is found within and between on-farm populations (here referred to as local varieties) collected from farmers in different geographical regions of Nicaragua. Seed color is an important commercial trait in Nicaragua, and farmers predominantly grow small red seeded beans, although they also have landraces that have brown or cream colored seed. Within these seed classes there are slight variations in seed size and pigment tones. The genes underlying these differences in seed appearance are not well understood, but the discovery of allelic variation at molecular markers may give some clues as to how farmers maintain the diversity in their seed stocks.

Materials and Methods

Thirty-three local varieties representing three seed classes (30 small red, 3 brown/cream) were genotyped with bean microsatellite markers. In the first step, DNAs from 12 individuals each of the 33 local varieties, were pooled and tested with 20 microsatellites. In the second step, the seven most polymorphic markers on the intital set of genotypes were chosen to assay the separate DNAs from each individual from 21 local varieties (Table 1). Seven Nicaraguan varieties that are part of the CIAT core collection were used as controls for gel-to-gel variation. The microsatellite alleles were resolved on silver-stained polyacrylamide gels and sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

Results and Discussion

The results indicate that microsatellites were ideal markers for detecting DNA polymorphisms in the closely-related but somewhat divergent genotypes of Nicaraguan farmers. In the bulk samples, an average of 3.2 alleles were identified per locus with a range from 1 to 12 alleles depending on the individual microsatellite. In the full array of individuals tested with the seven most polymorphic markers, an average of 6.9 alleles were identified per locus with a range of 2 to 21 alleles. The average polymorphism information content (PIC) values across Nicaraguan varieties ranged from 0.211 for the marker BMy-4 to 0.915 for the marker GAT91, and averaged 0.541 (Table 2). The average PIC values within each Nicaraguan varieties (ie among the 12 individuals of the variety) were lower than across all the varieties together and ranged from 0.085 to 0.514 for the same markers mentioned above and averaged 0.253. Within varieties some of the markers detected no polymorphism (PIC values of 0.0).

The genetic markers facilitated the differentiation of sub-populations within all the local varieties even when the individuals within the variety were all similar in terms of seed color and other phenotypic characteristics. This intra-population diversity was not correlated with agro-climatological zones or other variables. Although most of the individuals were homozygous for most loci, the frequency of heterozygous individuals in several of the populations was higher than 10%. This suggests that out-crossing had occurred at the farm level and that gene-flow between diverse individuals within the population or between adjacent populations from neighboring farms might be important. The presence of individuals with different alleles is proof that farmers are planting genetic mixtures or populations rather than pure line varieties. These results suggest that traditional farmer-managed local varieties are reservoirs of genetic diversity and highlight the importance of preserving genetic resources by *in situ* conservation.

Future activities

On-farm trials of single-plant selections (pure lines) from the mixed varieties are being grown in Nicaragua to determine the morphological variability associated with the genetic diversity identified in this study. The introgression and mixing of improved, BGMV-resistant, modern varieties into the local populations will be studied by

comparing the microsatellite fingerprints of varieties released in Nicaragua in the last twenty years to the alleles observed in this study. The amount of genetic variability present in today's landraces will be compared to that of the "same" varieties stored in genebanks in CIAT and in Nicaragua to determine the changes that occur over time with *in vitro* germplasm storage.

Table 1. Characteristics of the 21 local varieties from Nicaragua tested in this study.

Var	NombreLocal	SitioColecta	Departamento	Grain Color
V1	Chile Rojo	Santa Rosa, Condega	Estelí	Red
V5	Rojo Criollo	La Zopilota, Diriomo	Granada	Red
V6	Rojo Criollo	Palo Quemado, Diriomo	Granada	Red
V7	Rojo Criollo	Palo Quemado, Diriomo	Granada	Red
V9	Rojo Criollo	El Guarumo, Nandaime	Granada	Red
V10	Rojo Criollo	La Orilla, Nandaime	Granada	Red
V11	Rojo Criollo	La Granadilla, Nandaime	Granada	Red
V12	Kaki	El Horno	Matagalpa	Dark Brown
V16	Rojo Criollo	Santa Cruz, Estelí	Estelí	Red
V17	Chile Rojo	Condega	Estelí	Red
V18	Rojo Criollo	San Fco del Gamalote, Juigalpa	Chontales	Red
V19	Mono	Pantasma	Jinotega	Light Brown
V21	Gualiceño	Pantasma	Jinotega	Light Green
V22	Chile Rojo	Yali, Condega	Estelí	Red
V23	Vaina Roja	Estelí	Estelí	Red
V24	Chile Pando	Yali, Condega	Estelí	Red
V26	Rojo Criollo	Monte Grande, Nandaime	Granada	Red
V27	Rojo Pajiso	Santa Lucía	Boaco	Red
V28	Rojo Criollo	Santa Lucía	Boaco	Red
V29	Ligero Nacional	Santa Lucía	Boaco	Red
V30	Bayo	El Loro, San Juan del Sur	Rivas	Light Yellow

Table 2. Polymorphism information content of seven microsatellites tested on 21 local varieties of common bean from Nicaragua (average of 11 individuals per variety)

Variety	Polymorphism Information Content							
	AG-1	BM114	GAT54	GAT91	BMd-36	BMy-4	BMy-1	Average
No. of Alleles	2	4	2	21	12	2	5	6.9
Average PIC Within var.	0.085	0.261	0.172	0.514	0.376	0.110	0.251	0.253
Total PIC Between var.	0.316	0.547	0.481	0.915	0.803	0.211	0.509	0.541

1.1.6 Molecular taxonomy of the genus *Phaseolus* through ITS sequencing

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Introduction

Molecular data play an essential role in the reconstruction of evolutionary relationships among many organisms. For assessing molecular phylogenetic relationships among plants, the nuclear genes coding for the 18S and 25S ribosomal RNA components of the cytosolic ribosomes have been used extensively at family and higher taxa levels (Hamby and Zimmer, 1991). To study the phylogeny among closely relate genera or species, however, a more rapidly evolving gene is needed for the analysis. Internal transcribed spacer (ITS) regions have been shown to be relatively conservative in length and can be used as a source of nuclear DNA characters for phylogenetic reconstruction in plants (Baldwin, 1992). Both ITS regions (ITS1 and ITS2) are part of the rRNA genes (rDNA), which are organized in a tandemly repeated manner. Each rDNA repeat unit consists of the genes coding for the 18S, 5.8S, and 25S rRNA, which are separated from the next repeat unit by the intergenic spacer region (IGS). The coding regions are interrupted by two internal transcribed spacers: ITS1 and ITS2.

We have initiated a molecular phylogenetic study of the different section of the *Phaseolus* genus starting with the group of species related to the common bean (*Phaseolus vulgaris*). The second group to be analysed is of the Lima bean. A third target for this project is a total phylogeny for the genus including all species available at CIAT.

Methodology

Plant material and PCR reactions:

Total DNAs were isolated from fresh or frozen leaves following the protocol of Afanador et al. (Afanador, L. pers comunication). PCR amplifications of the ITS sequences were done using a total volume of 50µl, containing 2.5mM $MgCl_2$, 0.1uM each primer, 1mM dNTPs mixture and 10X *Taq* polymerase buffer and 1 unit *Taq* DNA polymerase was added to each reaction. PCRs were performed in a MJ research thermocycler. PCR products were purified using Wizard PCR Preps (Promega Corp.) or by elution in a low melting point agarose. Fragments were sequenced on a ABI Prism 377 (Perkin-Elmer) using Big-dye terminators and AmpliTaq FS (Perkin-Elmer).

Phylogenetic Analysis:

Nucleotide sequences were aligned using the CLUSTALX program. Phylogenetic analyses are carried out in PAUP version 4 (Swofford, 1998) with a Power Macintosh G3, and used maximum-parsimony and maximum-likelihood to search for optimal trees.

Maximum-parsimony analyses were conducted with characters weighted equally (MP) using 100 heuristic searches employing tree bisection-reconnection (TBR) branch swapping and random addition sequence, with the following settings: all characters of type unordered, one tree held at each step during stepwise addition, steepest descent option not in effect, branches collapsed if minimum branch length = 0, MAXTREES unlimited, and MULPTARS option in effect. To evaluate branch robustness in the parsimony trees, bootstrap (Felsenstein, 1985) analyses is being conducted. Bootstrap supports is going to evaluate using 100 bootstrap replicates, each replicate consisting of 10 heuristic search with simple addition sequences and TBR branch swapping. Maximum-likelihood ratio test (LTR) is going to be employ to identify a simple and robust substitution model for each data set (the "best-fit model")

Results and Discussion

ITS sequencing shows us the organization of the genus *Phaseolus* into several phyla. As expected, the American Phaseolinae *Vigna adenantha* and *Macroptilium atropurpureum* fall outside the genus, and serve as outgroups. Check is needed about sp10, a Mexican Phaseolinae with hooked hairs that has not bloom so far. The phylum that includes the Lima bean and its Andean and Mesoamerican gene pool also comprises the secondary gene pool of Andean species, confirming early results (Caicedo et al. 1999). The tertiary gene pool of Lima bean with taxa such as *maculatus, jaliscanus, polystachyus*, indeed links with the group of *P. lunatus* (Fofana et al. 1999; Maquet et al. 1999). If the link is through *P. leptostachyus* and *P. micranthus* remains however a point to be checked. Because they share the unique trait of rugose testa (Debouck 2000), it was expected that *P. filiformis, P. angustissimus* and *P. carteri* would be grouped together at some distance between the *P. lunatus* phylum and that including the common bean, and this is confirmed by ITS sequencing. The phylum of P. vulgaris includes *P. vulgaris, P. polyanthus, P. costaricensis,* and *P. coccineus*. The parsimony analysis thus confirms many former molecular studies (Schmit et al. 1993; Llaca et al. 1994; Hervieu et al. 1994; Jacob et al. 1995; Delgado et al. 1999). We need however to check further through bootstrap analysis the robustness of the present analysis, because the differences between cpDNA (e.g. Schmit et al. 1993) and nuclear DNA (Llaca et al. 1994; Jacob et al. 1995; Delgado et al. 1999) analyses are not fully revealed as such. Interspecific hybridization has long shown the remote but existing linkage between the common bean phylum and the tepary; this is also confirmed by ITS sequencing. Interestingly, *P. parvifolius* that was thought to belong to the secondary gene pool of *P. acutifolius* is indeed shown as such. The last group would need additional bootstrap analysis: it includes species as morphologically diverse as *P. xanthotrichus, P. chiapasanus*, or *P. neglectus*. Certain affinities revealed in previous studies (Delgado et al. 1993, 1999) appeared once again. For instance, *P. glabellus* appears close to the *P. pedicellatus* group; we know it to be quite separate from the *P. coccineus* group (Schmit et al. 1996). The differences between the *P. pedicellatus* group and the *P. neglectus* group although morphologically important would not be very strong on the basis of ITS sequencing data; both are however confirmed as groups including a couple of taxa (Debouck 2000). Some synonymy that was inferred from morphological studies would be validated by ITS sequencing.

As compared to a similar recent study (Delgado et al. 1999), our work 1) confirms the different phyla existing in the genus such as the *P. lunatus* phylum, the *P. vulgaris* phylum, etc, 2) validates the identity and position of several new species discovered by one of us mainly in Mexico, and 3) indicates better species relationships as a preamble to successful future breeding.

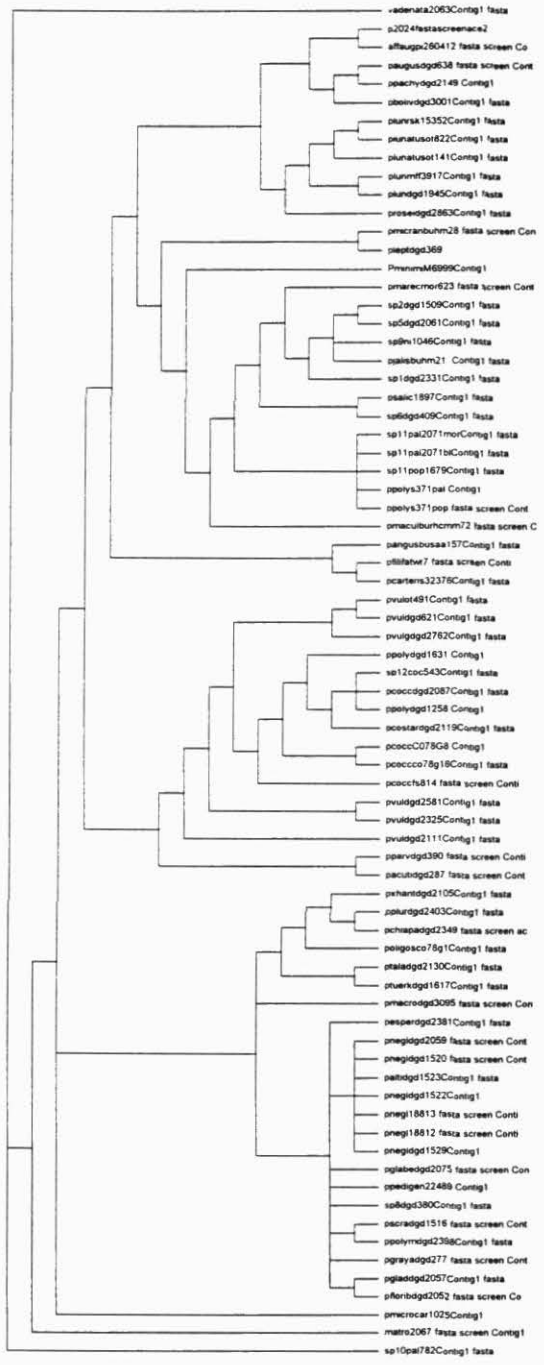

Fig. 1. Preliminary tree for selected *Phasesolus species* based on the maximum parsimony analysis

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1.1.7 Root quality genes from wild relatives of cassava for broadening the crop genetic base

N. Morante, M. Fregene

Introduction

For several years now, it has been shown that the "tremendous genetic potential locked up in germplasm banks can be released by shifting the paradigm from searching for phenotypes to searching for superior genes with the aid of new tools of "genomics" (Tanksley and McCouch, 1997). The value of exotic species as a source of useful alleles, while predicted by Vavilov, the founder of modern gene banks, more than 50 years ago, is only now being recognized and exploited using molecular genetic maps and the advanced back cross mapping scheme (Tanksley and McCouch, 1997). The tools, in particular a molecular genetic map of cassava, and easily assayed PCR-based molecular markers are now available in cassava (Fregene et. al. 1997, Mba et al., 2000), opening new horizons for the efficient use of exotic germplasm. Root quality traits known to exist in the wild and not in cassava include higher levels of protein content from *M.tristis* and *M. carthaginensis* and waxy starches from *M. crasisepala*. Resistance to post-harvest deterioration have also been reported to occur in *M. walkerae*

Materials and Methods

An inventory was made of *Manihot* species and their hybrids with cassava available as seeds and plants at CIAT in collaboration with the genetic resources unit. A representative sub-set of germplasm available at CIAT was germinated from seeds and stakes in the green house and transferred to the field at the UniValle experimental farm in August. At 9 months after planting, two roots will be "milked" from the wild *Manihot* accessions and interspecific hybrids and evaluated for the following root quality traits according to standard procedure established at CIAT:

1. Protein content
2. Dry matter percentage
3. Amylose/amylopectin content
4. Percent post-harvest deterioration, evaluated at 3 days after harvest

Genotypes that show high root protein or dry matter, or amylopectin content or resistance to PHD will be crossed or backcrossed (for inter-specific hybrids) to elite parents of the cassava genepools, these parents have also been established nearby for this purpose.

Highlights of Outputs

1. Inventory of available seeds and plants of *Manihot* species and inter-specific hybrids available at CIAT
2. Field establishment of a representative sub-set of seeds of *Manihot* species and inter-specific hybrids for evaluation of root quality traits.

Plans for Next Year

1. Evaluation of root quality traits on roots of the ***Manihot*** species and inter-specific hybrids

2. Crosses and backcrosses to initiate the advanced backcross scheme.

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1.1.8 SSR marker assessment of the genetic structure of cassava from principal growing regions in sub-saharan Africa and Latin America

M. C. Suarez R., M. Fregene

The principal objectives of the cassava molecular genetic diversity study of cassava in major growing countries in Sub-Saharan Africa and Latin America is to elucidate the genetic structure of cassava land races, determine the salient factors responsible for the structure and use the information to rationalize cassava improvement strategies. A pilot genetic diversity assessment study was conducted in an important cassava growing area of Southern Tanzania, a region that has one of the highest per capita consumption of cassava in the entire African continent. A sub-set of 92 SSR markers from the Cassava MapPairs of SSR markers was multiplexed in the Tanzanian collections and 110 genotypes from CIAT. To graphically represent relationships between the genotypes, the raw SSR data was converted into genetic distance matrices and analyzed by a principal coordinate analysis (PCA. The experienced gained in the pilot study will be extended to an assessment of genetic diversity of cassava land races in subsequent studies which

includes studies of cassava collections from Nigeria, Uganda and the Amazonian region of Colombia.

Materials and Methods

A survey of genetic diversity in cassava was carried out in the Mtwara, Newala, Masasi, and Nachingwea districts of Southern Tanzania, a region between latitude 10^{o} and 20^{o} and longitude 30^{o} and 40^{o}. A total of 10 villages were visited. At each village farmers were invited to share their knowledge on the cassava land races grown by them and a few stakes, usually 4 to 5 500cm stakes, were then requested for each land race from the farmer. A total of 96 genotypes were collected. Another 100 land races were collected from the cassava germplasm banks at the Agricultural Research Institute (ARI) at Naliendele, and at Kibaha, where land races from all over Tanzania are kept for breeding purposes. Woody stakes of all genotypes were planted in the green house in 20litre pots at the Kibaha agriculture research station and young fresh shoots were harvested after 3-4 weeks for DNA isolation. DNA extraction, according to Dellaporta et. al (1983), was carried out at the biotech laboratories of ARI Mikocheni (Dr Allois Kulaya). Quarantine restrictions due to the African cassava mosaic disease (ACMD), prohibits the shipment of cassava tissue from Africa to Latin America. Leaf tissue was from 3-4 week old plants of the collection established in Kibaha, and DNA extraction was. The DNA samples were shipped to CIAT, Cali, Colombia, quantified and diluted in preparation for the SSR Marker analysis. DNA from the core collection genotypes was isolated from the CIAT and IITA accessions by the same method.

A subset of 92 SSR markers, with broad coverage of the genome, from the 186 SSR markers developed at CIAT (Mba et. al. 2000), were organized into 23 quadriplexes (multiplexes of 4 markers). The quadriplexes were designed by searching for sets of 4 markers, from the pool of 186 SSR markers, with the condition that their primers do not form heteroduplexes at the 3' end. The primers were multiplexed on all the 315 genotypes using flourescent primer pairs. PCR product was denatured and electrophoresed on 4% polyacrylamide gels using an automated DNA sequencer ABI model 377 (Perkin Elmer Inc.). Extraction of the raw gel data was done using the ABI PRISM Gene Scan analysis software (Perkin Elmer Inc), and the Genotyper software.
The SSR raw allele data obtained from genotyper was transformed into genetic similarity matrices by the Nei''s standard distance (Gst), and proportion of shared alleles (PSA) using the computer software microsat (http:www.lotka.stanford.edu/microsat.html). Prinicipal Component Analysis (PCA) (Sneath and Sokal 1973) to test the degree of clustering among land races was performed on the similarity matrix using the JMP computer software (SAS Institute 1995).

Results

Raw SSR allele data could be extracted for 68 SSR markers; 24 markers yielded complex non-disomic marker genotypes or had too many missing data points. The PCA analysis of relationships from similarity indices of the Tanzanian genotypes is shown in Fig 1.

The PCA reveals genetic differentiation amongst the land races that is not strictly according to taste or location. The other basis for the clusterings may be the source of the land races; attempts are therefore being made to trace the sources of the land races. The clusters may also represent heterotic groups which is of benefit to cassava breeding. An effort is being made with collaborators in Africa to perform crosses between representatives of the clusters.

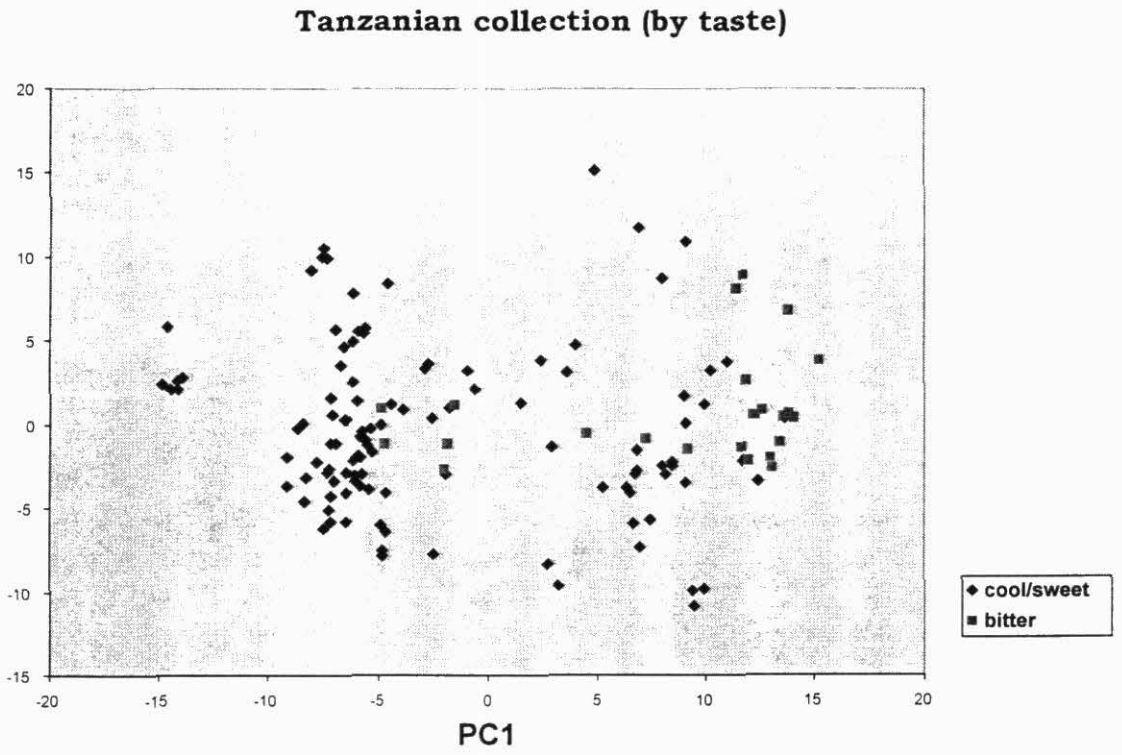

Fig. 1. Principal Component analysis of similarity indices derived from allele sizes From 68 SSR markers of 173 cassava land races from Tanzania

Highlights of Outputs

1. Elucidation of the genetic structure of cassava land races from a major growing region of Southern Tanzania
2. Development of a set of 68 SSR markers, with broad coverage of the cassava genome, for molecular characterization of cassava
3. Establishment of a procedure for the collection and SSR marker analysis of cassava germplasm

Future Activities

1. Completion of the comparison of the Tanzanian materials and core collections from CIAT using an analysis of molecular variation (AMOVA).
2. Search for parent-offspring relationships in the Tanzanian genotypes using SSR allele data.
3. Analyze similar collections from Nigeria, Uganda and the Amazonian region of Colombia with SSR markers.

1.1.9 Genetic fingerprinting of *Brachiaria* germplasm accessions and selected hybrids

A. Bohorquez, E. Gaitan, , M. C. Duque, J.W. Miles and J. Tohme

Introduction

The expression of hybrid vigor is a feature that has been extensively used in agriculture to increase of crop yields in corn, rice, etc. Unfortunately this vigor is quickly lost in subsequent generations after the F_1. Due to this, plant breeders have been trying to create hybrids with good agronomic features which don't lose the hybrid characteristics and on the other hand are able to retain their vigor. In order to achieve this goal the exploitation of a natural phenomenon called apomixis has been proposed. Apomixis is found in a number of families of higher plants, is an asexual mode of reproduction by seed. During apomictic processes, both meiosis and egg fertilization are omitted in embryo formation making the offspring exact genetic replicas of the mother. Apomixis is of immense potential value to agriculture because it can be used to fix hybrid vigor, which would make hybrid seed production much more economical.

The common mode of reproduction of commercial cultivar of *Brachiaria* is by facultative apomixis. Apomixis is not yet well understood genetically, although research efforts have increased significantly in recent years. Apomixis has significant potential as a tool in plant breeding, in that even highly heterozygous genotypes breed true through seed.
Brachiaria pastures rely essentially on a few genotypes of seven species. Five accessions of four species have been the source of 20 cultivars, released in different tropical American countries. Consequently, the genetic base of cultivated *Brachiaria* is extremely limited (Keller-Grein et al. 1998). Probably the best known and most widely used *Brachiaria* cultivar is *B. decumbens* cv. Basilisk which is well adapted to infertile acid soils, forming high-yielding sward that withstands heavy grazing and trampling. Cultivar Basilisk is apomictic and tetraploid (Valle and Savidan, 1998). Probably the best known and most widely used cultivar, its susceptibility to spittlebugs (Homoptera: Cercopidae) reduces its value as a pasture plant in areas where this pest is a major constraint. Another widely used species is *B. brizantha* cv. Marandu which is tetraploid and apomictic. This grass provides a palatable forage of nutritional quality similar to that *B. decumbens* cv.

Basilisk. However it does not tolerate poor soil drainage and requires higher soil fertility; but it has antibiotic resistance to spittlebug.

Reproduction of *B. ruziziensis* cv. Kennedy its is sexual; it is the only obligate sexual diploid one of the commercial species of *Brachiaria* (Ferguson and Crowder, 1974; cited by Pineda, 1999). *B. ruziziensis* provides palatable forage of high nutritive quality, out requires fertile soils and is highly susceptible to spittlebug and less productive than *B. decumbens*. Its reproduction and autoincompatibility made it a potential female parent for breeding programs by means of interspecific crosses with the apomictic species previously named. Successful interspecific has been achieved hybridization using a tetraploidized sexual *B. ruziziensis* and two apomictic tetraploid accessions of *B. decumbens*, or *B. brizantha* as pollinators.

Objectives of existing *Brachiaria* breeding programs follow from recognized deficiencies in existing commercial cultivars and germplasm accessions. The initial objective is to develop an apomictic cultivar combining the persistence, productivity and adaptation to infertile acid soils of common *B. decumbens* cv. Basilisk with durable antibiotic resistance to spittlebugs, which are its principal biotic constraint (Valério et al., 1996). Additional attributes would be desirable in any bred *Brachiaria* cultivar.

The principal objective this work was to characterize with SSR marker of 41 new sexual clones and apomictic clones to select new potential parents of hybrid apomictic cultivar. We also sought marker loci that would unequivocally identify germplasm accessions and apomictic hybrids with commercial potential.

Materials and Methods

The SSR or microsatellites are random short repeated sequences found all eukaryotic genomes. The simple repeated unit can range from two to six base pairs (bp). SSRs show high polymorphism, are locus specific and multiallelic; they have mendelian inheritance with codominance. This kind of marker is useful for population studies, germplasm characterization, genetic mapping, and gene tagging of traits of agronomic importance. The *Brachiaria* SSRs developed at CIAT, (Gaitán, personal communication) BRCT13A, BRCT14, GM18, GM34, GM37, GM40, GM44, GM80, GM88 and GM90 were used. These SSRs were selected because they presented polymorphism among the parents (*B. decumbens* cv. Basilisk, *B. brizantha* cv. Marandu and *B. ruziziensis*) of a sexual hybrid population developed at CIAT (J. W. Miles, personal communication). We used the correspondence analysis procedure (Joseph, et al., 1992).

Plant Material: Sixty-seven *Brachiaria* genotypes (clones) were used (Table 1)

Table 1: *Brachiaria* genotypes

Entry code	Entry ID	Sp.	Cv.	Observations
FBR/001	BR93NO/1371	Ap. Hyb.		
FBR/002	BR98NO/1251	Ap. Hyb.		Promising ap. selection
FBR/003	BR99NO/4015	Ap. Hyb.		Promising ap. selection
FBR/004	BR99NO/4132	Ap. Hyb.		Promising ap. selection
FBR/005	BRUZ4X/4402	B. ruziziensis		4x B. ruziziensis
FBR/006	BRUZ4X/4403	B. ruziziensis		4x B. ruziziensis
FBR/007	CIAT 00606	B. decumbens	Basilisk	Parental, sexual pop.
FBR/008	CIAT 06294	B. brizantha	Marandu	Parental, sexual pop.
FBR/009	CIAT 26110	B. brizantha		Soon to be released
FBR/010	FM9201/1873	Ap. Hyb.	Mulato (Papalotla)	
FBR/011	FM9503/S046/024	Ap. Hyb.		Promising ap. selection
FBR/012	CAQNS/003	Hyb.		Selection, Caquetá
FBR/013	CIAT 06387	B. brizantha		Selection, Brach network.
FBR/014	CIAT 16322	B. brizantha		Selection, Brach network.
FBR/015	CIAT 16327	B. brizantha		Selection, Brach network.
FBR/016	CIAT 16488	B. brizantha		Selection, Brach network.
FBR/017	CIAT 16497	B. brizantha		Selection, Brach network.
FBR/018	CIAT 26124	B. brizantha		Selection, Brach network.
FBR/019	CIAT 26318	B. brizantha		Selection, Brach network.
FBR/020	CIAT 26556G	B. brizantha		Selection, Brach network.
FBR/021	CIAT 16107	B. brizantha		Parental, sexual pop.
FBR/022	CIAT 16126	B. brizantha		Parental, sexual pop.
FBR/023	CIAT 16152	B. brizantha		Parental, sexual pop.
FBR/024	CIAT 16296	B. brizantha		Parental, sexual pop.
FBR/025	CIAT 16827	B. brizantha	Similar or identical to Marandu	Parental, sexual pop.
FBR/026	CIAT 16829	B. brizantha	Similar or identical to Marandu	Parental, sexual pop.
FBR/027	SX99/0029	Sx. Hyb.		New sexual parentals,
FBR/028	SX99/0164	Sx. Hyb.		New sexual parentals,
FBR/029	SX99/0236	Sx. Hyb.		New sexual parentals,
FBR/030	SX99/0246	Sx. Hyb.		New sexual parentals,
FBR/031	SX99/0275	Sx. Hyb.		New sexual parentals,
FBR/032	SX99/0497	Sx. Hyb.		New sexual parentals,
FBR/033	SX99/0574	Sx. Hyb.		New sexual parentals,
FBR/034	SX99/0711	Sx. Hyb.		New sexual parentals,
FBR/035	SX99/0731	Sx. Hyb.		New sexual parentals,
FBR/036	SX99/0823	Sx. Hyb.		New sexual parentals,
FBR/037	SX99/0835	Sx. Hyb.		New sexual parentals,
FBR/038	SX99/1145	Sx. Hyb.		New sexual parentals,
FBR/039	SX99/1260	Sx. Hyb.		New sexual parentals,
FBR/040	SX99/1345	Sx. Hyb.		New sexual parentals,
FBR/041	SX99/1370	Sx. Hyb.		New sexual parentals,
FBR/042	SX99/1513	Sx. Hyb.		New sexual parentals,
FBR/043	SX99/1616	Sx. Hyb.		New sexual parentals,
FBR/044	SX99/1622	Sx. Hyb.		New sexual parentals,
FBR/045	SX99/1630	Sx. Hyb.		New sexual parentals,
FBR/046	SX99/1805	Sx. Hyb.		New sexual parentals,

FBR/047	SX99/1833	Sx. Hyb.	New sexual parentals,
FBR/048	SX99/2030	Sx. Hyb.	New sexual parentals,
FBR/049	SX99/2115	Sx. Hyb.	New sexual parentals,
FBR/050	SX99/2162	Sx. Hyb.	New sexual parentals,
FBR/051	SX99/2173	Sx. Hyb.	New sexual parentals,
FBR/052	SX99/2200	Sx. Hyb.	New sexual parentals,
FBR/053	SX99/2280	Sx. Hyb.	New sexual parentals,
FBR/054	SX99/2341	Sx. Hyb.	New sexual parentals,
FBR/055	SX99/2349	Sx. Hyb.	New sexual parentals,
FBR/056	SX99/2354	Sx. Hyb.	New sexual parentals,
FBR/057	SX99/2514	Sx. Hyb.	New sexual parentals,
FBR/058	SX99/2606	Sx. Hyb.	New sexual parentals,
FBR/059	SX99/2621	Sx. Hyb.	New sexual parentals,
FBR/060	SX99/2663	Sx. Hyb.	New sexual parentals,
FBR/061	SX99/2822	Sx. Hyb.	New sexual parentals,
FBR/062	SX99/2857	Sx. Hyb.	New sexual parentals,
FBR/063	SX99/2927	Sx. Hyb.	New sexual parentals,
FBR/064	SX99/3488	Sx. Hyb.	New sexual parentals,
FBR/065	SX99/3564	Sx. Hyb.	New sexual parentals,
FBR/066	SX99/3690	Sx. Hyb.	New sexual parentals,
FBR/067	SX99/3770	Sx. Hyb.	New sexual parentals,

Results

One hundred eighteen different alleles were obtained in the 67 *Brachiaria* genotypes with the 10 SSRs analized (Fig.1), (table 2).

With the correspondence analysis procedure, using all SSR loci and alleles, we clasified the genotypes in nine groups. These explain 99% of the total variability. The biggest group includes the sexual hybrid population, and parentales *B. ruziziensis, B. decumbens* cv. Basilisk (48 total individuals). This group presents a high level of cohesion; only one individual (#2) is present out of this group. Interestingly the sexual hybrid population has more fixed alleles from the apomictic *B. decumbens* cv. Basilisk and from sexual parental *B. ruziziensis* than from parental *B. brizantha* cv. Marandu. This is proved by the fact the latter is clustered in another group with other varieties of *B. brizantha,* (numbers 9, 20, 25 and 26; 25 and 26 are similar or identical to Marandu), this group (6 individuals total) also includes the apomictic hybrid Mulato. The rest of the genotypes from *B. brizantha* separate forming 4 groups showing a great divergence and high variability (Table 3). Individual #15 (*B. brizantha* CIAT 16327) is separated in another group which might be the result of this individual being the only one with pentaploid features (5 alleles in the SSRs GM44 y GM80).

In another approach constructed starting with similarity (Nei-Li similarity coefficient) we found sexual hybrids forming a group closer to both *B. ruziziensis* and the apomictic hybrid 4, different from the results obtained with the correspondence procedure analysis. After joining Basilisk and the apomictic 12, finally they join the other apomictic hybrids, then the accessions of *B. brizantha* (that include Mulato, 26, 25, Marandu y el hibrido 11) form an isolated subset. We found other groups with the remaining accessions of *B. brizantha*: 13, 14, 16, 18, 20, 23, 24, 9, 21.

Both analyses, correspondence and similarity, agree in forming a compact group with the hybrid sexual population and have more affinity with *B. ruziziensis* followed by Basilisk and Marandu. Both analyses agree in identifying accessions of *B. brizantha* as an outgroup. In conclusion given the level of similarity expressed by Nei-li coefficient and the X^2 distance which assigns different informative value to the allele, we found similar population schemes (Fig. 2).

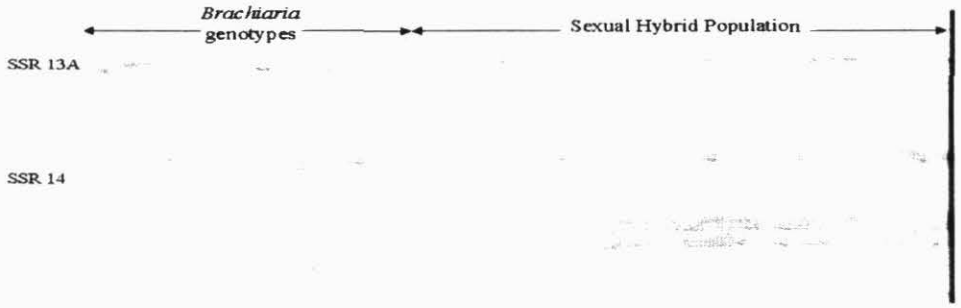

Fig. 1: Silver stained polyacrylamide gel showing SSRs 13A and 14 in the 67 *Brachiaria* genotypes

Table 2: Number of alleles from ten SSRs markers from 67 *Brachiaria* genotypes.

SSR Locus	Total Alleles	# Alleles in Parentals (Basilisk, Marandu, Ruzi)	# Alleles in the Sexual Hybrid Population (SHP)	Varieties (Ap. Hyb., *B. brizantha* (Brach network, etc)
BRCT13A	5	3	2	2 unique alleles, 2 shared with SHP
BRCT14	18	7	5	11 unique alleles, 5 shared with SHP
GM18	6	5	4	1 unique allele, 3 shared with SHP
GM34	14	4	4	10 unique alleles, 4 shared with SHP
GM37	13	5	5	8 unique alleles, 5 shared with SHP
GM40	14	6	4	8 unique alleles, 4 shared with SHP
GM44	15	6	6	9 unique alleles, 6 shared with SHP
GM80	9	6	6	3 unique alleles, 6 shared with SHP
GM88	13	5	4	8 unique alleles, 4 shared with SHP
GM90	11	6	5	5 unique alleles, 5 shared with SHP
	118			

Fig. 2: Cluster analysis of *Brachiaria* genotypes, using UPGMA, of pairwise distances calculated with the Nei-Li similarity coefficient.

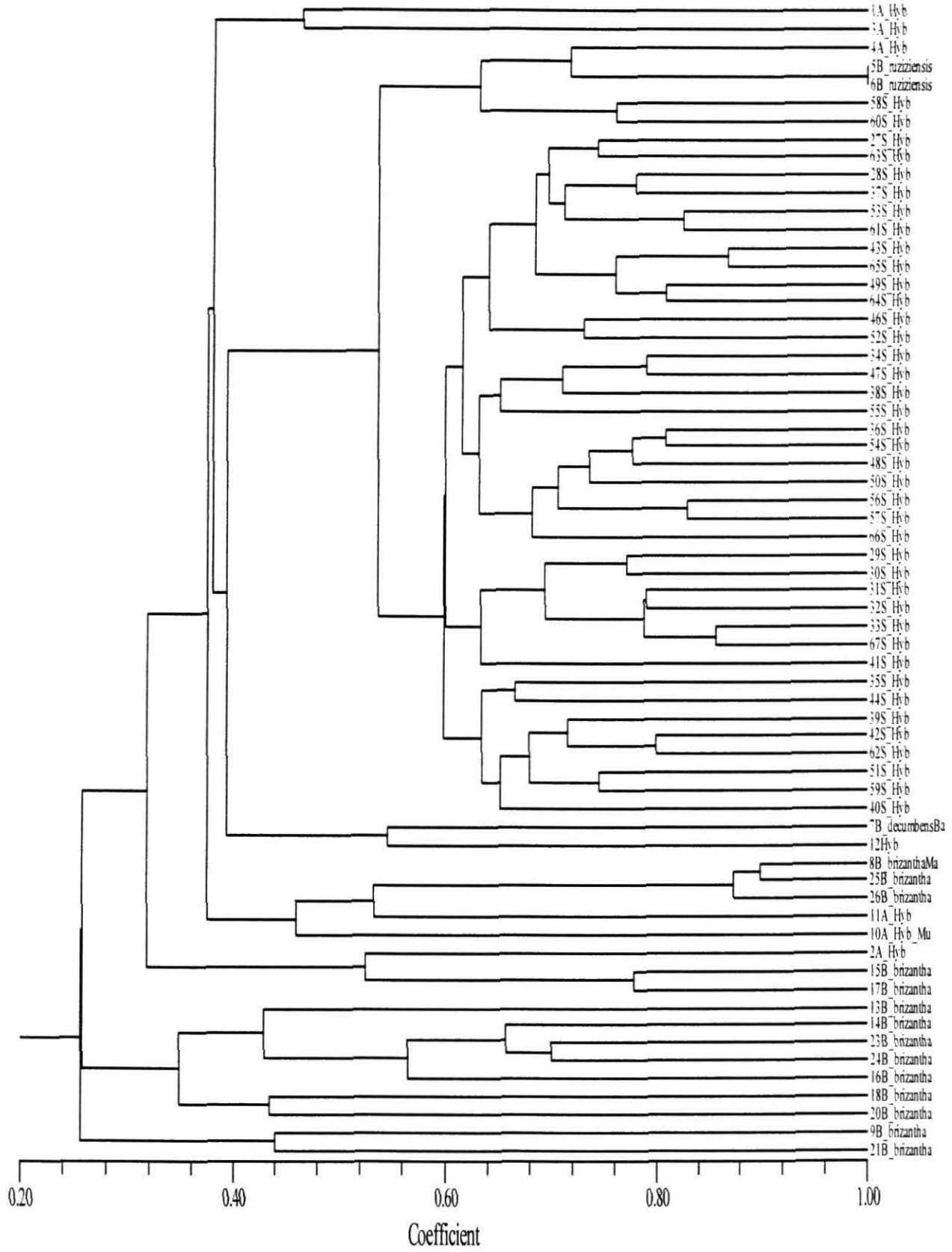

Table 3: Nine groups obtained from correspondence analysis procedure in the 67 *Brachiaria* genotypes number of individuals per group and rare alleles.

Group	# individuals/ group	Rare Alleles	SSR
1	48	46, 53	37
		81	44
		95	88
2	6	17	14
		27	18
		43	34
		69	40
		98	88
3	3	65	40
		76	44
		103, 105, 106	88
		109	90
4	2	19, 20, 21	14
		34, 38	34
		64	40
		76, 77, 80	44
		102	88
		111, 117, 118	90
5	1	18	14
		56	37
		76, 77	44
		96	88
		117	90
6	1	37	34
		52	37
		93	80
		118	90
7	1	98	88
8	1	37	34
		81, 85	44
		93, 94	80
		100, 104	88
		118	90
9	1	37	34
		77	44
		93, 94	80
		102	88
		118	90

Conclusions

With the correspondence analysis procedure for all SSRs we classified the genotypes in nine groups. These explain 99% of the total variability. . In this analysis we observed that the sexual hybrid population has more fixed alleles from the apomictic *B. decumbens* cv. Basilisk and from sexual parental *B. ruziziensis* than from parental *B. brizantha* cv. Marandu.

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1.1.10 Characterizing *Xanthomonas axonopodis* pv. *manihotis* strains from Africa.

R. Pineda, C. González, Verdier, V.

Introduction

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) is a worldwide disease that affects cassava crops in Africa. RFLP analyses have shown that *Xam* is more genetically diverse in South America than in Africa. However, genetic diversity was recently detected among strains collected in Africa leading to the characterization of 14 haplotypes (Assigbétsé et al. , 1996).

The main objective of this study was to characterize the genetic and pathotypic diversity of a *Xam* population recently collected in Togo in experimental or farmer fields. Results will be compared to the *Xam* database available at CIAT.

Materials and methods

Xam strains

Infected cassava leaves and stems samples were collected in Togo from which 137 *Xam* strains were isolated. For long-term storage, bacteria colonies were kept in glycerol at -80^{0}C.

RFLP analyses

Genomic DNA was extracted and digested by *Eco*RI according to the manufacturer instructions (Gibco BRL, Life Technologies, Md.). Electrophoresis of DNA was carried out in 1% agarose in TAE buffer for 15-18 h. Alkaline transfer were performed to Hybond N+ membranes (Amersham). All of the strains were analyzed by RFLP using the plasmid probe *pth*B. The DNA probe was labeled with ^{32}P(dATP), according to the manufacturer instructions (Multiprime DNA labeling system, Amersham). Hybridization and washing were conducted as described previously. Autoradiography was done at -80 ^{0}C, using Kodak film with intensifying screens. Then each RFLP banding pattern were compared with others banding patterns.

AFLP reactions

AFLP markers were analyzed according to Restrepo et al (1999). DNA was digested with the combinations of enzymes *Eco*RI/*Mse*I, then ligated to the respective adapters. The selective amplifications were performed using two selected primer combination *Eco*RI+T/*Mse*I+T, *Eco*RI+T/*Mse*I+A. After completing the second amplification; products were separated on a 6% polyacrylamide denaturing gel, as described by Restrepo et al (1999) and stained using the silver staining technique. The banding pattern of each strain was coded in binary form, representing the presence or absence of each band.

Results

137 *Xam* strains were analyzed by RFLP using the *pth*B gene as a probe. 13 haplotypes were characterized and will be compared to the *Xam* haplotype database available at CIAT. This will provide information on the origin and evolution of *Xam* strains in Africa. First results obtained with AFLP fingerprinting allowed a better definition of the genetic relationships between *Xam* strains.

Ongoing Work

- Evaluation of AFLP *Eco*RI+T/*Mse*I+A primer combination between 137 *Xam* strains.
- Assessing the genetic diversity among *Xam* strains. Select a set of strains for further pathotypic characterization .
- Select a group of strains representing the haplotypes for disease resistance screening assays.

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ACTIVITY 1.2 Identification and mapping of useful genes and gene combinations

Main Achievements

- Marker assisted selection was pursed this year for bean, rice and Brachiaria. A total of 5616 bean plants from 109 crosses were screened for the bgm-1 Scar marker. In the case of Brachiaria, 96 hybrid clones were screened with the N14 SCAR linked to apomixis. In rice bulks from 159 genotypes with different level of resistance to Sogata were screened with 330 RAPD primers resulting the identification of putative five markers tightly linked to the resistance to Sogata.

- The mapping of resistance to *Thrips palm*, a damaging pest in bean resulted in the identification of QTLs for damage and reproductive adaptation.

- The mapping of bean angular leaf spot and anthracnose disease resistance genes was completed using the cross DOR 364 x G19833. QTSl for disease resistance were found in five basic regions of the bean genome. A common QTLs for both disease wwas identified suggesting a genes cluster.

- The identification of a SSR and RFLP markers that flank at a distance of 10 cM and 6 cM respectively the single dominant gene controlling resistance to Cassava mosaic disease (CMD) providing with the tools for marker assisted breeding of CMD resistance at CIAT in the absence of the disease.

- A comparison of marker assisted selection and conventional methods for the rapid conversion of African gene pools to CMD resistant lines was initiated in collaboration with IITA. About 6000 progeny from 12 crosses were generated to start the comparison.

- The QTLs analysis of early bulking in cassava resulted in the identification of QTLS associated with harvest index and dry foliage weight with strong influence on early bulking. F1S1 population for marker inheritance and fidelity studies were developed in vitro and established in the field.

- The QTL analysis of the advanced backcross rice 288 B2F2 families of BG 90-2 by *O rufipogon* was completed. QTLS for yield and yield components were identified. Association between markers and yield on chromosomes 3, 5, 6, 9 and 12 were detected. Based on these results 87 families associated with QTLs were selected for the development of near isogenic lines.

- Collaboration with WARDA was pursued by sending 1283 segregating lines from *O. sativa* x *O. glaberrima* interspecific crosses devolved for the advanced backcross program.

1.2.1 Marker-assisted selection (MAS) for bean *golden mosaic virus* (BGMV) resistance in common beans

C. Quintero, H. Terán, S. Beebe, F. Morales and J. Tohme

Introduction

During 1999 MAS was successfully implemented for beans using a SCAR marker for BGMV resistance. The methodology was fast enough to provide results before flowering time; thus breeders can use only the plants having the SCAR for developing multiple crosses. The efficiency of MAS was demonstrated by a reduction in breeding effort and total planted area by 60%.

Development of new crosses and population advancing. In three separate plantings a total of 5616 plants (109 crosses) were screened for the *bgm-1* SCAR marker from Oct. 1999-June 2000.

Some modifications were made to the protocols in order to reduce time-consuming activities such as sampling and DNA extraction. Briefly, leaf disks were cut and placed in the microtiter plates directly in the field, thereby eliminating the need to prepare plastic envelopes and organize samples in Elisa plates. Also the utilization of an apparatus to macerate 96 tissue samples at the same time made it possible for one person instead of two to isolate the DNA easily. In comparison with 1999 the total time for selecting the *bgm-1* gene in a 3000-plant nursery was reduced by approximately two days (Table 1).

Table 1. One year progress in time-consuming activities for selecting the *bgm-1* gene in a 3000-plant screening.

Task	July 1999		Feb. 2000	
	No. People	Time (days)	No. People	Time (days)
1. Preparation of stickers, envelopes, labels	4	2	4	2
2. Labeling plants in the field	3	2	3	2
3. Field sampling	10	0.4	7	0.6
4. Sample organization in Elisa plates	3	2	-	-
5. DNA extraction	2	2	1	2
6. DNA dilution	1	2	1	2
7. PCR	1	2	1	2
8. Electrophoreses	2	3	2	3
9. Reading gels	1	0.5	1	0.5
Total Time		15.9		14.1

The SCAR selected 45.6% of the total number of plants in the nurseries evaluated. Plants having the marker were advanced and used in the generation of new crosses.

BGMV selection in red-seeded lines. The effectiveness of MAS for the *bgm-1* SCAR was shown in a group of red-seeded lines. F_1 plants previously selected (1998) with the SCAR were used to develop the 18 final crosses.

The populations were advanced using gamete selection and evaluated for resistance to CBB, ALS, BSMV, BCMV and low phosphorous tolerance (n=826) in complementary nurseries (Mesoamerican Bean Genetics Program). Then in 1999, one F_5 plant per line was screened with the BGMV SCAR, and those that had the resistant band were selected (n=194). In the F_6 (Feb. 2000), 5 plants per line were sampled, and DNA bulks were used to amplify the SCAR (Fig. 1). It was concluded that the same result is obtained when evaluating 1 or 5 plants in F_5 or F_6 progenies, which means that they are already homozygous for the marker.

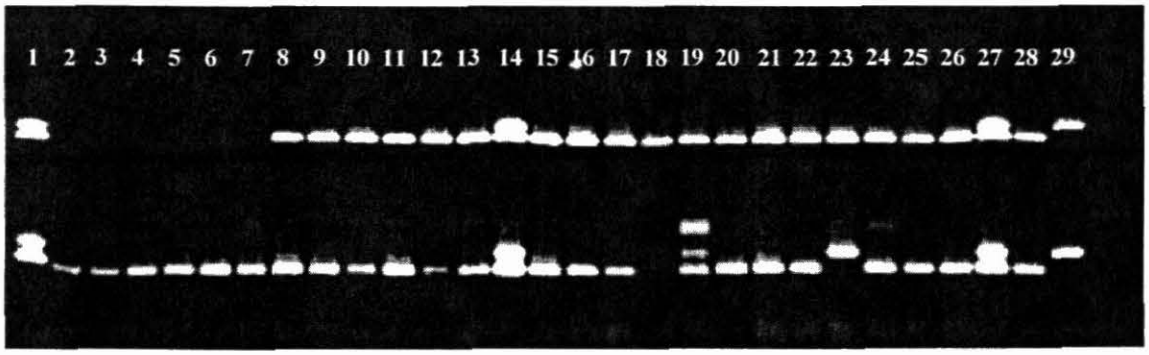

Figure 1. Selecting the *bgm-1* marker in red-seeded lines. Lanes 1, 14 and 27 correspond to PCR products of a susceptible ($DOR21_{570}$) and a resistant ($DOR21_{530}$) variety used as size marker. In both rows, bands appear in lane 28 from Tio Canela 75 (BGMV-resistant line) and lane 29 from Orgulloso (BGMV-susceptible variety).

Two sets of lines with commercial seed quality were selected and inoculated with BGMV in the Virology Research Unit greenhouse. The first set of 61 lines had the *bgm-1* SCAR, but the second set of 22 lines did not. Ten plants per line were inoculated with BGMV. The results were as follows: A comparison can be made between the SCAR and the evaluation in the greenhouse. Both agreed on 66.3% of the lines (55/83), of which 50.6% had the marker and high or intermediate levels of resistance and 15.7% lacked the marker and were susceptible. For the remaining 33.7%, the SCAR did not match the result of the greenhouse evaluation. If the *bgm-1* gene were present in susceptible lines, it would not be expressed; and if absent in resistant lines other genes would probably be involved in the expression of resistance to BGMV (Table 2).

Table 2. Relation between BGMV *infection* in red-seeded lines and the SCAR linked to BGMV resistance.

BGMV infection (%)	*bgm-1* SCAR	
	Present (+)	Absent(-)
0%	16	5
10-20%	14	3
22-44%	12	1
50-100%	19	13

For this nursery, MAS proved to be an efficient tool for breeding and developing commercial bean varieties. Attempts were also initiated to use W12 SCAR linked with a QTL for BGMV resistance derived from DOR 364. The utilization of markers linked with other resistance genes would make it possible to select all the plants potentially resistant to BGMV.

1.2.2 Marker assisted selection in *Brachiaria*

C. Quintero[1], J.W. Miles and J. Tohme
[1] IP-1 Project

Introduction

As a breeding tool, apomixis offers several advantages because it associates fixation of hybrid vigor with seed propagation (do Valle and Savidan, 1996). Breeding initiatives aim to create apomictic genotypes combining desirable characteristics such as spittlebug resistance and high productivity (Miles and do Valle, 1996). Several efforts have been made to tag the apomixis gene; and RFLP, AFLP, RAPD and SCAR markers have been identified (Rocha *et al.*, 1997). The objective of this project is to use those markers for selecting apomictic plants in order to improve Brachiaria breeding.

Materials and methods

A population of 96 hybrid clones was screened with a SCAR derived from RAPD N14, which is located at 6cM from the apomixis gene (Rocha et al, 1997). These clones were obtained from crosses between clones selected in 1997 from the sexual breeding population and elite apomictic genotypes including both accessions and hybrid-derived clones. DNA was extracted using the protocol described by Rocha et al. (1997). Because of the complexity of this DNA extraction, an effort was made to develop a rapid and suitable method for large-scale screening of markers. The protocol of Edwards *et al.* (1991) was modified as follows: one gram of young leaf tissue was collected and dried at

45-50°C for 20 h approximately. The dry tissue was ground and stored at –20°C until its utilization. DNA was extracted from 30-50 mg of tissue as follows: 600µl of 200mM Tris-HCl pH 8.0, 250mM NaCl, 25mM EDTA, 0.5% SDS and 1% β-mercaptoethanol were added and mixed in a vortex genie for 10 sec. Then 200µl of 5M cold potassium acetate were added, and tubes were held on ice for 10 min. They were then centrifuged at 14000 rpm, and the DNA was precipitated overnight at –20°C by adding 400µl of cold isopropanol to 500µl of the supernatant. The DNA was pelleted by centrifugation at 14000 rpm for 10 min., washed with 70% ethanol, dried in a Speedvac and dissolved in 50µl of 10mM Tris-HCl pH 8.0. Five microliters of the extracted DNA were diluted in 45µl of sterile water. Then 5 microliters of the diluted DNA were used for the PCR. When the N14 SCAR was amplified using both DNA extraction methods, no difference was observed.

In a subsequent trial, nine open-pollinated progenies derived from the aforementioned hybrid clones were planted, and their DNA was extracted using the short protocol. Not only was the N14 marker amplified in the total number of 202 plants, but also five microsatellite markers were developed from the apomictic genotype CIAT 606 (E. Gaitán, pers. comm.). Simultaneously, a progeny test was conducted in the field.

The progeny test showed the apomictics BR98NO/1251, BR98NO/4015, BR98NO/4132. Also, the N14 marker was found only in these hybrids and their progeny, being absent in the sexual ones (Fig. 2).

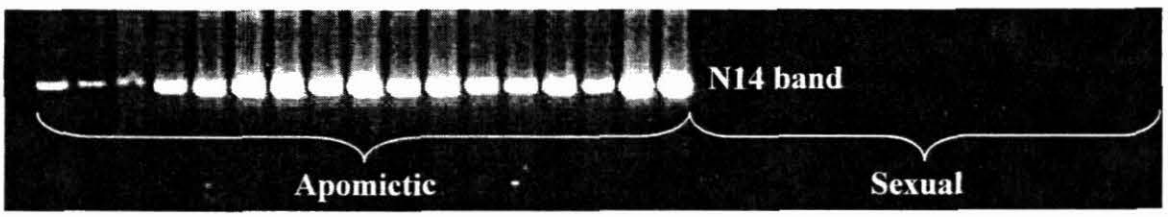

Figure 2. Identification of apomictic and sexual families, using the N14 SCAR marker.

When microsatellite markers were amplified, similar results were obtained. A high degree of monomorphism was observed in the three hybrids listed above and in their progeny. Plants in sexual families, when compared to the maternal genotype, showed absence of some alleles (Fig. 3). Only BR98NO/1251 was absolutely monomorphic for the five microsatellites. In this group of families the utilization of molecular markers was useful in the identification of apomixis.

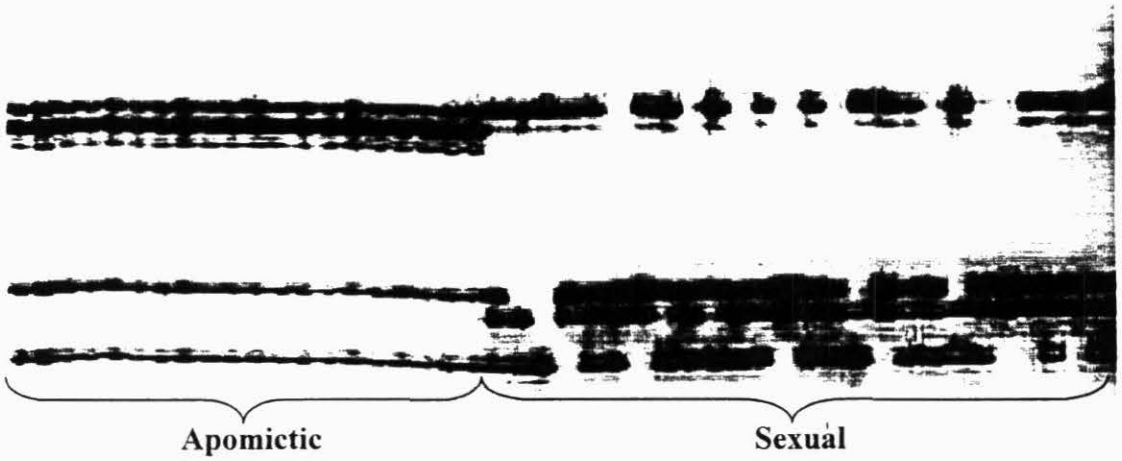

Figure 3. Identification of apomictic and sexual families, using the GM6 microsatellite.

In another trial, 44 hybrids with their offspring (at least 9 plants) were screened for the SCAR marker and three of the microsatellites. Although N14 was found in 18 maternals, only BR98NO/0313, BR98NO/0314, BR98NO/0627 and BR98NO/0861 were monomorphic with the microsatellites (GM6, GM7 y GM80). A MAS scheme for apomixis in Brachiaria will be established.

1.2.3 Marker assisted selection in rice

C. Quintero, F. Escobar[1], C. Martínez, F.Correa[1] and J. Tohme
1 IP-4 Project

Introduction

Hoja blanca virus (HBV) is one of the most important diseases in rice, transmitted by the insect *Tagosodes orizicolus* (Muir) known as *Sogata*. Common symptoms are chlorosis, dwarfism and necrosis, which finally reduce yield. In 1981 HBV caused economic losses in Colombia calculated at 85-100%. From 1995-1996 a new epidemic started in Colombia but was partially controlled; and during the last three years a severe attack of HBV has caused 70% yield losses in Peru (Calvert and Reyes, 2000). Although a number of varieties resistant to both insects and viruses are available, looking for molecular markers linked to the resistance genes would be useful in the breeding program for a rapid development of new lines.

Materials and methods

Over several evaluations of rice germplasm, 159 lines were chosen for the consistency of their reaction to the virus and its vector. DNA was extracted individually and then eight groups (bulks) were formed according to resistance and origin (Table 3).

Table 3. Distribution of rice genotypes for tagging Sogata and HBV-resistance genes

Bulk No.	Reaction to		Description
	Sogata	HBV	
1	R	R	Highly resistant
2	R	R	Highly resistant
3	R	R	IRAT varieties
4	R	S	IRRI varieties
5	R	S	CIAT varieties
6	S	R	Different origin
7	S	R	Indica varieties
8	S	S	Different origin

The RAPD technique was used and approximately 600 ten-mer oligonucleotide primers were amplified in the eight DNA bulks. Up until September, 330 primers were electrophoresed. For Sogata, five RAPD bands were found consistently in all the resistant bulks (Fig. 4) and 27 in the susceptible ones. When two of the primers showing resistant bands were screened in each of the resistant varieties, only CT8447-5-6-3P-1X did not have the OPZ-19 marker. For HBV, some RAPD markers that are also linked with resistance or susceptibility were found, but the results were not as consistent as those for Sogata. This work is still ongoing.

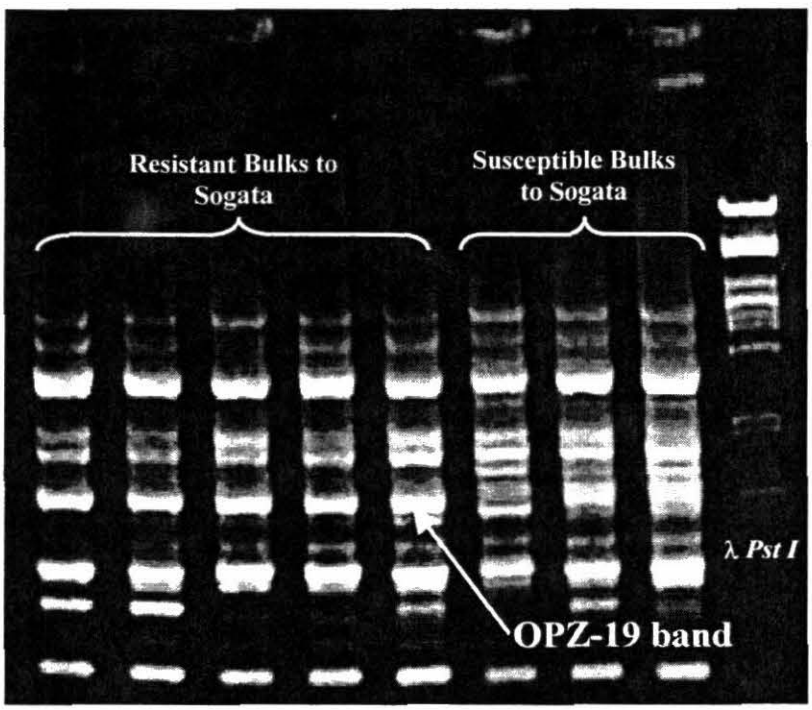

Figure 4. Amplification of OPZ-19 RAPD primer in rice varieties with different levels of resistance to Sogata and HBV.

Ongoing activities

- Continue with the MAS scheme for beans
- Evaluate a large set of microsatellites for MAS in Brachiaria
- Continue with RAPD screening in rice and cloning polymorphic DNA fragments linked with the resistance to Sogata and HBV

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1.2.4 Marker-assisted breeding of resistance to CMD in Latin American cassava gene pools

A. Akano, I. Lozano, L.Calvert, H. Ceballos, M. Fregene

Introduction

The availability of molecular markers for CMD resistance has made possible for the first time its breeding at CIAT. As a first step to CMD resistance breeding at CIAT, the new source of resistance to CMD, found in a group of closely related Nigerian cassava land races were imported from IITA to CIAT. Due to quarantine restrictions, the land races themselves could not be imported into Colombia, rather F_1 progeny obtained crossing the resistant parent to an improved line and resistant to CMD maintained as *in vitro* culture of embryo axes were imported as the source of CMD resistance. The CMD donor lines were imported with the approval of the Colombian quarantine authorities. The *in vitro* plantlets were tested on arrival by ELISA and PCR methods for the presence of ACMV or EACMV before sub-culturing and transfer to the green house. Permission to transfer the plants to the field will be obtained from the Colombian quarantine authorities, and the plants will be transferred to a healthy site for hybridization with the elite parents of Latin American cassava gene pools at CIAT.

Materials and Methods

Twenty cassava lines from an F_1 mapping population having TME3, one of the land races with the new source of CMD resistance as parent, and resistant to CMD were shipped to CIAT from IITA as *in vitro* plantlets. The plantlets were obtained from embryo axes cultures that have always been kept *in vitro* and have never been in the field, neither have they been in contact with the virus or its vector. The plantlets were sub-cloned using 2 nodal cuttings cultured in Murashige and Skoog's (1962) basal medium supplemented with 2% sucrose, $1mgl^{-1}$ GA3 (growth hormone) and 0.7% agar, pH adjusted to 5.6± 0.1 before sterilization. All plant cultures were maintained at 25-29oC with 12 hours photoperiod and 5,000 lux in culture rooms. The plantlets were tested for presence/absence of cassava mosaic virus (CMV) using enzyme linked immunosorbent assay (ELISA) and PCR-based diagnostic methods.

Plantlets were transplanted to the screen house after 5-6 weeks of culturing. Plantlets were carefully shaken out of culture tubes into a plastic bucket containing clean water and culture medium was washed off from the roots and then placed in jiffy peat pots three-quarters full of peat moss mixture. They were kept for hardening under a humidity chamber with an initial relative humidity (RH) of 100% for the first 3 days at 25-35oC in a shaded screen house. The RH in the chamber was gradually reduced, by perforating the chamber increasingly until it was equal to atmospheric humidity, by the 6th-8^{th} day after transplanting. After 3 weeks of hardening, the plants were transplanted into polythene plastic bags filled with sterile topsoil and kept in the screen house.

Results

An average of 5 plants each, per genotype, was successfully sub-cloned *in-vitro* (Table 2). A copy each from the 20 genotypes is maintained *in-vitro* in the tissue culture lab, while the remainder have been moved to the screen house.

Table 1: List of resistant cassava lines sub-cloned *in-vitro* and currently available in the green house and the tissue culture room of the BRU

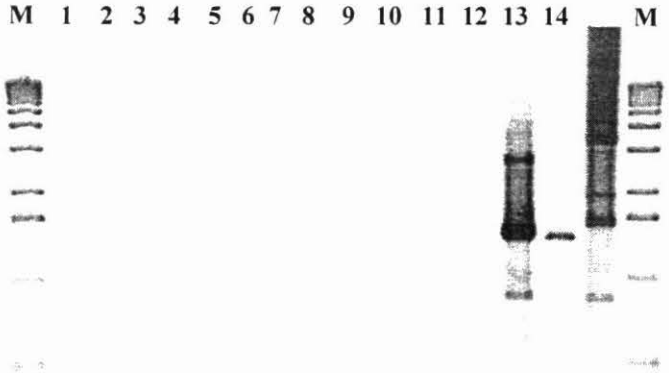
Genotypes	Numbers sub-cloned *in-vitro*	Genotypes	Numbers sub-cloned *in-vitro*
C54	6	C18	7
C35	4	C127	8
C39	7	C43	5
C22	3	C373	9
C41	3	C227	6
C33	6	C101	8
C413	5	C400	5
C24	5	C377	2
C6	8	C151	3
C243	7	C19	8

The results from ELISA and PCR diagnostics tests carried out on samples showed absence of the cassava mosaic virus and are shown below (Table2 and Fig 1).

Table 2: Result of the *ELISA test to* detect ACMV in cassava plantlets introduced from IITA, Nigeria using Monoclonal antibibody 4C1-3F7

Sample numbers	Genotypes	Absorbance (405nm)*	ELISA reaction (±)
1	C33	-0.016	-
2	C41	-0.016	-
3	C43	-0.018	-
4	C54A	-0.017	-
5	C54B	-0.016	-
6	C71	-0.016	-
7	C227	-0.017	-
8	C243	-0.017	-
9	C373	-0.015	-
10	C401	-0.003	-
11	Blanco	-0.096	-
12	Negative (healthy) control	-0.011	-
13	Positive control	1.488	+

*Any absorbance value more than twice that of the healthy control is considered positive



1. C33
2. C41
3. C43
4. C54A
5. C54B
6. C71
7. C227
8. C243
9. C373
10. C401\
11. Control healt (-)
12. Control positivo (BGMV)
13. Control positivo (potato)
14. Control positivo (plasmido Q3)

M. Marcador de peso molecular (1Kb)

Fig 1. PCR for the detection of the African Cassava Mosaic Virus (ACMV)

The CMD resistance donor plants are currently in the green house and will be moved to the field for hybridizations with elite parents of Latin American cassava gene pools once approval for this is obtained from the Colombian quarantine authorities.

Highlights of Outputs

1. Cassava plantlets from 20 resistant lines to CMD successfully transferred *in-vitro* from IITA, Nigeria to CIAT.
2. Cassava plantlets free from CMD from the 20 lines subcloned and copies maintained *in-vitro* in the tissue culture room of CIAT's BRU.
3. Cassava plants from 20 resistant lines successfully established in the green house in CIAT.

Plans for Next Year

- Cassava plants from the 20 resistant lines to be planted on the field and hybridized with elite LA elite.
- Hybridized lines to be used in marker-assisted selections in collaboration with IITA, Nigeria in Africa.

1.2.5 QTL mapping of micronutrient content in two populations of common bean

M.W. Blair, S. Beebe, J. Rengifo, C. Astudillo

Introduction

Legumes provide essential micronutrients that are found only in low amounts in the cereals or root crops. An ongoing project, has shown that bean seeds are variable in the amount of minerals (iron, zinc and other elements), vitamins and sulfur amino acids that they contain and that these traits are likely to be inherited quantitatively. The objective of our most recent studies has been to tag some of the quantitative trait loci (QTLs) controlling mineral content in beans.

Materials and Methods

We began this study with the parents of four recombinant inbred line (RIL) populations that were developed for micronutrient studies (Table 1). These eight parents were chosen because they had contrasting amounts of micronutrients. As an initial step for genetic mapping, we analyzed whether the parents were polymorphic using 100+ RAPDs and 25 microsatellite markers. The two populations that were selected for genetic mapping, represented both major gene pools of common bean: one population was derived from an Andean x Andean cross (G 21657 x G 21078); while the other was from a Mesoamerican x Mesoamerican cross (G14519 x G4825). Miniprep DNA was extracted from all the

lines in both populations and all the individuals were genotyped for RAPDs and microsatellites. The marker information was used to construct separate genetic maps for each of the populations, using the software program MAPMAKER. The phenotypic data was obtained by analyzing both the parents and the recombinant inbred lines for mineral and sulfur amino acid content by ICP (Inductive coupling plasma) and HPLC analysis, respectively. Quantitative trait locus (QTL) analyses were conducted by regressing the phenotypic data for micronutrient content onto marker genotypes with the software program qGENE.

Results and Discussion

In general the rate of polymorphism was low in these crosses since they were from within a single genepool, however both types of markers used detected sufficient polymorphism to allow us to proceed. RAPD primers were used to generate a large number of markers, while microsatellites were used because they have several advantages including occupying unique mapped positions in the genome and a somewhat higher rate of polymorphism. The two most polymorphic populations were chosen for the mapping effort and two genetic maps were constructed. For the Mesoamerican population, eight linkage groups could be detected, while the Andean population had a total of 14 linkage groups. As common bean has 11 homologous chromosomes, the present maps are unlikely to be complete. The single locus nature of microsatellite markers was useful for anchoring the RAPD markers to known chromosomes.

In both the populations both iron and zinc content in the RILs presented a continuos distribution, suggesting that mineral content behaved as a quantitative trait. Highly significant correlations were observed among iron and zinc content in the recombinant inbred lines (Table 1). QTLs were found for iron and zinc content in both populations. The positive markers varied in their level of significance and the proportion of variance in mineral content that they explained (R-squared value). The most significant QTLs explain up to 19% of the variance in mineral content. In some cases the QTLs for both minerals occurred jointly at the same marker, in other cases there were QTLs specific for each mineral. It can be concluded that some of the QTLs for the accumulation of both minerals may be genetically linked or pleiotropic, controlling both traits at once. If the same QTLs contribute simultaneously to both iron and zinc content, it may be easy to select for these traits jointly. Of 29 markers that were positive for a significant effect on mineral content in the Andean population, 10 controlled both Fe and Zn content, 7 controlled Fe alone and 10 controlled Zn alone. These results agree well with what is suggested by the number of segregating lines that presented iron contents similar to the high parent. Positive QTLs were found on linkage group 2 for the G14519 x G4825 population, and on chromosome B3 and linkage groups 2 and 5 for the G21242 x G21078 population. The majority of the positive QTLs are associated with alleles from the high mineral parent. Therefore, it appears that high mineral content parents provide most of the genes for high mineral content to their progeny, while low mineral parents provide only a few additional genes for mineral content. This may explain why the distribution of

mineral content in the progeny was similar to the range between the parents and why transgressive segregation was minimal.

Future Activities

At the moment is difficult to integrate the results from the two populations without using common markers across the separate maps through a process called comparative mapping. Progress in linking the two maps derived from both populations will depend on the development and mapping of more microsatellite markers. Since these markers are single-copy and map to specified regions of chromosomes they can be used in comparative mapping to anchor two maps to each other and to previously published maps that have these markers. By next year, we hope to be able to identify and anchor the chromosomes and to compare the QTLs located on each map with a full set of microsatellites.

The present work will hopefully permit us to focus on certain parts of the genome to determine if desirable alleles for higher mineral content are located at the same loci in the different populations developed specifically for this purpose. Future analysis will also include the detection of QTLs for the amount of sulfur containing amino acids (SAA), as well as for the amount of the other minerals analyzed in the ICP study, which include Mn, Ca, Mg, K, P and S. The QTL analysis will help us to consider the possibility of genetically increasing SAA (methionine or cysteine) or Lysine as uptake promoters of iron or decreasing antinutritional factor such as tannins or lectins that reduce the uptake of iron. We also plan to integrate the information about the map locations of QTLs for micronutrients with those for other agronomic traits that we have been studying, so that we can select for the best recombinants from crosses between high micronutrient lines and the best varieties.

Table 1. Parents and crosses used for the QTL analysis of micronutrients content and correlation between iron and zinc content in three datasets (the populations of recombinant inbred lines and the Andean core collection).

Cross	Origin	Traits	Correlation (r)
G 21242 X G 21078	Andean x Andean	Fe, Zn and SAA	0.63**
G 11350 X G 11360	Meso x Meso	Fe, Zn	--
G 14519 X G 4825	Meso x Meso	Fe, Zn	0.55**
G 21657 X G 21078	Andean x Andean	Fe	--
Andean core collection	Landraces - Andeans	Fe, Zn	0.52**

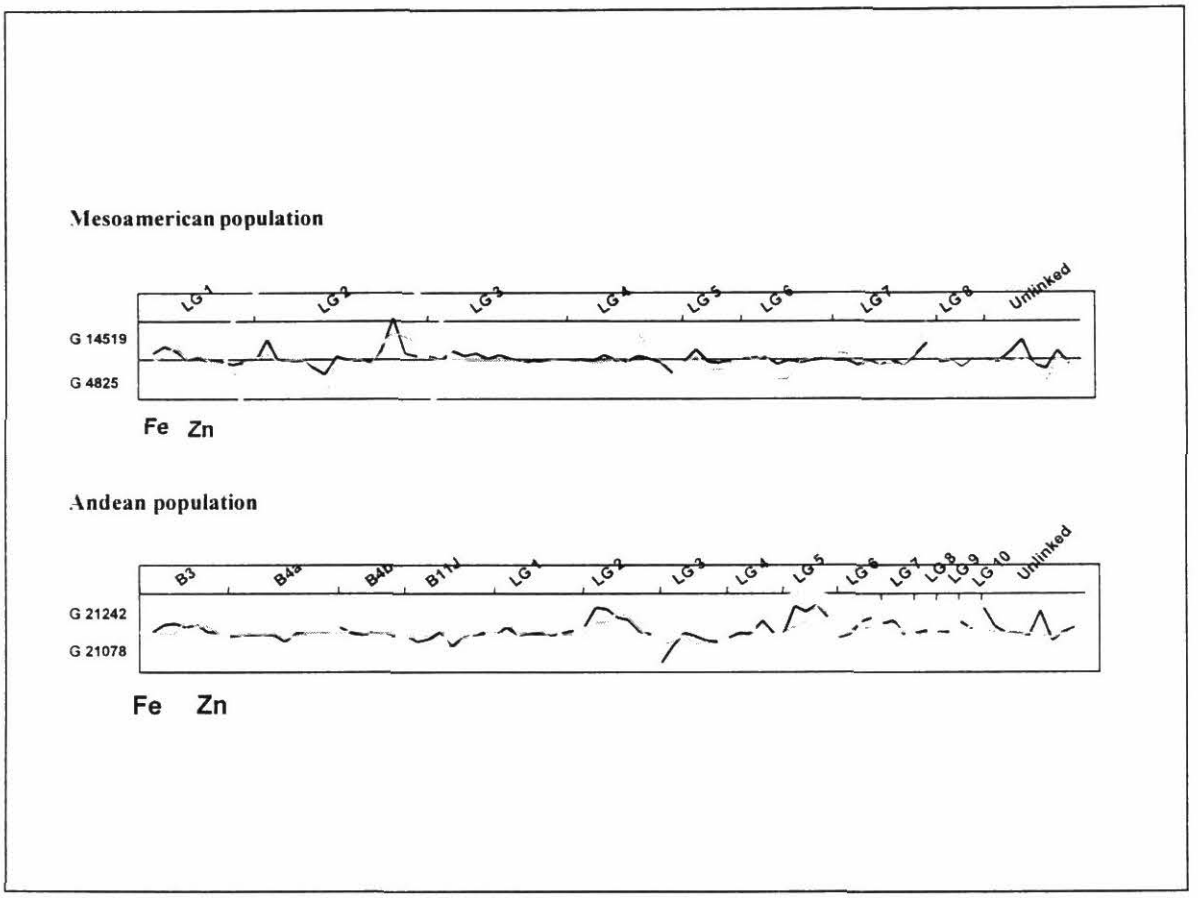


Figure 1. Analysis of quantitative trait loci (QTLs) for iron (Fe) and zinc (Zn) micronutrient content in two populations (Mesomaerican and Andean). Chromosomes (B) or unidentified linkage groups (LG) are identified and significant QTLs are circled.

1.2.6 QTL mapping of resistance to *Thrips palmi* in common bean

M.W. Blair, C. Cardona, S. Beebe, J.M. Bueno

Introduction

Thrips palmi is a damaging insect pest of common bean and other dicotyledenous crops that was introduced from Asia (Java, Indonesia) into the Americas during the last decade. Starting in the Caribbean, (Cuba, Dominican Republic, Haiti and Puerto Rico) the species spread rapidly into the United States and northern South America (Brazil, Colombia, Ecuador and Venezuela). The greatest damage inflicted to common bean production in Colombia is seen in climbing bean varieties that are grown for the fresh market (including snap beans and Cargamanto dry beans). Sequential plantings, common in the production of snap beans is very conducive to heavy infestations of thrips and whiteflies,

which are synergistic in the damage that they inflict. Misuse of insecticides also can lead to resurgence in thrips populations. The first studies in integrated pest management of thrips have been conducted recently at CIAT (see IP-1 project annual report). One of the bean genotypes with the best thrips resistance has been BAT881. The objective of this research was to study the inheritance and location of quantitative trait loci controlling the resistance derived from this variety in a recombinant inbred line (RIL) population derived from BAT881 x G21212.

Materials and Methods

The BAT881 x G21212 population, consisting in 139 RILs was evaluated over two seasons at a field site in Pradera, Valle, Colombia. In the first season (semester 1999A - April), the population was planted as an un-replicated trial; while in the second season (semester 1999B - July) it was planted in a randomized complete block design with three repetitions. The parents of the population were included in both seasons. The bean genotypes, PVA773 and RAZ 136, were used as check varieties in the first season while PVA773 was used alone in the second season. Infestation by *Thrips palmi* was increased with an initial planting adjacent to a heavily affected snap bean field. The lines were evaluated on a per row basis using a 1-9 scale according to the CIAT standard evaluation (1=resistant; 9=susceptible). DNA was extracted from 95 of the RILs by a standard miniprep procedure. One hundred and fifty one RAPD markers were run on these individuals and the segregation information was analyzed to construct a genetic map using the software program MAPMAKER. Quantitative trait loci (QTL) were identified through single-point regression analysis of the phenotypic data onto the marker genotypes using the software program qGENE.

Results and Discussion

The population of RILs was normally distributed for thrips resistance, suggesting that the inheritance of resistance was truly quantitative. Some of the RILs outperformed either parent suggesting transgressive segregation had ocurred, however the best lines were different in each season. BAT881 was the more resistant parent for both resistance scores (damage score = 6.0 and 7.5 in first and second seasons, respectively; reproductive adaptation = 7.0 and 7.1), however G21212 was somewhat similar for its reproductive adaptation (9.0 and 7.6) but inferior for its susceptibility to thrips damage (8.0 and 8.2). Overall the correlation between seasons was moderate ($r = 0.277$ for damage score and $r = 0.371$ for reproductive adaptation), while the correlation between the two resistance scores within a season were high ($r = 0.873$ for the first season and $r = 0.751$ for the second season).

Positive QTLs were found for thrips resistance in both seasons and were associated with both parents (Table 1). However, it appeared that G21212 was the principal source of QTLs for resistance to thrips damage, while both BAT881 and G21212 were the sources of QTLs for reproductive adaptation under thrips infestation. However this varied from the first season when a mix of both genotypes provided QTLs for reproductive adaptation to the second season when all the positive QTLs except one were provided by G21212.

An additional single QTL from BAT881 provided resistance to damage in the second season but was unlinked to other markers. The existence of additive QTLs from both parents may explain why transgressive segregation was observed in some of the individual progeny.

In general different markers were associated with QTLs for the two traits. Indeed the QTLs for the two traits were usually found on separate linkage groups. The only exceptions to this rule were for the markers AJ901 and E401 in the first and second seasons, respectively. In the first season QTLs for both traits were found together on the linkage group BG9. However in this season additional QTLs, albeit with low significance were found on several additional linkage groups. Meanwhile, it was interesting that the marker that detected the only QTL from G21212 that was significant for reproductive adaptation in the second season, was the same marker associated with the top QTL for damage score in that season. This suggest that resistance to thrips damage from G21212 does allow for pod set under thrips infestation but is not the only factor controlling it.

Because the trial was replicated in the second season, QTLs tended to be more highly significant for the second season's data (up to F-value of 11.2) than for the first's (maximum F-value of 6.5). In addition, in the second season the inheritance of resistance appeared to be fairly simple: only one QTL each could be identified for resistance to damage and reproductive adaptation. The QTL for resistance to damage consisted in six significant markers on linkage group BG1 with the strongest association with the G21212 allele at E401. The QTL for reproductive adaptation was found on linkage group BG7 and was associated with the BAT881 allele at the loci for AG1002, H1202, P403 and L205. This QTL was also observed in the data from the first season but at a slightly different location along the linkage group BG7 with the markers, I702 and Q302.

Future Activities

Another population derived from the cross BAT477 x DOR364 will be analyzed for thrips resistance. The results from the BAT881 x G21212 and BAT477 x DOR364 populations will be integrated by comparative mapping using PCR-based microsatellite markers. The four genotypes used to produce the two population are from the same race of Mesoamerican beans, therefore it will be interesting to see how well the genes for thrips resistance identified in these studies can be transferred to other genepools.

Table 1. RAPD markers positively associated with resistance to *Thrips palmi* in an unreplicated trial of the population BAT881 x G21212 in Pradera, Valle in two seasons.

Marker	Linkage Group	N	Source	F	RSq	LOD	P
Season 1 - 1999A							
Damage Score							
O1603	BG3	95	BAT881	5.56	0.0564	1.2	0.0205
AJ901	BG9	94	BAT881	4.48	0.0464	0.97	0.037
M1501	BG3	95	BAT881	4.33	0.0445	0.94	0.0402
S1001	BG3	95	BAT881	4.1	0.0422	0.89	0.0458
M1503	BG8	95	BAT881	4.06	0.0418	0.88	0.0468
Reproductive Adaptation							
AJ901	BG9	94	BAT881	6.45	0.0655	1.38	0.0128
Z1901	BG9	95	BAT881	6.07	0.0613	1.3	0.0156
L1103	BG6	95	BAT881	6	0.0606	1.29	0.0162
L1102	BG13	95	G21212	5.34	0.0543	1.15	0.0231
T701	BG5	94	BAT881	4.55	0.0471	0.99	0.0356
I702	BG7	94	G21212	4.48	0.0464	0.97	0.037
L1104	BG6	95	BAT881	4.34	0.0446	0.94	0.04
Q302	BG7	95	G21212	4.12	0.0424	0.89	0.0452
Season 2 - 1999B							
Damage Score							
E401	BG1	95	BAT881	11.2	0.1075	2.35	0.001
E402	BG1	95	BAT881	10.47	0.1012	2.2	0.002
O1505	unlinked	95	G21212	10.4	0.1006	2.19	0.002
AL401	BG1	94	BAT881	8.75	0.0868	1.85	0.004
AE101	BG1	94	BAT881	8.47	0.0843	1.8	0.005
C706	BG1	95	BAT881	7.86	0.0779	1.67	0.006
AG1301	BG1	61	BAT881	5.26	0.0819	1.13	0.025
Reproductive Adaptation							
AG1002	BG7	94	G21212	7.55	0.0758	1.61	0.007
H1202	BG7	95	G21212	7.02	0.0702	1.5	0.010
O403	BG7	94	G21212	6.12	0.0624	1.31	0.015
L205	BG7	95	G21212	5.91	0.0598	1.27	0.017
E401	BG1	95	BAT881	5.11	0.0521	1.1	0.026

1.2.7 QTL mapping of angular leaf spot and anthracnose disease resistance in the *common* bean cross DOR364 x G19833

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1. IP-1 Project

Introduction

All together, several dozen major resistance genes have been tagged in common bean mostly through bulked segregant or simple genetic linkage analysis with either RAPDs or SCARs. Reliable markers have been developed for the well known genes for resistance to bean common mosaic virus (*I, bc-3, bc-12*), bean golden mosaic virus (*bgm-1*), rust (*Ur-3, Ur-5, Ur-11*) and anthracnose ($Co\text{-}4^2$ and *Co-2*). Most of the resistance genes studied have been simply-inherited. Relatively fewer studies have looked at the quantitative trait loci (QTLs) controlling partial resistance to diseases. The objective of this research was to identify QTLs for resistance to two pathogens of common beans, *Colletotrichum lindemuthianum*, the causal agent of anthracnose and *Phaseoisariopsis griseola*, the causal agent of angular leaf spot (ALS) in a well-characterized genetic mapping population based on the cross DOR364 x G19833. Recombinant inbred lines (RILs) from this population have been a very useful resource for field and greenhouse studies because they are a genetically-stable set of advanced lines that can be tested in replicated trials with a series of disease isolates.

Materials and Methods

The 87 RILs of the DOR364 x G19833 population were tested with six isolates each of ALS (PG3COL, PG260COL, PGCRI, PG3ELS, PG14HND and PG12MEX) and anthracnose (CL5DOM, CL20COL, CL43COL, CL77CRI, CL235COL and CL289COL) by artificial inoculation in the greenhouse. Disease evaluation was done qualitatively by treating disease reaction as a binary trait; and assigning the RILs into a susceptible or resistant category. For six of the disease isolates (PG260COL, PG3ELS, PG14HND, CL20COL, CL43COL and CL77CRI) data was also taken quantitatively; and the individual plants were scored for resistance on a 1-9 scale according to the CIAT standard evaluation (where 1= resistant and 9= susceptible). The genetic map for the population consisted of 417 markers (AFLPs, microsatellites, RAPDs and RFLPs) and was constructed using the software program MAPMAKER. The microsatellite markers were placed at a minimum LOD score of 2.5, while the rest of the markers had a minimum LOD score of 2.0. Quantitative trait loci (QTL) were identified using the software program qGENE by 1) single-marker contingency analysis using chi-square tests comparing the genotypic classes to the categorical data from the qualitative disease evaluation; and 2) single-point regression analysis of the quantitative disease score data onto the marker genotypes. A probability threshold of $P = 0.0001$ was used for the individual marker tests to reduce overall type I error rate to $P = 0.05$, based on the full set of genetic markers used in the experiment.

Results

For anthracnose, significant QTLs were found on chromosome 3, 4, 10 and 11 (Figure 1). Three isolates (CL77CRI, CL20COL and CL5DOM) uncovered the same resistance QTL on chromosome 3. One of these isolates (CL5DOM) also uncovered a QTL on chromosome 4. Another isolate (CL235COL) uncovered a second QTL for resistance on chromosome 4 proximal to the previous QTL. A fifth isolate (CL43COL) uncovered two QTLs, one on chromosome 10 and another on chromosome 11. No significant QTLs were detected when using the isolate CL289COL.

The QTLs on chromosome 4 coincided in location with known resistance factors for anthracnose (*Co-8*, *Co-y* and *Co-z* cluster) and BGMV resistance. Meanwhile the QTL on chromosome 11 was probably a previously undescribed allele of the *Are* gene (renamed *Co-2*). The resistance QTLs on chromosomes 3 and 10 do not coincide with any previously mapped resistance genes in common bean but may be allelic to the anthracnose genes that have been characterized and tagged but not mapped (*Co-1*, *Co-4*, *Co-5*, and *Co-6*).

For angular leaf spot, significant QTLs were found on chromosomes 3, 4 and 10. Four isolates (PG12MEX, PG3ELS, PG14HND and PGCRI) uncovered QTLs on chromosome 10 in the vicinity of the QTL for anthracnose described above. The isolate PG12MEX also detected a QTL at one end of chromsome 3. All along chromosome 3 minor genes appeared to be proportioning resistance against several isolates but their effects were less significant. The isolate PG260COL uncovered a significant QTL on chromosome 4, while the isolate PB3COL uncovered no QTLs for resistance. The QTLs uncovered in this study may be alleles of the two angular leaf spot resistance genes, *Phg-1*, and *Phg-2*, which have been tagged with SCARs but have not been localized on the genetic map of common bean.

Analysis of the quantitative data showed the same QTLs as those of the qualitative evaluation described above. For all the QTLs identified in this study, the positive effect was always from alleles of the resistant parent G19833 not from those of the susceptible parent DOR364. In contrast a similar study showed that the DOR364 provided resistance QTLs for bean golden mosaic virus.

Discussion

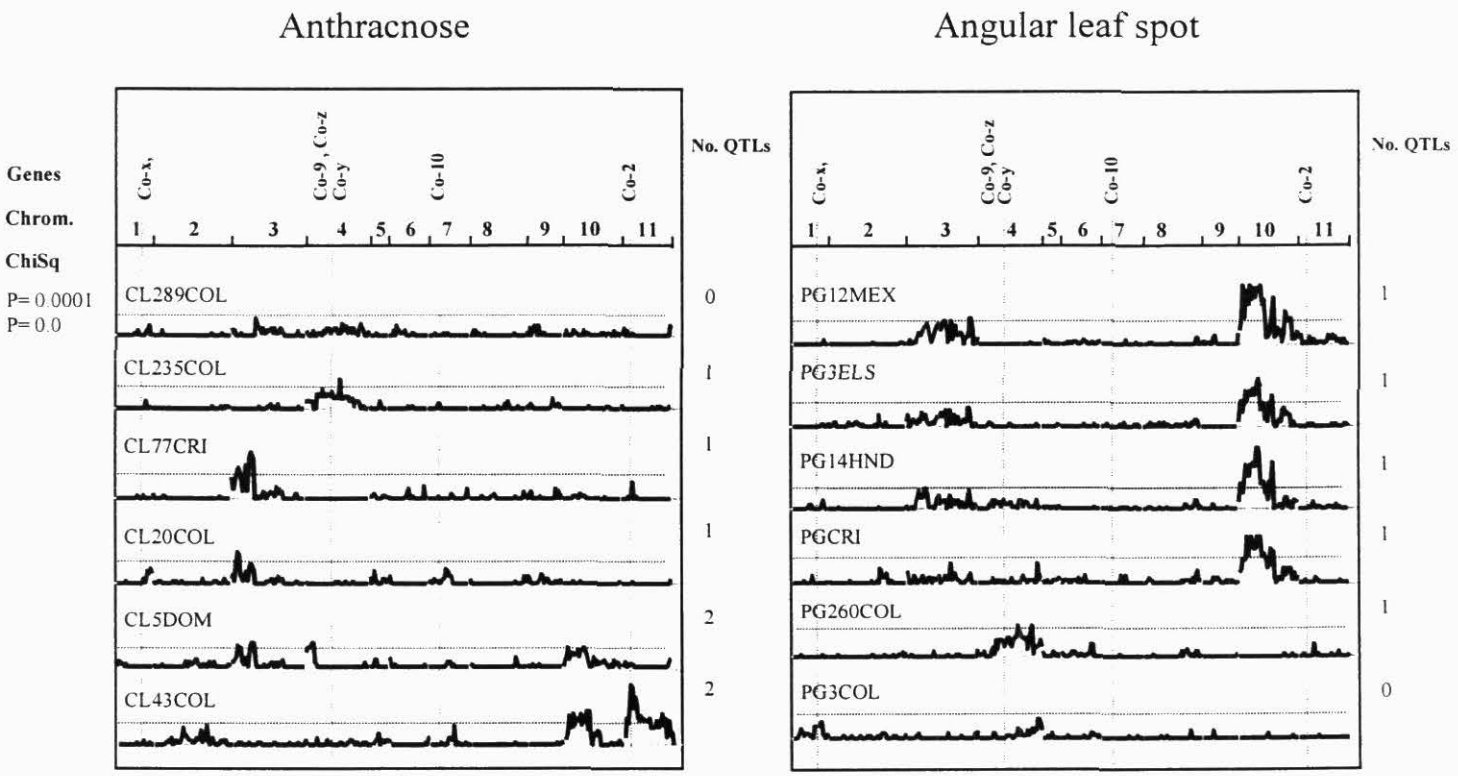
The inheritance of resistance to both anthracnose and angular leaf spot appeared to be surprisingly simple in the DOR364 x G19833 population. A maximum of two chromosomes were involved in resistance to any single isolate and on average only one significant QTL could be identified per inoculation. QTLs for disease resistance were found in five basic locations in the genome; both in areas that are already known to contain resistance genes and in areas where no resistance genes have been previously identified. A common QTLs was found for resistance to both diseases. At resistance gene clusters it is common to find resistance genes that function against multiple diseases and to find minor genes associated with major genes. Therefore, it is not surprising that

the QTLs for resistance to the two diseases provided by G19833 are clustered together with known resistance genes or with each other.

Future Plans

We have made crosses between other sources of ALS resistance, inlcuding a set of Mesoamerican and Andean differential varieties and plan to study the inheritance of resistance. It will be interesting to see whether the genes found in these studies are at the same locations of QTLs found in the DOR364 x G19833 population.

Figure 1. Location of QTLs for disease resistance identified in the population of DOR364 x G19833



1.2.8 Analysis of Andean advanced backcross populations for yield traits derived from wild *P. vulgaris*

M.W. Blair, S. Beebe, A.V. Gonzalez, G. Iriarte, J. Tohme

Introduction

The genetic diversity of cultivated *P. vulgaris* is thought to be larger than that of wild common bean due to a genetic bottleneck that occurred during domestication. Yield increasing alleles may still reside untapped in the wild accessions that could be exploited to improve cultivated beans. The advanced backcross method has been shown to be a useful method for incorporating wild germplasm into cultivar breeding programs for tomato and rice. Although wild beans have been used before to transfer resistance to diseases and insects, as in the noted case of the Arcelin gene that provides resistance to bruchids, the studies presented here are among the first to attempt to obtain a higher yield potential from wild beans. The objective of this research was to conduct a molecular analysis of two advanced backcross populations to find quantitative trait loci derived from wild beans that can be useful in the improvement of cultivated beans.

Materials and Methods

Two Andean advanced backcross populations were analyzed. The populations were derived from G24390 and G24404, which are wild beans from Mexico and Colombia, respectively, that were backcrossed into the background of a Colombian, large red-seeded, "Radical" type, commercial variety, ICA Cerinza. Each population consisted of 95 selected lines, while the first and second populations had 215 and 62 additional lines, respectively. The population Cerinza /2/ G24390 had a total of 309 lines; while the population Cerinza /2/ G24404 had a total of 157 lines. The selected and additional lines were yield tested in three locations for each population. The field sites were Puerto Rico, Palmira and Popayán for the Cerinza /2/ G24390 population and Darien and twide in Popayán for the Cerinza /2/ G24404 population. Three repetitions in a lattice design were used for each of the experiments, except in Puerto Rico where a randomized complete block design with two repetitions was used. An array of RAPD, SCAR and microsatellite markers was used to evaluate the introgression level in the full set of lines for each population. Quantitative trait loci (QTL) were identified through single-point regression analysis of the phenotypic data onto the marker genotypes using the software program qGENE. Map positions of markers were inferred by their locations on other genetic maps of beans.

Results and Discussion

In the BC2F1 generation, 25% of plants are expected to contain an introgressed allele for a given locus, but all of these plants are expected to be heterozygous. Upon self-pollination, the loci are expected to segregate in a Mendelian ratio whereby in each generation heterozgosity is lost and the loci are fixed to homozygosity. In this study we analyzed B2F3 derived families, in which the total amount of introgression for a given

locus is expected to be 15.675% of which, 9.375% should be homozygous and 6.25% heterozygous for the introgression.

The selected lines had a significantly lower introgression rate than the additional lines (Table 1), indicating that selection had eliminated large amounts of introgression. The observed rate of introgression was lower than expected for the selected lines for each of the Andean populations. However the additional lines had rates of introgression that were not significanltly different from those expected. By comparison the rate of introgression in the Mesomaerican population, DOR390 x G19892, was much lower than expected for both selected and additional lines (IP-1 Annual report, activity 1.1).

The microsatellites were ideal for this study because they had the advantage of being co-dominant and single copy and therefore could diagnose the families derived from heterozygous genotypes and differentiate them from those derived from homozygous individuals. For each microsatellite marker, the percentage of genotypes that represented heterozygous or homozygous introgressions was close to that expected for the BC2F3 generation. Meanwhile, the RAPD markers were dominant and could only diagnose the full number of introgressed individuals when the polymorphic band was present in the wild parent and absent in the cultivated parent. Given the opposite situation, band present in the cultivated parent and absent in the wild parent, the RAPD marker only detected introgression in those individuals that were homozygous for the marker locus. As expected, the detectable rate of introgression was lower when the RAPD band was absent in the wild parent than when it was present.

Significant QTLs for yield were found in both the Cerinza x G24390 and Cerinza x G24404 crosses (Table 2). However, the majority of the QTLs associated with the wild alleles were negative, in effect, positive QTLs whose source was Cerinza. This indicates that there were still a large number of the alleles transmitted from the wild parent that had negative effects on yield and that remained to be eliminated from the progeny. Only negative QTLs were detected in Puerto Rico, Palmira and Darien, which are environments that are atypical for the production of Cerinza or other Radical type varieties. In these environments, yields were low and differences were not significant in the replicated trials. The negative QTLs detected in each season were at different positions in the genome, indicating that there is a significant QTL x environment interaction for this trait.

Positive QTLs were found for both populations when grown in Popayán. This field site at 2100 masl was closer to the ideal growing environment for the Cerinza variety therefore a more appropriate testing site for advanced backcross progeny derived from Cerinza. In Popayán, the positive QTLs had phenotypic effects that ranged from 99 to 225 kg/ha increased yield and these significant QTLs explained from 7 to 22% of the variance for yield in these seasons. The QTLs were not associated with later flowering or reduced seed size. This suggests that wild beans can be a useful source of genes for higher yield in cultivated beans and that advanced backcross strategies can be successful at transferring these yield genes into commercial seed -types.

Future Plans

We will compare the location of QTLs found for the two Andean and one Mesoamerican populations by their association with homeologous RAPD and microsatellites markers that have been positioned on the DOR364 x G19833 reference map. We will analyzed for the QTLs controlling seed size, days to flowering and days to harvest to determine if these are associated with the yield QTLs identified here.

1.2.9 Gene tagging of resistance to whiteflies in cassava

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1 IP-1 Project

Introduction

The whitefly is one of the most serious pest and disease vectors affecting agricultural production around the world. In cassava (*Manihot esculenta* Crantz), the whitefly causes from 70-80 percent economic losses. The principal symptoms in the plant are: total chlorosis of the leaves, curling of the apical leaves, yellowing and drying of the basal leaves and retardation of plant development.

Different sources of resistance to whitefly have been reported (CIAT, 1995). The most important source of resistance genes was genotype Ecu-72. Due to the importance of the whitefly as a pest and virus vector, it is necessary to know about the nature of genes that confer resistance to whitefly in genotypes like Ecu-72. For this purpose it would be useful to know the F1 segregation of the cross Ecu-72 (resistant genotype) x any highly susceptible genotype, using molecular markers. This would help accelerate selection of materials resistant to whitefly and the isolation of the resistance genes.

Materials and Methods

For this work the cross Ecu-72 (as the resistant parent) x M Col 2246 (as the susceptible parent) was used. Although the latter has advantages such as tolerance to other pests including mites and thrips, and good flowering, it seems to be very susceptible to the whitefly. An F1 offspring of 282 individuals was produced by this cross.

Sexual seeds of this cross were cultured in sterile soil in plastic dishes with 67 spots under greenhouse conditions for 6-8 weeks. The temperature was ±30°C. The seedlings were transferred to the field for multiplication.

For the greenhouse evaluation, the seeds were multiplied in vitro to obtain enough material in a short period of time (approx. 3 mo); This period is brief compared to the normal 6-month period that cassava requires to obtain stems. Optimal health conditions were also achieved.

In vitro propagation methodology, developed by Escobar (1991), will be used in this work. This methodology is based on cutting plant tips, which are transferred to the lab, disinfected first by washing them with sterile deionized water, ethanol 70%, hypochlorite 0.25% and finally washed three times with sterile deionized water. The tips are cultured in 4E medium (Roca, 1984) in 16-ml assay tubes. The calculated growing period is from 60-80 days. Following this period a second in vitro propagation in 4E medium in small, 100-ml flasks will be performed to increase the amount of material per clone. After this the tips of each clone will be cut for culturing in 17N rooting medium (Roca, 1984) for 30-40 days. Finally the plants will be transferred to the greenhouse. This methodology will allow the conservation of material under optimal health conditions, and it will supply sufficient material in a reduced space.

The parents Ecu-72, M Col 2246 and their offspring will be evaluated in the greenhouse, using the "clip cage" methodology, which consists in two polyethylene cylinders of different height joined by forceps. Both cylinder bases are covered by muslin, and the higher cylinder has a small hole through which flies are introduced. With this evaluation it is expected to be able to identify the gene segregation in the offspring and identify the resistant and susceptible materials.

Simple sequence repeats (SSRs) are being used to find markers associated with resistance for mapping and ultimately cloning the resistant genes. The SSRs are random repeat sequences across all eukaryotic genome. These simple repeats can range from 2-6 base pairs (bp). SSRs show high polymorphism, are locus-specific and multiallelic, they have a mendelian inheritance and also are codominant. Silver staining is being used to visualize the allelic segregation of the markers.

Results

Both parents Ecu-72 and M Col 2246 were evaluated with 218 cassava SSRs including 32 recently developed cDNA SSRs (Mba et al., submitted). Approximately 60% of the SSRs were polymorphic (Fig. 1, Table 1).

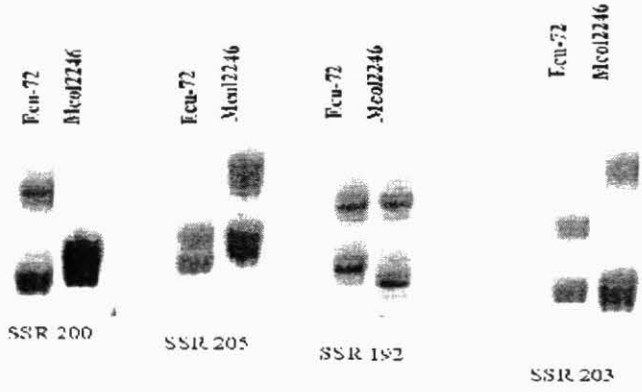

Figure 1. Silver-stained polyacrylamide gel showing SSRs of cDNA in both parents Ecu-72 (female) and M Col2246 (male).

Table 1: SSRs in parents Ecu-72 x M Col 2246.

SSR #	Size (bp)	Anneal. T. (°C)	Polymorphism	SSR #	Size (bp)	Anneal . T. (°C)	Polymorphism
SSRY1	197	45	X	SSRY51	298	50	X
SSRY2	225	55	X	SSRY52	266	55	X
SSRY3	247	45	X	SSRY53	138	55	Monomorphic
SSRY4	287	45	X	SSRY54	151	55	X
SSRY5	173	55	X	SSRY55	145	50	X
SSRY6	298	45	X	SSRY56	137	50	Monomorphic
SSRY7	250	45	X	SSRY57	293	55	X
SSRY8	288	45	X	SSRY58	217	55	X
SSRY9	278	55	Monomorphic	SSRY59	158	55	X
SSRY10	153	55	X	SSRY60	137	55	X
SSRY11	265	55	X	SSRY61	233	55	Monomorphic
SSRY12	266	55	Monomorphic	SSRY62	250	55	Monomorphic
SSRY13	234	50	X	SSRY63	290	55	Monomorphic
SSRY14	300	55	Monomorphic	SSRY64	194	55	X
SSRY15	215	50	Monomorphic	SSRY65	299	55	X
SSRY16	218	55	X	SSRY66	261	55	Monomorphic
SSRY17	277	50	X	SSRY67	278	55	Monomorphic
SSRY18	198	44	Monomorphic	SSRY68	287	55	X
SSRY19	214	50	X	SSRY69	239	55	X
SSRY20	143	55	X	SSRY70	249	55	X
SSRY21	192	55	X	SSRY71	217	55	X
SSRY22	299	43	Monomorphic	SSRY72	141	55	X
SSRY23	247	45	X	SSRY73	265	50	Monomorphic
SSRY24	100	45	Monomorphic	SSRY74	114	55	X
SSRY25	296	45	Monomorphic	SSRY75	284	55	X
SSRY26	121	55	X	SSRY76	273	55	X
SSRY27	277	50	X	SSRY77	275	55	X
SSRY28	180	55	Monomorphic	SSRY78	248	55	X
SSRY29	281	55	Monomorphic	SSRY79	210	55	X
SSRY30	220	50	X	SSRY80	299	55	X
SSRY31	188	50	X	SSRY81	204	55	Monomorphic
SSRY32	298	50	Monomorphic	SSRY82	211	55	X
SSRY33	273	50	Monomorphic	SSRY83	239	55	Monomorphic
SSRY34	279	55	X	SSRY84	203	55	X
SSRY35	282	55	Monomorphic	SSRY85	292	50	X
SSRY36	134	55	X	SSRY86	296	50	X
SSRY37	187	50	Monomorphic	SSRY87	102	55	X
SSRY38	122	55	X	SSRY88	243	55	X
SSRY39	293	50	X	SSRY89	120	55	X
SSRY40	231	50	X	SSRY90	193	55	Monomorphic
SSRY41	271		X	SSRY91	300	55	Monomorphic
SSRY42	221	50	X	SSRY92	171	55	Monomorphic
SSRY43	255	43	Monomorphic	SSRY93	289	55	X
SSRY44	194	50	Monomorphic	SSRY94	268	55	X
SSRY45	228	50	X	SSRY95	282	55	X
SSRY46	268	50	Monomorphic	SSRY96	149	55	X
SSRY47	244	55	X	SSRY97	194	55	X
SSRY48	178	50	Monomorphic	SSRY98	209	55	Monomorphic

SSRY49	300	50	Monomorphic	SSRY99	192	55	X
SSR #	Size (bp)	Anneal. T. (°C)	Polymorphism	SSR #	Size (bp)	Anneal. T. (°C)	Polymorphism
SSRY50	271	50	X	SSRY100	210	55	X
SSRY101	213	55	X	SSRY153	117	45	X
SSRY102	179	55	Monomorphic	SSRY154	318	55	X
SSRY103	272	55	X	SSRY155	158	55	X
SSRY104	258	52	Monomorphic	SSRY156	160	44	Monomorphic
SSRY105	225	55	Monomorphic	SSRY157	500	45	Monomorphic
SSRY106	270	55	X	SSRY158	224	45	Monomorphic
SSRY107	120	45	X	SSRY159	159	45	Monomorphic
SSRY108	203	55	X	SSRY160	151	50	X
SSRY109	125	55	X	SSRY161	220	55	X
SSRY110	247	55	Monomorphic	SSRY162	126	43	X
SSRY111	235	55	Monomorphic	SSRY163	231	44	Monomorphic
SSRY112	117	55	X	SSRY164	187	55	X
SSRY113	187	45	X	SSRY165	243	55	X
SSRY114	167	55	X	SSRY166	244	55	X
SSRY115	296	.	No amplified	SSRY167	183	45	X
SSRY116	167		No amplified	SSRY168	277	55	Monomorphic
SSRY117	142	55	X	SSRY169	100	55	X
SSRY118	169	55	Monomorphic	SSRY170	299	55	X
SSRY119	155	55	X	SSRY171	291	55	X
SSRY120	139	55	X	SSRY172	201	55	X
SSRY121	168	43	X	SSRY173	281		NO
SSRY122	273	45	X	SSRY174	136	43	X
SSRY123	136	55	X	SSRY175	136	55	X
SSRY124	146	55	Monomorphic	SSRY176	112	45	Monomorphic
SSRY125	247	55	Monomorphic	SSRY177	268	55	X
SSRY126	245	55	Monomorphic	SSRY178	104	55	Monomorphic
SSRY127	130	44	Monomorphic	SSRY179	226	55	X
SSRY128	243	45	X	SSRY180	163	55	X
SSRY129	205	55	Monomorphic	SSRY181	199	55	X
SSRY130	223	55	X	SSRY182	253	50	Monomorphic
SSRY131	111	45	Monomorphic	SSRY183	221	50	X
SSRY132	196	45	Monomorphic	SSRY184	163	50	X
SSRY133	295	55	Monomorphic	SSRY185	243	50	X
SSRY134	213	55	Monomorphic	SSRY186	297	55	
SSRY135	253	55	X	SSRY187	160	55	
SSRY136	296	55	Monomorphic	SSRY188	198	55	Monomorphic
SSRY137	157	55	Monomorphic	SSRY189	185	55	X
SSRY138	129	50	Monomorphic	SSRY190	164	55	
SSRY139	129	44	Monomorphic	SSRY191	186	55	Monomorphic
SSRY140	212	43	Monomorphic	SSRY192	183	55	X
SSRY141	262	55	X	SSRY193	218	55	X
SSRY142	206	55	X	SSRY194	196	55	
SSRY143	153	55	Monomorphic	SSRY195	186	55	X
SSRY144	117	55	X	SSRY196	188	55	
SSRY145	143	45	X	SSRY197	209	55	X
SSRY146	139	45	X	SSRY198	219	55	
SSRY147	113	45	Monomorphic	SSRY199	205	55	
SSRY148	114	55	Monomorphic	SSRY200	205	55	X

SSRY149	500	45	X	SSRY201	197	55	X
SSR #	Size (bp)	Anneal. T. (°C)	Polymorphism	SSR #	Size (bp)	Anneal. T. (°C)	Polymorphism
SSRY150	175	45	Monomorphic	SSRY202	191	55	
SSRY151	182	55	X	SSRY203	246	55	X
SSRY152	233	45	X	SSRY204	182	55	X
SSRY205	201	55	X	SSRY212	238	55	
SSRY206	219	55		SSRY213	199	55	
SSRY207	199	55		SSRY214	234	55	
SSRY208	198	55		SSRY215	204	55	X
SSRY209	195	55		SSRY216	210	55	
SSRY210	219	55	Monomorphic	SSRY217	181	55	X
SSRY211	202	55	Monomorphic	SSRY218	203	55	X

At present 130 polymorphic SSRs have been obtained for the parents. After isolating the DNA in 282 individual offspring, the polymorphic SSRs will be evaluated.

Conclusions and ongoing work

- A high percentage (>60%) polymorphism was found between parents Ecu-72 and M Col-2246.
- Segregation data from the SSR and greenhouse evaluation of the 282 F1 individuals will allow the construction of a linkage map for whitefly resistance.

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1.2.10 Molecular genetic markers for the new source of resistance to the cassava mosaic disease (CMD)

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Introduction

Cassava mosaic disease (CMD) remains the most economically damaging disease of the crop in Africa, and it is a threat to cassava production in the Americas. The whitefly vector biotype of CMD has recently invaded the New World increasing the possibility that the cassava mosaic virus (CMV) or a native geminivirus will cross over to cassava in the near future. CMD also prevents the transfer of germplasm from the crop's center of diversity in Latin America to Africa; resistance to CMD has not, until recently, been emphasized in gene pool development goals in LA. Host-plant resistance to CMD is the best method of containing the disease and currently deployed resistance was originally obtained from a wild relative of cassava, *M. glaziovii*. A more durable source of resistance controlled by a single dominant gene (Akano et al. 2000) was identified in closely related Nigerian cassava land races in IITA. Given CIAT's global cassava mandate it is necessary to develop gene pools adapted to CMD for Latin America should the CMD disease makes its debut in the region, and for the transfer of useful variability to Africa, such as high carotene content, and tolerance to drought. CMD breeding at CIAT requires a method to select in absence of the disease agent a task for which molecular makers are best suited.

To develop markers for marker-assisted breeding of CMD resistance, populations segregating for genes controlling both sources of resistance were developed and evaluated under disease pressure in Nigeria over a period of two years under the auspices of a Rockefeller Foundation funded CMD resistance gene mapping project project. Last year a SSR marker was found for the currently deployed CMD resistance introgressed from *M. glaziovii*, which is recessive in nature. We report here identification of a simple sequence repeat marker and an RFLP marker that are tightly linked and flank the dominant R gene controlling the new source of resistance to CMD. This result opens up the possibility of marker-assisted selection for breeding resistance to CMD in the absence of the pathogen as in CIAT and for rapidly introducing the gene into African cassava genepools.

Materials and Methods

The mapping population was a F_1 progeny from a cross between a Nigerian land race (TME3) that represent the new source of ACMD resistance and a susceptible improved line (TMS 30555). The F_1 progeny, of 240 individuals, was established from embryo axes, multiplied and six copies per genotype were transferred to the field in a low CMD pressure site in Nigeria and two copies kept *in-vitro*. For field evaluation of CMD resistance, the mapping population was established from woody cuttings 15-20cm long, in two CMD high-pressure sites, Onne, a high humid forest agroecology and Ikenne, a

low humid forest agroecology, both in Nigeria. The experimental design was an augmented randomized complete block design with 10 plant rows per genotype per block. CMD disease resistance was evaluated at 3 and 6 months after planting (MAP). Visual assessment of symptom intensity was conducted according to the IITA scale, ranged from 0 for no observable symptom to 5 for very severe chlorosis and reduction in leaf area, for each leaf on each plant and averaged by genotype. Genotypes were observed to fall into two broad classes of no symptom score of 1, and very severe symptom score of 4, in both sites, suggesting a qualitative inheritance. Genotypes were therefore scored as resistant and susceptible and a chi square test was performed to test a single heterozygous gene model of resistance.

A bulk segregant analysis (BSA) using two pools of 40 susceptible and 40 resistant genotypes from the mapping progeny was used as a quick method to identify markers associated with the resistance gene. The two bulks and two parents were screened for with the 186 Cassava MapPairs SSR markers using PCR conditions as described by Mba et al. (2000). Any marker found to be polymorphic in the two parents and the two bulks was employed to evaluate members of the bulks individually and markers that were still polymorphic in the resistant and susceptible progeny were used to genotype a subset of 162 genotypes from the progeny. Single point marker analysis using a simple linear regression of CMD resistant data on the SSR marker genotypic classes as independent variable was conducted to determine how much of the phenotypic variation was explained by the marker.

Results

The ratio of resistant to susceptible genotypes in both sites was not significantly different from a ratio of 1:1 in the mapping progeny by a Chi square test at a probability level of 0.01. This suggests a single dominant gene heterozygous in the ACMD resistance parent. In the bulk segregant analysis, only one SSR primer SSRY 28 showed an allele that was present in the resistant parent and in the resistant bulk but absent in the susceptible bulk and the susceptible parent. The polymorphism was confirmed when members of the bulks were individually screened although there were 6 recombinants (Figure 1). Analysis of a total of 162 progeny revealed 16 recombinants, suggesting the marker is at least 10cM from the CMD resistance gene. A simple regression analysis also revealed that the marker explains 70 % of the phenotypic variance of CMD resistance. SSRY 28 was found to located on linkage group R of the male-derived map of cassava linked in coupling to marker GY1 (RFLP), and in repulsion to marker Ai19 (RAPD). See Fig 2. RFLP analysis of the mapping progeny with GY1 revealed the CMD R gene is located between SSRY 28 and GY 1 at a distance of 10cM, and 6cM respectively.

Highlights of Outputs

- Identification of a SSR and RFLP marker that flank, at a distance of 10cm and 6cM respectively, the single dominant gene controlling resistance to CMD.
- Tools for marker-assisted breeding of CMD resistance at CIAT.

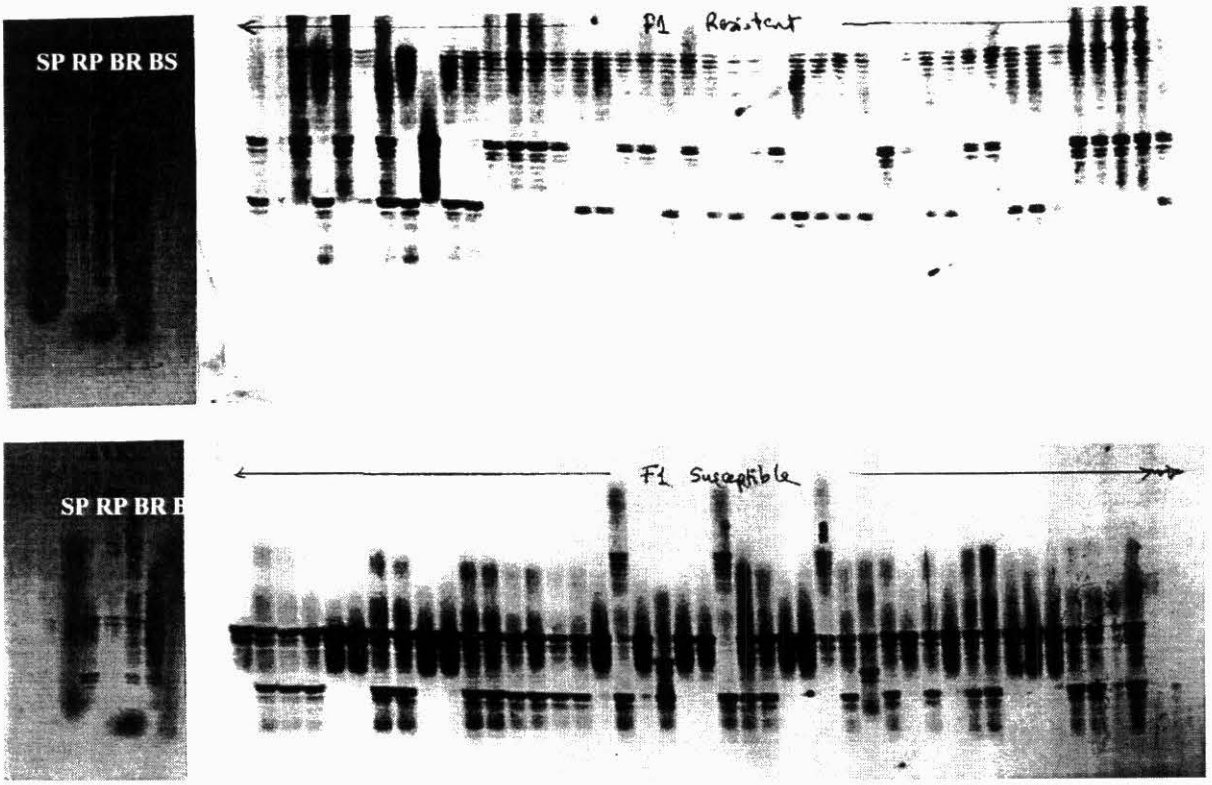

Fig 1 Bulk segregant analysis (BSA) of CMD susceptible and resistant progeny with SSR Marker SSRY28 (SP, SB: susceptible parent, susceptible bulk; RP, RB: resistant parent, resistant bulk)

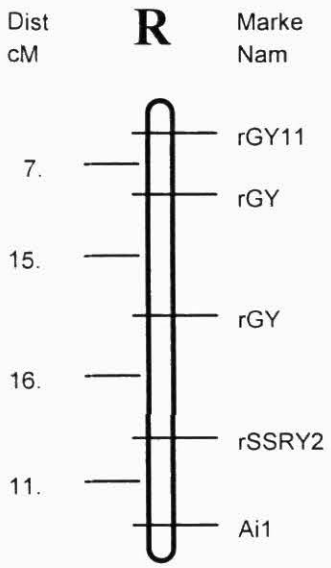

Fig 2 Linkage group R of the male-derived Map showing the location of the dominant gene, CMD 2, controlling the new source of CMD resistance

Future Activities

The two markers that flank the CMD resistance gene are linked in coupling and can be used together to determine genotypes which bear the CMD resistance gene. However, the RFLP marker does not readily lend itself to high-throughput genotyping required for MAS compared to the SSR marker. It is therefore planned to convert the RFLP marker to an SSR by identification of BAC clones that contain GY1, and an SSR sequence.

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1.2.11 Introgress resistance to ACMV into Latin American elite cultivars and select for resistance into the segregating progeny using molecular markers.

H. Ceballos

Introduction

Cassava mosaic disease (CMD) remains the most economically damaging disease of the crop in Africa, and it is a threat to cassava production in the Americas. The whitefly vector biotype of CMD has recently invaded the New World increasing the possibility that the disease may eventually cross over to cassava. Breeding efforts in Latin America have cassava not placed an important value on resistance to the disease for two reasons: a) the disease is not present in the Americas and, therefore, there was no need for resistance against it and b) since the disease is not present, we cannot select for it. Latin American varieties, consequently, are very susceptible to the disease. Resistance to CMD is the only reliable method for disease control. The first source of resistance was probably derived from *M. glaziovii* and its mode of action was reported to be a recessive trait. A better source of resistance, controlled by a single dominant was recently identified in Nigeria.

Materials and Methods: Until recently several thousand crosses between the *M. glaziovii* source of resistance and elite Latin American germplasm have been produced. However, given the excellent level of resistance conferred by the new gene and its dominant mode of action, we will start using this new source. The advantage is that being dominant, the F1 thus produced will also show high levels of resistance. The old source of resistance required a back-cross to the donor which complicated greatly the breeding

process and made considerably more difficult the recovery of the desirable traits of the Latin American elite lines.

To fulfill the requirements of the Colombian plant quarantine authorities for introducing cassava gerrmplasm from Africa into Latin America we introduced F_1 progeny from the cross between **TME3** (source of the new resistance) and **TMS 30555** (and improved variety from IITA) rather than TME3, the CMD resistant land race. Seeds of TME3 by TMS30555 cross were germinated *in vitro* from embryo axes and sub cloned; two copies were kept permanently *in vitro* to avoid any risk of infection by the virus (back-up copies) while 6 were sent to the field for replicated evaluation of CMD resistance. Tissue culture plantlets, obtained from the back-up *in vitro* copies, of genotypes showing field resistance to CMD comparable to the TME3 parent were then shipped to CIAT. The *in vitro* plantlets were tested on arrival by ELISA and PCR methods for the presence of gemni virus including ACMV before transfer to the green house. When permission to transfer the plants to the field is obtained from the Colombian quarantine authorities, and the plants will be transferred to a healthy site for hybridization

Once we have plants in the field we will start the crosses between these sources of resistance and about 20 elite varieties from tropical (low and highlands) and subtropical adaptation. In addition we will also used these materials for crosses to be shipped to Africa. CIAT and IITA are interested in continuing the introgression of germplasm from Latin America into Africa. Particular goals here will be to combine resistance to ACMV with: **a)** high carotene levels in the roots, a trait present in several Latin American materials with yellow/orange coloration; and **b)** drought tolerance, also present in Latin American genotypes (particularly from North East Brazil).

1.2.12 The utility of molecular genetic markers for the rapid conversion of african gene pools to CMD resistant lines: a comparison of MAS and conventional methods approaches

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Introduction

One of the primary objectives of genetic mapping and gene tagging efforts in cassava is to provide tools that can increase the efficiency and cost-effectiveness of cassava breeding. Markers increase the efficiency of breeding by the elimination of inferior genotypes at the seedling stage allowing the breeder concentrate on fewer genotypes for the crucial single row trial stage. Fifteen years of cassava breeding at the CIAT-Asia program has shown that that negative selection at the seedling stage, combined with indirect selection for yield, using harvest index, is most the efficient breeding scheme for yield in cassava. The availability of genetic markers for the new and more durable source

of CMD resistance provides a unique opportunity to test the cost effectiveness and efficiency of marker-assisted breeding compared to conventional breeding for the introduction of CMD resistance gene into African cassava genepools. The advantage of genetic markers for CMD resistance is that it enables the breeder to eliminate, at an early stage inferior genotypes and thus considerably reduce, by 50%, the materials to be established in the field, thus cutting his task and costs by half. Provided a cheap, slab gel-free method of genotyping can be developed, the cost-savings and increase in efficiency can be considerable. A joint project to test marker-assisted selection of CMD resistance was initiated with IITA this year.

Materials and Methods

The Nigerian cassava land races TME 3, TME4 and TME28, that carry the single dominant CMD resistance gene, were crossed to TMS 53808, TMS 30572, and TMS71173, and TMS91934, important parental lines at IITA. A target of about 500 seeds for each of the 12 crosses, making a total of 6000 seeds, has been set for the experiment. Seeds from all 6 crosses will be divided equally into two and designated as population A and population B. Seeds from populations A will be planted in the seedling nursery and evaluated with markers associated with the CMD resistance gene at 3 weeks after planting, while seeds from B will be planted but not evaluated. Plants bearing the CMD resistance gene from population A and all plants from population B will be introduced into the regular IITA cassava breeding scheme of seedling trial (ST), and single row trial (SRT). At the end of SRT the cost of maintaining populations A and B, and of genotyping population A will be estimated. The selection efficiency of markers against no markers will be also estimated for population A and B by determining the mean of both populations for traits of agronomic interest such as resistance to CMD, CBB, dry matter content and dry matter yield. The above activities are the objectives of a Ph.D dissertation to be conducted by a student at IITA with technical and partial financial support from CIAT.

Results

More than 1500 pollinations each were performed for all possible unique crosses between the CMD donor parentsTME 3, TME9, TME28, and the IITA breeding parents TMS 53808, TMS 30572, TMS71173 TMS91934 at the IITA crossing block in Ubiaja, Nigeria. The total number pollination are more thant 18,000. A minimum of about 500 seeds is expected per cross. The seeds will be harvested beginning late October and will be established in the seedling nursery by early January 2000.

Highlights of Outputs

- Generation of 12 crosses of about 6000 progeny in total for the comparison of MAS and conventional approach to cassava breeding

Plans for Next Year

- Establishment of the 12000 progeny, divided into population A and B
- Marker evaluation of seedlings of population A
- Field establishment of plants having the CMD resistance gene in population A and all of B
- Seedling trial evaluation of both populations

1.2.13 QTL Mapping of Early Bulking in Cassava

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Introduction

Based upon the results of a preliminary evaluation of early bulking in the F_1 mapping progeny, a second study, to study the genetics of early bulking was initiated January 1999 and concluded late last year. Early bulking is one of the most important traits in the acceptance or rejection of cassava varieties in Sub-Saharan Africa based on the findings of the collaborative study of cassava in Africa (COSCA) (Nweke et al, 1994). It is also a much sought trait in Latin America due to the increased flexibility of supply of raw material it offers in the industrial production of cassava for animal feed and starch. The objectives of the early bulking study were to identify traits strongly associated with early bulking, and subsequently find markers linked to genes or QTLs controlling such traits for marker-assisted breeding of earliness. Results obtained from the early bulking study are presented in this report. To verify QTLs identified in the early bulking study, a new F_1 S_1 mapping population of 240 individuals was developed and established *in vitro* from embryo axes and transferred to the field. The population will be genotyped on a genome-wide basis with SSR markers and evaluated in replicated trials for traits found to be most strongly associated with early bulking next year.

Materials and Methods

A preliminary assessment of early bulking was conducted in 1998 by harvesting the F_1 mapping population at 7 MAP in CIAT Palmira location (Fregene et. al. 2000). Dry matter yield was determined on three plants per genotype. Based on results from this evaluation, 40 early bulking genotypes and 40 late bulking groups were selected. Carefully picked healthy cuttings of the 80 selected genotypes were planted in a new experiment in December 1998 in CIAT Palmira. A early bulking cassava land race (Mandioca de tres meses) introduced from Brazil was used as control. Field layout was a randomized complete block design of two replications. Each plot had 60 plants of each genotype in a 6 x 10 (column by row) arrangement; the central 32 plants, arranged as eight rows of four plants, were used in the sequential harvest with an interval of three weeks, beginning at 6WAP through to 30 WAP. A total of nine harvests were done within a seven month duration after which the experiment was terminated (July 1999).

At each harvest, four plants in a row within a plot, per genotype, were evaluated for root yield and other traits assumed related to bulking. The traits evaluated were: plant height, plant vigor, leaf area index, fresh root yield, fresh foliage, number of roots per plant, root diameter of the biggest five storage roots. Others were harvest index, measured as the ratio of root yield to total harvested biomass, root dry matter and dry foliage. Plant vigor was evaluated on a visual rating scale of 1 – 5 (1 = poor; 5 = best). Dry matter assessment for root and foliage were carried out by taking samples of each plant and oven-drying to a constant weight to determine dry matter content. Starch initiation (or commencement in bulking) was also evaluated. Samples from roots were randomly picked, sectioned and then stained with iodine for blue black coloration test for starch presence.

Multiple regression analyses were performed to determine the linear relationships between evaluated traits (independent variable) and dry matter root yield (dependent variable) for each harvest time based on the linear equation:

$$Y = a + b_1X_1 + b_2X_2 + ...b_kX_k + e$$

Where Y is the dependent variable (dry matter root yield); a is the intercept and b (1,2...k) is the partial regression coefficient of the corresponding independent variable and X (1,2,...k).refer to the independent variables, with e as the error term.

Regression coefficient of each trait variable was accepted as significantly associated to early bulking at $P < 0.05$. Single linear regression of dry matter root yield with time (i.e. progressive increase in dry matter root yield (bulking) over the nine harvest periods) per genotype was done to determine rate of bulking given by the regression coefficient. QTL analysis was done for traits significantly linked to early bulking using QGENE (Nelson, 1997) and markers linked to the traits associated with early bulking were declared significant at $P< 0.005$. The PGRI computer package (Liu, 1995) was also used for QTL analysis.

Results

At 6WAP when sequential harvesting commenced, it was observed that 79% of the early bulking population had already started synthesizing starch implying little genetic variation in starch initiation within the population but only 16% of the population had shown development of storage roots based on visual observation. At the next evaluation, 9WAP, over 75% of the population had storage roots. Results showed that yield was significantly correlated to all other traits evaluated. The multiple linear regression model using all other traits as independent variable adequately explained variation observed for early bulking. The proportion of variation accounted by the independent variables, given by the multiple coefficient of determination (R^2), was high and the adjusted R^2 varied from 0.67 – 0.86 between 12 WAP and 30WAP. Our analysis reveal that foliage and harvest index are the two most important factors influencing early bulking. These traits were evaluated between 12WAP and 30 WAP and were found to be significantly associated to early bulking for most of the times (Table 1). Within the evaluation period

of 12WAP and 30 WAP, dry foliage and harvest index were highly significant in their partial regression coefficients for early bulking in five of the six evaluation times or 83% of the evaluation period. All other traits were most of the times non-significant in their partial regression coefficients and thus weakly related to early bulking (Table 1). Based on our result, root dry mater yield is a function of total plant biomass, harvest index and dry matter percentage of fresh root yield.

QTL analysis for harvest index and dry foliage showed genomic regions with significant effects for these traits associated with early bulking. Three QTLs each were found for foliage dry weight and harvest index (Table 2). An RFLP marker CDY76 on linkage group J alone explains about 33% of the phenotypic variance for foliage at 24 WAP and hold great potential for marker assisted breeding given its major effect for foliage/shoot development. For harvest index, a major QTL (rBEST-2) chromosome A in the male *map accounted* for 32% of the phenotypic variance at 18WAP (Table 2). Kawano et. al. (1998) showed that selection of harvest index in breeding scheme is an efficient *indirect* selection parameter for root yield. Based on the analysis and information from the early bulking study, it is apparent that early bulking (and by extension yield), can be increased more effectively by using a selection criteria based on foliage (or total plant biomass), and harvest index in addition to yield potential of the genotype.

Table 1. *P* levels of traits evaluated at each harvesting stage of early bulking assessment at Palmira over a 30 week period

Variables	6 WAP	12 WAP	12 WAP	15 WAP	18 WAP	21 WAP	24 WAP	27 WAP	30 WAP
Starch initiation	0.0040 *	0.9275	0.5489	0.5575	0.0463	0.7878	0.1399	0.7663	0.3926
Size differentiation	0.0857	0.0192*	0.1056	0.5250	0.4214	0.5839	0.8279	0.0995	0.2152
Root diameter	0.0001 *	0.0001*	0.0002*	0.2195	0.0788	0.3129	0.2702	05153	0.3672
Dry foliage	-	-	0.0254*	0.0701	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Harvest index	-	-	0.0018*	0.1418	0.0001*	0.0001*	0.0001*	0.0096*	0.0001*
Number of roots	-	-	0.0638	0.0254*	0.4147	0.9160	0.0100*	0.4294	0.1197
Plant height	-	-	0.4876	0.7258	0.2932	0.5566	0.1149	0.2296	0.0980
Plant vigor	-	-	0.1393	0.9957	0.4120	0.3618	0.7240	0.2124	0.1332
Adj. R^2	0.51	0.83	0.82	0.72	0.84	0.82	0.86	0.67	0.82

*Significant variables at each evaluation stage

Table 2. Markers identified to dry foliage, harvest index and bulking rate in the early bulking trial

Trait	Marker	Chromosome	WAP	R^2	P
Dry foliage	CDY 76	NgJ	24	0.33	0.0002
	CDY131	NgL	24	0.25	0.0018
Harvest index	GY142		15	0.29	0.0009
	GY212		27		
	rCDY106	NgK	15, 27	0.23	0.0026
	rGY55	CmF	15	0.19	0.0044
	rBEST-2	CmA	18	0.32	0.0002
	GY68	CmA	18	0.19	0.0050
	nGY162	CmE	18	0.19	0.0047
Bulking rate	GY202			0.14	0.0012
	GY142			0.19	0.0038
	rP3	NgQ		0.23	0.0032

*Chromosomes with prefix "Ng" are for the female derived map while chromosomes with prefix "Cm" are for the male derived map.

Identification of putative QTLs for traits associated with early bulking provides a first step in the understanding of the genetics of these complex traits. Deploying these marker tools in crop improvement requires further testing. To confirm putative QTLs identified for early bulking and to propose a model of genetic control for these traits, a F_1 S_1 population was developed by selfing a genotype from the F_1 mapping population possessing high foliage and high harvest index, and favorable QTLs alleles for these traits. Self pollination resulted in 725 seeds, which were then tested for viability by soaking seeds in water. After the viability test, embyro culture was carried out for 473 seeds in the 17N culture medium (1/3 medium, supplemented with 0.01 mg l^{-1}NAA, 0.01 mg l^{-1} GA_3, 1.0 mg l^{-1} thiamine-HCL, 100 mg l^{-1} inositol, 2% sucrose, 0.7% agar (Sigma Co.) and 25 mg l^{-1} of a commercial fertilizer containing: N 10, P 52, K 10, pH 5.7-5.8 (Roca 1984) as follows. Mature seeds were treated with sulfuric acid for 50 minutes, then thoroughly washed and rinsed before soaking in water for 30 minutes. Seeds were surface-sterilized by immersion in 70% alcohol for 5 minutes followed by immersion in 5% sodium hypochlorite and tween for 20 minutes, and then rinsed thrice. Under aseptic condition, the seeds were split along the longitudinal axis and embryonic axes removed by means of sterile forceps and scapel.germinated. Excised embryonic axes were placed radicle down on 17N medium. The embryo cultures were incubated in darkness for three days to promote radicle growth and then transferred to growth chambers with a 12hr photoperiod. A total of 240 progeny were hardened and established in the field.

Highlights of Outputs

- Identification of traits (harvest index and dry foliage weight) with strong influence on early bulking
- QTLs associated with harvest index and dry foilage weight
- Development, in vitro and field establishment of F_1 S_1 population for marker inheritance and fidelity studies.

Future Activities

The F_1 S_1 population will be genotyped on a genome-wide basis with SSR markers and evaluated in replicated trials for traits found to be most strongly associated with early bulking next year.

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

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Introduction

Wild *Oryza* species represent a potential source of new alleles for improving yield, quality and stress resistance of cultivated rice. Nevertheless, the effective use of wild species genes remains unexplored. Advanced backcross-breeding schemes using molecular mapping techniques represent an alternative for reducing the genetic background from wild species parentals and detecting these alleles in segregating populations more easily.

This collaborative project between CIAT, WARDA and Cornell University is aimed at characterizing and utilizing the genes from rice wild species to improve cultivated rice. This report focuses on progress made in identifying quantitative trait loci (QTLs), associated with yield increase in *Oryza rufipogon* and *Oryza barthii*, and the selection of families with these QTLs.

Materials and methods

Experiments for identifying segregant alleles from advanced backcrosses were set up in the field, greenhouse and the CIAT Biotechnology laboratory. The BC_2F_2 segregant families (288) from the cross BG90-2 x *O. rufipogon* and BC_3F_2 segregant families from the cross Lemont x *O. barthii* (326) were planted in a randomized complete block design (RCBD) with two replications. Planting was done in a 4-row plot, 5 m long each, at CIAT HQ from June-Dec. in 1996 and 1997. Data on 13 traits were taken from 10 randomly selected plants. Based on yield potential and agronomic performance, 38 BC_2F_2 families from the Bg90-2/*O. rufipogon* cross were selected for grain yield evaluation, using a RCBD design with four replications.

The DNA of young leaves from the parental genotypes and the segregating populations in both crosses was extracted using the Dellaporta Method (McCouch et al., 1988) and modified for the PCR assay by the CIAT Biotechnology Research Unit. A total of 127 markers (83 RFLPs and 44 simple sequence repeats (SSRs) were used to evaluate the segregants from the Bg90-2 x *O. rufipogon* cross, while the offspring of the Lemont x *O. barthii* cross were evaluated with 85 SSR markers. These markers, which were used in the evaluations and the QTL analyses, were selected from the rice molecular framework linkage map (10-20 cM intervals throughout the genome) (Causse et al., 1994; Chen et al. 1997).

Based on the screening of 210 SSRs in the parents of both crosses (Temnykh et al., 2000), the PCR assay protocols for 40 of them were standardized for use in the screening the progeny of the Lemont x *O. barthii* cross.

Based on the results obtained in the molecular analyses of the 288 BC_2F_2 families from the Bg90-2/*O. rufipogon* cross, 87 families associated with QTLs from *O. rufipogon* were selected to start the development of NILs. A total of 40 plants from each family were planted in the greenhouse and later transplanted in the field. Data on 13 agronomic traits were taken and used to select more promising families for the traits of interest.

While the plants were still in the greenhouse, leaf discs (5 mm in diam.) were taken from each plant of the selected BC2F2 families for DNA extraction using the Alkali Method (Klimyuk et al., 1993). Approximately 40 SSRs were analyzed in a total of 235 PCR assays done with the 87 families. Plants carrying homo- or heterozygote alleles for the *O. rufipogon* genotype were chosen and backcrossed to BG90-2. BC3F1 seed was obtained to continue the development of NILs carrying specific QTLs. Several cross combinations were made to combine different QTLs derived from *O. rufipogon.*

Results and discussion

Based on the 83 RFLPs and 44 SSRs from the RF-Cornell framework map screened in 288 BC_2F_2 families from the cross BG90-2 x *O. rufipogon*, putative linkages were

identified with yield and yield components from replicated data available for the whole mapping population. Using the Qgene software for molecular breeding (version 3.0 - Nelson 2000), associations between markers and yield on chromosomes 3, 5, 6, 9 and 12 were detected. Results obtained for chromosomes 5 and 12 showed similar associations to those reported by Xiao et al. (1996; 1998) (Fig. 1, Table 1).

Table 1. Statistical analyses of BC_2F_2 population, multiple regression for yield and plant height traits, threshold value 5% (Qgene, Nelson 2000)

Quantitative Trait	Chr	Markers	Adjusted R^2 Total	F	Coeff.	T (2 tails)	P	Increment Effect	Add%
YIELD		*Intercept*	0.164	8.2	2166.325	2.46	0.0146		
	3	RG100			403.2821	2.848	0.0048	BG90	12.84
	5	RM13			-317.629	-2.047	0.0418	Rufipogon	-13.39
	6	RM217			742.3548	2.886	0.0043	BG90	13.01
	9	RM215			-404.861	-2.686	0.0078	Rufipogon	-13.34
	12	RG901			630.5277	2.871	0.0045	BG90	12.92
	12	G1112			441.4269	2.506	0.0129	BG90	12.9
PLANT		Intercept	0.746	137.84	152.313	47.03	0		
HEIGHT	1	RZ538			-22.1423	-24.603	0	Rufipogon	-48.09
	2	RM233A			-3.4199	-4.695	0	Rufipogon	-6.76
	6	RM3			-4.2506	-3.577	0.0004	Rufipogon	-10.1
	8	RM38			-1.9829	-2.267	0.0243	Rufipogon	-10.83
	12	RG341			1.78	2.411	0.0167	Rufipogon	-5.88

In addition, another 61 QTL associations with yield and the various yield components were detected. For example, five markers were found associated with plant height on chromosomes 1, 2, 6, 8 and 12. RZ538 explained 70% of the phenotypic variation in plant height in this BC_2F_2 cross (Fig.1, Table 1) and showed a similar location on the chromosome as reported by Xiong et al. (1999). These results suggest that each QTL derived from *O. rufipogon* has a different effect on plant height, which gives breeders the opportunity to develop improved breeding lines having different plant heights to suit diverse growing conditions.

Research highlights with collaborating institutions

WARDA. In 1997 WARDA initiated the Africa/Asia joint research project on interspecific hybridization between the African and Asian rice species for developing improved rice varieties having greater weed competitiveness, resistance to the African rice gall midge, drought and iron toxicity, and good grain quality. CIAT was invited to participate in the development of improved breeding populations through interspecific crosses and evaluation of breeding lines.

Seeds of 100 interspecific *O. sativa*/O. *glaberrima* progenies were received from WARDA in 1998 and planted in observational plots under upland, acid soil conditions at La Libertad Experiment Station, Villavicencio (Meta). Oryzica Sabana 6 and 10 and Line 30 were planted as control checks. Nearly 60% of the lines were susceptible to rice blast,

but most of the lines showed good tolerance to leaf scald and Helminthosporium. Based on preliminary data on field performance and yield potential, 36 lines were selected for further evaluation in 1999. These selected progenies continued to show high resistance to principal diseases such as leaf and neck blast (P. oryzae), brown spot (H. oryzae) and grain discoloration, as well as tolerance to acid-soil conditions. Some of them had better seedling vigor and earliness than local checks. Lines WAB450-1-B-P-82-2-1, WAB450-1B-P-91-HB, WAB450-1-B-P-133-HB, WAB450-1-B-P-6-2-1 and WAB450-1-B-P-92-3-1 yielded as well as local checks (2 t/ha). In terms of grain quality, however, these interspecific progenies do not meet consumer preferences in Latin America for a long, slender, translucent grain type. Some of these lines are being used as progenitors in our breeding program. A CIAT rice breeder traveled to WARDA in Sept., selecting 300 interspecific lines from their nurseries for evaluation in Villavicencio in 2001.

In 1998, 305 lines derived from the BC2F2 generation of BG90-2 and *O. rufipogon* were sent to WARDA for evaluation under the different ecologies found in West Africa. Many promising lines were identified and selected by their breeders. Nearly 1283 segregating breeding lines derived from interspecific crosses were sent to WARDA this year for evaluation.

Cornell University. A pilot project on rice was initiated between CIAT and Cornell in 1994. The basis of this project is a forward-looking strategy for improving crop performance using existing collections of genetic resources and biotechnology tools. Crosses involving *Oryza glaberrima, O. rufipogon* and *O. barthii*, in combination with elite irrigated and upland varieties, were made; and several improved populations have been developed thus far. An advanced backcross-breeding strategy was used to identify QTLs associated with eight agronomic traits in a BC2F2 population derived from a cross between Caiapo, an upland *Oryza sativa* subsp. Japonica rice from Brazil, and an accession of *O. rufipogon* from Malaysia. Based on analyses of 125 SSLP and RFLP markers distributed throughout the genome and using single-point, interval and composite-interval mapping, two putative *O. rufipogon*-derived QTLs were detected for yield, 13 for yield components, 4 for maturity and 6 for plant height. It was concluded that advanced backcross QTL analysis offers a useful germplasm-enhancement strategy for the genetic improvement of cultivars adapted to stress-prone environments. Although the phenotypic performance of the wild germplasm would not suggest its value as a breeding parent, it is noteworthy that 51% of the trait-enhancing QTLs identified in this study were derived from *O. rufipogon*. A paper (Moncada et al., 2000) was submitted and accepted for publication by Theoretical Applied Genetics.

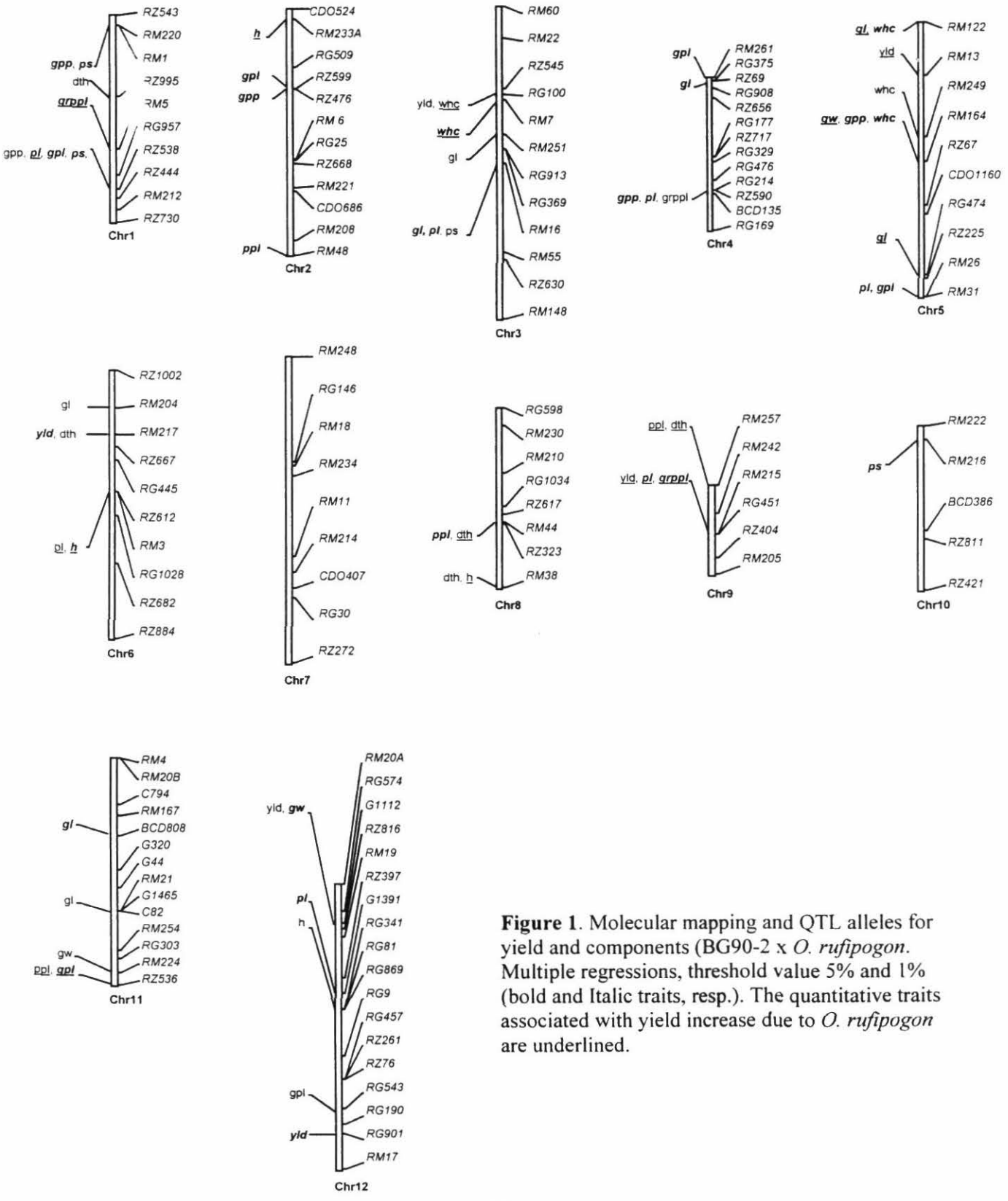

Figure 1. Molecular mapping and QTL alleles for yield and components (BG90-2 x *O. rufipogon*. Multiple regressions, threshold value 5% and 1% (bold and Italic traits, resp.). The quantitative traits associated with yield increase due to *O. rufipogon* are underlined.

Ongoing activities

- Complete the characterization of agronomic and molecular data, and QTL analyses to determine the number of QTLs associated with yield increase across environments for Bg90-2/*O. rufipogon* and Lemont x *O. barthii* crosses.

- Develop NILs carrying specific QTLs for use in breeding programs from the cross Bg90-2/*O. rufipogon*.

- Develop a mapping population (CG14/ WAB56-104) in collaboration with WARDA.

- Prepare draft papers for publication of results.

- Fingerprint advanced lines from Bg90-2/*O. rufipogon* and Lemont/*O. barthii* crosses.

- Initiate agronomic and molecular characterization of several other populations involving crosses with *O. glaberrima* to identify QTLs associated with yield increase.

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Moncada, P.; Martinez, C.P.; Borrero, J.; Chatel, M.; Gauch, H.; Jr; Guimaraes, E.; Tohme, J. and McCouch, S.R. 2000. Quantitative trait loci for yield and yield components in an *Oryza sativa* x *Oryza glaberrima* BC2F2 population evaluated in an upland environment. Theor. Appl. Genet (in press).

Trips

1. 28th Rice Technical Working Group. Feb. 27-March 1. Biloxi, Mississippi. A paper entitled "Advanced backcross analysis for the transfer of QTLs from *Oryza rufipogon* and *Oryza barthii*" was presented.
2. Chandler Memorial Symposium. June 15-17. Cornell University. Ithaca. NY.
3. Visit to Yale University at New Haven, University of Minnesota at St Paul and Novartis. June 20-28.
4. Visit to WARDA. Sept. 10-18. Bouake, Ivory Coast. Coordination of CIAT/WARDA collaborative activities in rice.
5. 4th International Symposium on Rice Genetics. Oct. 23-27. IRRI, Los Baños, Philippines. A paper entitled "Utilization of new alleles from the wild rice *Oryza rufipogon* to improve cultivated rice (*Oryza sativa*) in Latin America" will be presented.

1.2.15 A cDNA subtraction library for tagging the apomixis gene in *Brachiaria*

D.F. Cortés, J. Miles & J. Tohme

Introduction

Apomixis is an asexual mode of reproduction by seed, which occurs in a number of families of higher plants. During apomictic processes, both meiosis and egg fertilization are omitted, making offspring exact genetic replicas of the mother. Apomixis is of value to agriculture because it can be used to fix hybrid vigor, which would make hybrid seed production much more economical.

The common mode of commercial-scale reproduction of *Brachiaria* cultivars is by facultative apomixis. Apomixis is not yet well understood genetically although research efforts have increased significantly in recent years. An additional step toward a better understanding of apomixis in *Brachiaria* is the establishment of a cDNA library, which has been developed with a new, highly effective method known as suppression subtractive hybridization (SSH). Using this approach, two mRNA populations, extracted

from both apomictic and sexual genotypes, were examined to elucidate the differential gene expression between them.

Materials and Methods

Plant material. Two different species of *Brachiaria*, which are parents of the apomixis gene- mapping population, were used to develop the cDNA library: *B. decumbens* (CIAT 606), tetraploid apomictic; and *B. ruzziciensis* (CIAT 44-3), colchicine-induced tetraploid sexual. Several plants from each species were maintained in pots in a greenhouse at a temperature ranging from 25°C during the day to 20°C at night.

RNA isolation. Inflorescences from several pre-anthesis stages of development were excised from five apomictic and five sexual plants. The different tissues were polled for every genotype and immediately put into liquid nitrogen. Total RNA was extracted from 100 mg of fresh material using the RNeasy™ Plant Total RNA kit (Qiagen, Dorking, UK). DNA contaminants were removed using RQI DNase (1U/µl). The mRNA was isolated using Dynabeads® mRNA purification kit (Dynal 610.06), following the manufacturer's instructions.

cDNA synthesis and SSH. Both cDNA synthesis and SSH were done with the CLONTECH PCR-Select™ cDNA Subtraction Kit, following the manufacturer's instructions. SSH compares two populations of mRNA and obtains clones of genes that are expressed in one population but not in the other. First both populations of mRNA are converted into cDNA. The cDNA of *B. decumbens*, which contains specific (differentially expressed) transcripts, is referred to as the "tester," and the reference cDNA of *B. ruzziciensis*, as the "driver." Both the tester and driver were hybridized; and hybrid sequences, removed. The remaining unhybridized cDNAs represent genes that are expressed in the tester but are absent in the driver mRNA.

In order to confirm that the sequences really expressed differentially, two sets of dot-blots were prepared with the clones obtained in the cDNA library and hybridized with cDNA of both the apomictic and sexual genotypes. The products from the SSH were inserted into the pGENTeasy vector plasmid (Promega), and ligated cDNAs were transformed into *E. coli* DH5α with selection for ampicillin resistance. Random transformant clones were picked to 3 ml of LB medium with ampicillin in wells of a microtiter plate and grown at 37°C for 12 h. After the plasmid isolation, the inserts were amplified using primers M13 forward and reverse in 25-µl vol. for 25 cycles and sequenced using an ABI Prism 377 sequencer. Homology searches were carried out using BLAST programs through servers at the National Center for Biotechnology Information and the National Center for Genome Research.

Results and Discussion

A total of 288 random transformant clones were picked and grown, and the plasmid isolated. The PCR product of these clones was then submitted to restriction with enzymes *AluI* and *MspI* in order to identify duplicates. Different restriction patterns were,

however, found among them: thus each one corresponds to particular sequences. With the dot-blot hybridization, 45 clones were identified as being expressed differentially between the apomictic (35 = 78%) and sexual (10) genotypes. This means that at least 12% of the transcripts in the cDNA library are expressed only in the apomictic parent. Finding sequences that are expressed exclusively in the sexual genotype in the cDNA subtractive library could be explained because the genotypes used in this study are very closely related. Thus the genomes are similar to each other, and few sequences among them are different.

Of the 22 clones sequenced using BLAST search, 12 contained patches that closely matched sequences related to a stress response, reproductive organs and embryonic development in plants (Table1). In particular, sequences expressed during pre-anthesis, anthesis, embryo and endosperm development in grasses, as well as pathogen response. Another ten clones had little or no significant DNA sequence match in the database to genes of known function. Some function-unknown genes might encode completely new classes of proteins with specific roles in apomictic or sexual development in *Brachiaria*. Others might be vestigial and thus make little or no contribution to apomictic or sexual development, which would involve other pathways.

Table 1. Features of apomictic-specific transcripts with significant database matches.

Query	Genebank Accession	**Description Homology**
BR10 (250 bp)	gb|BE599256.1|BE599256	Pathogen-induced 1 (PI1) *Sorghum bicolor* cDNA
BR14 (436 bp)	gb|AW982962.1|AW982962	*Hordeum vulgare* pre-anthesis spike EST library HVcDNA0008 (white to yellow anther) *H. vulgare* cDNA clone
BR74 (365 bp)	gb|BE361813.1|BE361813	Dark-grown 1 (DG1) *S. bicolor*: 5-day-old dark-grown seedlings
	gb|AI820230.1|AI820230	Endosperm cDNA library from Schmidt lab Zea mays cDNA. Tissue type: nucellar, embryo and endosperm Development stage: 10-14 days post-pollination
BR110 (474 bp)	gb|BE400692.1|BE400692	Wheat meiotic stage library *Triticum aestivum* cDNA clone AWB006.A04
	dbj|C72446.1|C72446	Rice panicle at flowering stage *Oryza sativa* cDNA clone E1635_1A.
BR230 (93 bp)	gb|BE598918.1|BE598918	Pathogen-induced 1 (PI1) *S. bicolor* cDNA
BR255 (420 bp)	gb|BE598979.1|BE598979	Pathogen-induced 1 (PI1) *S. bicolor* cDNA
	gb|AW258135.1|AW258135	Early embryo from Delaware *Z. mays* cDNA. Developed from pool of equal amounts of RNA from developing embryos sampled at 14, 21, 28 and 35 days after pollination
BR60 (105 bp)	gb|AW499462.1|AW499462	Mixed stages of anther and pollen
BR91 (164 bp)	dbj|AU075852.1|AU075852	Rice mature leaf *O. sativa* cDNA clone S20430_2Z
BR94 (110 bp)	gb|AW671858.1|AW671858	Light-grown 1 (LG1) *S. bicolor* cDNA
BR165 (465 bp)	gb|AW520091.1|AW520091	Mixed stages of anther and pollen *Z. mays* cDNA
BR174 (520 bp)	dbj|D23588.1|D23588	Rice callus *O. sativa* cDNA clone C3051_1A
BR179 (132 bp)	gb|AW924323.1|AW924323	Water-stressed 1 (WS1) *S. bicolor* cDNA
	gb|AI396275.1|AI396275	Apical meristem cDNA library from Hake lab *Z. mays* cDNA

Ongoing work

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

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

Main Achievements

- Bean microsatellites were obtained either from gene sequences deposited in public databases or from enriched library developed and screened at CIAT. A sub set has already been mapped onto the CIAT bean mapping population of Dor 364 x G 19833 as a first steps towards their use in MAS.

- Bean cDNA and small-insert genomic libraries for microsatellite development were established. A set of 768 cDNA clones were sequenced. This collection of new gene sequences for common bean represents as many sequences as are currently in the Genbank public database for all *Phaseolus* species together.

- Cassava micro satellites set was expanded this year by the development and mapping of new SSR markers coming from either enriched genomic or cDNA libraries. The cDNA library consisted of 80,000 clones picked and arrayed onto high density filter using the Clemson University facilities.

- Brachiaria microsatellites were developed and used in conjunction with AFLP markers to establish the first framework molecular map for the species opening the way for QTL analysis for complex traits.

- The Serial Analysis of Gene Expression (SAGE) method was implemented for cassava as part of a collaborative project with the Iwate Biotech Center in Japan. The SAGE protocol is being used to identify candidate genes for the cloning of the CMD resistance gene. In additiona to the SAGE method, the postional cloning of CMD was initiated.

- The cDNA- AFLP technique was implemented to study plant gene expression for Cassava - Xam interaction.

- Resistance gene analogs (RGAs) in cassava resulted in the characterization of 12 classes of RGS and one cDNA clones related to R gene.

- Bean RGA were mapped onto the Rils population of Dor 364 x G19833. Tight linkage with resistance genes was detected. The screening of a bean BAC library allowed the isolation of regions flanking the RGAs in order to clone the targeted R genes.

- The region of the Pi-2 gene cluster for resistance to rice blast was saturated with AFLP and RAPD maker as a first step toward cloning the gene. The markers mapped also provided new options for MAS.

1.3.1 Common bean cDNA and small-insert genomic libraries for microsatellite development

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Introduction

Microsatellites based on simple sequence repeats have been developed for a wide range of plant species. Various techniques exist for discovering new microsatellite markers from anonymous genomic sequence. All of these techniques rely on the availability of DNA libraries. It is best if these libraries consist of small-insert clones (< 1 kb) because they are easier to screen for microsatellites and to sequence if they are shown too be positive. Various enrichment procedures have been used to increase the prevalence of SSRs in genomic libraries. One method relies on the selective capture of small fragments with oligonucleotides on hybond membranes (Edwards et al., 1996). An enrichment method has been used at CIAT to develop two GAn and CAn microsatellite-containing libraries for common beans (CIAT Annual Report 1999). In this study, we were interested in developing non-enriched libraries to screen for microsatellite sequence motifs that have not been screened in the enriched libraries. The screening of non-enriched random genomic libraries will allow us to investigate the normal frequency with which different microsatellite motifs occur in common bean.

Materials and Methods

Seven small insert genomic libraries were constructed (Table 1): 1) *Taq*I library (from bean DNA of the genotype DOR364) 2) *Rsa*I library (DOR364) 3) *Alu*I library (DOR364); 4) *Hae*III library (DOR364); 5) *Dra*I/*Alu*I library (G19833); 6) *Dra*I/*Hae*III library (G19833) and 7) Sonicated library (DOR364). For the enzyme-digested libraries, bean DNA was digested with the appropriate restriction enzyme, size selected to the range of 0.4-1.2 kb and ligated into vectors using T4 DNA ligase. In the case of the *Rsa*I library the insert was first ligated to *Mlu*I adapters, amplified by PCR using the adapter primers and ligated into the pGEMTeasy vector from Promega; while for the *Taq*I library a modified pUC19 vector (pJV1) was used. The other libraries were made in the pBluescript vector. All ligation reactions were transformed into Electromax DH5alpha *E. coli* cells and plated onto the appropriate antibiotic containing LB agar media. DNA was extracted from 20 white colonies per library by standard alkaline lysis procedures using an Autogen robot and checked for insert presence and size by restriction digestion with appropriate enzymes. A leaf cDNA library (Stratagene, pCMV Sport 6.0 vector, EMDH125 cells) was also prepared for microsatellite screening.

The clones from the libraries were plated on Q-plates with Carbenicillin (100mg/L) and in the case of the small insert libraries, x-gal and IPTG as well. A Q-bot robot was used

to pick and array colonies for both the small insert and cDNA libraries. Automatic blue/white screening was used to pick the clones. All the clones were placed into 384-well plate format glycerol stocks, grown overnight and stored. The master plates for the library were copied twice into working copies of the library. The clones were spotted onto gridded Hybond N+ membrane filters containing 6 fields of 8 x 384-well plates each for a total of 48 plates per filter. Clones were arrayed in a double-replicate 4 x 4 pattern.

Results and Discussion

The three small-insert genomic libraries with the best transformation efficiency, the highest percentage clones with inserts and the average insert size closest to 0.5 kb were the libraries made for *Alu*I, *Hae*III and *Rsa*I. These were chosen for further analysis and plating. A total of 57,984 clones were arrayed from the three small-insert libraries (Table 1). These libraries each represent a total of 10 Mb of DNA which is equivalent to 0.015 X genome equivalents considering that the genome of common bean is 650 Mb in size. Taken together the three libraries contain approximately 30 Mb of bean DNA which should be equivalent to 5 % of the total genome. If the average distance between microsatellite loci is between 20 and 100 kb throughout the bean genome, as it is in other crops with small genomes, than this number of clones should provide between 600 and 3,000 SSR-containing clones which can be developed into new microsatellite markers. In addition, these clones will give an accurate picture of the relative frequency of different SSR motifs in the bean genome.

A total of 64,128 clones were arrayed from the cDNA library (Table 1). The average insert size of the cDNA clones was 1.3 kb which compares favorably to other cDNA libraries that have been used in sequencing projects of expressed sequence tag sites (ESTs). A set of 768 cDNA clones were sequenced from the Sp6 end (equivalent to the 5 ' end of the insert). The new sequences were a) searched for vector segments to check for insert integrity and b) BLAST searched against each other to check for sequence redundancy (redundant clones consist in 90% identity over half the nucleotides in a sequence). The vast majority (97%) of the sequences were unique and very few short clones were identified. The sequences were also compared to the Swissprot database and to all the soybean proteins downloadable from Genbank. Out of the 745 unique sequences, 240 were homologous to annotated genes form the Swissprot database and 170 were homologous to soybean genes, both with expectation values of at least 1×10^{-7}. This collection of new gene sequences for common bean represents as many individual sequences as are currently in the Genbank public database for all *Phaseolus* species together.

Future activities

Our future plans are to begin screening the libraries with different oligonucleotide probes representing microsatellite sequence motifs to give us an estimate of the relative abundance of microsatellites on an absolute scale per kb of genomic sequence. Given the small genome size of common bean, we hope to recover a large number of microsatellites

from these libraries. We plan to sequence all positive SSR containing clones to design microsatellite primers for the development of new markers. The sequenced cDNA clones represent the first substantial number of EST sequences in beans. Ultimately many of these cDNA sequences can be genetically mapped as RFLPs or SNP (single nucleotide polymorphism) based assays, especially as simple procedure such as dense chips, become available.

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Table 1. Libraries prepared for microsatellite screening in common bean

Subcloning method	Vector	Genotype	Insert Size	Clones with insert	Transformation Eff.	No. of Clones picked	Library name
TaqI	pJV1	DOR364	0.7	35	$5.15x10^5$	no	no
RsaI	pGEMTeasy	DOR364	0.5	100	$7.53x10^5$	19,584	PV-DEc
AluI	pBS	DOR364	0.5	71	$1.02x10^5$	18,432	PV-DEa
HaeIII	pBS	DOR364	0.5	75	$3.86x10^5$	19,968	PV-DEb
DraI/AluI	pBS	G19833	0.3	80	$1.73x10^7$	no	no
DraI/HaeIII	pBS	G19833	0.4	50	$1.61x10^7$	no	no
Sonicated	pBS	DOR364	0.5	56	$7.56x10^4$	no	no
CDNA	pCMVSport6	G19833	1.3	100	$3.85x10^6$	64,128	PV-GEa

1.3.2 Discovery and characterization of polymorphic simple sequence repeats in the common bean

E. Gaitán and J. Tohme

Introduction

Common beans (*Phaseolus vulgaris*), exhibit a considerable amount for morphological traits and for resistance to diseases. Identification of molecular markers has been of great help to bean breeders, geneticists and taxonomists. The objectives of this work are to identify simple sequence repeats (SSR) markers in the cultivated common bean and to test these markers for their ability for gene tagging and to discriminate among accessions.

SSR markers, which are reported to be more variable than RFLPs or RAPDs, have been widely adopted for genetics in plants such as soybeans (Rongwen et al., 1995) and rice (Penaud et al., 1996). SSRs are valued because they are genetically defined, highly variable, codominant markers, and because they are easily detected by PCR amplification of small amounts of DNA. Detection and analysis of SSRs can also be automated, facilitating high-throughput projects (Michell et al., 1997). The present study was

initiated to identify SSR markers in the common bean and determine whether these markers might exhibit enough variation to be useful for studies of genetic variation and for bean breeding.

Methodology

New bean libraries for dinucleotide (GA and CA) and trinucleotide (ATT_{14}, CAG_{14}, CAA_{14} and ACG_{14}) motifs were constructed, following Edward's methodology (Edwards et al., 1996), with some modifications. After enrichment, positive clones were identified by hybridization. Cells from hybridizing colonies were cultured individually, and DNA minipreps were prepared for automated sequencing using dye terminator chemistry. Samples were analyzed on an automated DNA sequencer (377 Perkin-Elmer).

PCR amplification and evaluation of polymorphism

Using the PRIMER computer program (version 3.0), 68 locus-specific primer pairs were designed and tested for PCR amplification and polymorphism, using DNA from the parents of mapping populations (DOR 363 * G19833). The PCR reaction was carried out in a 20-ul final vol. containing 20 ng of genomic DNA, 0.1 uM of each forward and reverse primer, 10 mM Tris-HCl (pH 7.2), 50 mM KCl, 1.5-2.5 mM MgCl2, 0.25 mM of each dNTP and 1 unit of Taq DNA polymerase. The temperature-cycling profile was an initial denaturation step for 3 min at 96°C, followed by 35 cycles of denaturation at 94°C for 15sec, annealing from 48-60°C for 15 sec, and primer extension at 72°C for 15 sec. From 3-4 ul of the PCR reaction were resolved on 6% polyacrylamide-sequencing gels for 2 h at 100 W, and DNA was visualized by silver staining according to the manufacturer's guide (Promega Inc., USA)

Results

For the dinucleotide library 1007 positive clones were identified, of which 479 were sequenced, and 379 were redundant (70%). From the 140 nonredundant clones, it was possible to design 64 primer pairs. Both the occurrence of short-tandem repeats (<6 core units) and the close proximity of the SSR to the end of the cloned insert DNA limited the design of primers. For the trinucleotide library, 3321 positive clones were identified, 86 clones of which were sequenced. There were 61 redundant positive clones (70%). It was possible to design 4 primer pairs.

The 68 primer pairs were assayed against parents of mapping populations (DOR 363 * G19833) to test the ability to detect polymorphism between them. Of these, 28 polymorphic and 11 monomorphic primers were found (Fig. 1), 16 did not amplify, and 13 remain to be tested.

Figure 1. **Amplification by PCR with parentals G19833 and DOR 364 using several Bean Microsatellites**

Ongoing activities

- Sequence more positive clones and design primer pairs
- Evaluate these primers on cultivated and wild *Phaseolus vulgaris* and other *Phaseolus species* to test their ability to detect polymorphic loci

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1.3.3 Developing microsatellites from gene sequences of common bean

M.W. Blair

Introduction

Microsatellite loci are often found in non-coding regions of the genome where the effect of their contraction and expansion will not affect gene expression or introduce mutations into open reading frames. However, in many organisms simple sequence repeats (SSRs) have also been found at a predictable frequency in gene coding regions. For example, a large number of SSRs have been discovered in expressed sequence tag (EST) database entries of rice. These SSRs are often located in introns, and untranslated regions of sequenced cDNAs. Our objective in this study was to develop microsatellite markers for common bean from sequences in the Genbank database.

Materials and Methods

In this study, we developed and tested a group of 44 common bean microsatellites derived from database searches for SSR-containing sequences. Eleven markers were available from a previous study by Yu et al. (1999) and were implemented at CIAT with our PCR conditions. Thirty-five additional microsatellites were obtained using database searches of sequences deposited in the Genbank database before September 1, 2000 for any *Phaseolus* species. SSR containing Genbank sequences were identified using Repeatmasker software that screens for all possible dimeric, trimeric and terameric repeats. Any SSR-containing sequence with more than five repeat units in the case of di-nucleotide repeat, four in the case of tri-nucleotide repeats, or three repeat units in the case of tetra, penta and hexa-nucleotide repeats were selected for microsatellite development. Compound repeats containing several adjacent SSR sequences were favored over SSRs that only had the minimum number of repeat units. Of the 612 *Phaseolus* sequences obtained from Genbank, microsatellite markers were derived from 43 *P. vulgaris* sequences and one *P. coccineus* sequences. Primers were designed using Primer 3.0 software to produce PCR amplification fragments that were on average 150 bp long, and to have melting temperatures of 55 C or above and an average length of 20 nucleotides.

Results and discussion

Some repeat motifs were more frequent than others in the Genbank sequences. Almost half of the microsatellites were di-nucleotide based SSRs (Table 1). Of these, the ATn / TAn or TCn / GAn motifs were the most common SSRs in the *Phaseolus* sequences, while GTn / CAn sequences were very infrequent. Several of the tri-nucleotide motifs, notably CCAn / TGGn, CTTn / AAGn and GATn / ATCn were represented by three or four sequences each, while four other trinucleotide motifs were represented by only one

sequence each. The highest number of repeats detected in the sequences was nine, while the average number of repeats was 6.7 among all the microsatellites identified.

SSR repeats were found in a range of coding and non-coding sequences. A total of 492 sequences represented partial or full-length cDNAs or genes. Over 130 of these sequences were of multiple alleles of the small subunit ribosomal RNA gene from different *Phaseolus* species none of which contained SSR motifs. A total of 230 sequences that were searched represented partial and complete genes from *P. vulgaris*, *P. lunatus*, *P. coccineus* and *P. acutifolius*. GAn, ATn and a variety of tri-nucleotide repeats were common in these sequences. Interestingly some trinucleotide repeat in gene sequences were associated with amino acid repeats in the corresponding protein, for example the gene for hydroxyproline rich glycoprotein contains multiple repeats of the CCA codon for proline throughout the open reading frame.

An additional 120 of the individual sequences searched in this study represented genomic RFLP clones from the Bng series, that have been mapped in common bean by Vallejos et al. (1992). Three of these sequences contained SSRs. Interestingly, these non-coding RFLP sequences contained two tri-nucleotide motifs (ATC and ATT) which were uncommon in the coding sequences described above. In addition a single RFLP clone contained a di-nucleotide GA repeat. These RFLP clones originated from *Pst*I restriction fragments which are often in areas of the genome that are under-methylated and have a higher than average GC-content. This may explain why none of the clones contained AT repeats, which are postulated to be the most common motif among plant genomes. The 120 entries contained a total 50.5 kb of sequence information. Therefore, the average density of microsatellites in a random sample of non-coding sequence can be predicted to be 1 SSR every 16.8 kb. These results confirm that the frequency of SSRs in common bean (total genome size of 650 Mb) is similar to the frequency of SSRs in other small-genome species such as rice (450 Mb) and Arabidopsis (150 Mb). This is promising for the further development of new microsatellites for beans.

Future Activities

In the future more EST and genomic sequences will be available for common bean. In this regard, CIAT should begin to develop and sequence cDNAs from tissues and genotypes that are expressing traits of interest for our bean breeding program, such as roots from low-phosphorous tolerant varieties or pods, leaves and roots of disease resistant varieties. Several new BAC libraries are being developed for common bean in the United States and it is likely that entire clones or insert ends will be sequenced. This new sequence information will give the Biotechnology program at CIAT a host of opportunities to develop additional microsatellite markers.

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Table 2. The repeat motifs and average number of repeats *in microsatellite* markers designed from sequences in the Genbank database.

SSR motif	frequency	avg. number of repeats	SD
di-nt			
ATn / TAn	9	10.3	5.8
TCn / GAn	11	7.1	1.9
GTn / CAn	1	na	na
tri-nt			
ATTn / TAAn	1	na	na
CCAn / TGGn	4	5.3	0.9
CTTn / AAGn	4	5.3	0.9
GATn / ATCn	3	5.0	1.0
CAAn / GTTn	2	5.0	0.0
CCTn / AGGn	1	na	na
AGCn / GCTn	1	na	na
GGCn / GCCn	1	na	na
tetra-nt			
CATG	2	4.0	0.0
AATG	1	na	na
TGAA	1	na	na
TTAA	1	na	na
hexa-nt			
CGCCAC	1	na	na

1.3.4 Legume microsatellites tested in common bean

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Introduction

Common bean (*Phasoulus vulgaris*) belongs to the Phaseoleae subtribe along with several other legume crops originally from the tropics or subtropics, like cowpea (*Vigna unguiculata*), mung bean (*Vigna mungo*), soybean (*Glycine max*) and pidgeon pea (*Cajanus cajan*). The Phaseoleae tribe contains the largest number of genera and most economically important crops of any tribe in the Legume family. The genetic similarity between Phaseoleae species has been confirmed by karyotyping and comparative mapping studies. Most of the species in this tribe share the same or similar basic number of chromosomes (either n=10 or 11), although they vary greatly in the size of their genomes. Polyploidy is sometimes found in this tribe, as is the case for soybean, which is a diploidized autotetraploid. The legumes that originated in temperate regions, such as peas, lentils, chickpeas, alfalfa and clover, belong to other tribes and typically have basic chromosome sets of 7 or 8. Peanuts are also from a different branch of legumes than

either the tropical or temperate legumes mentioned above. Variability in genome size is typical of legume genera, with the *Vicia* genus showing a huge range between small and large genome species.

A large number of microsatellite markers have been developed for soybean by the USDA (Cregan et al., 1999) and at companies such as Dupont (Peakall et al., 1998). Relatively fewer microsatellites have been developed for other tropical legume crops. However at IITA an effort is underway to develop cowpea microsatellites (C. Fatokun, pers. Comm.), and an initial set of peanut microsatellites is also available (Hopkins et al., 1999). Our objective in this study was to try to adapt the microsatellites available for other Phaseoleae legume crops (especially soybean and cowpea) to common bean. If successful, the transfer of microsatellites between legume species, would reduce the cost and time needed to develop markers specific for each crop individually.

Materials and Methods

The microsatellites tested included 423 from *Glycine max* (408 with ATTn motif, 3 with GAn motif and 12 from coding sequences) and 67 from *Vigna* (mixture of GA, CA, AT and compound motifs). These markers were tested against a panel of nine legumes, that included the soybean, Mesomerican and Andean common bean genotypes (Williams, DOR364 and G19833, respectively) that have been the sources of microsatellite libraries made for these crops. That way we would have a control in the size of the allele that was detected. For the other legume species we used genotypes that were representative of varieties grown in the Andean region. Primer amplification was tested with a range of conditions. The soybean microsatellites were tested initially with lax amplification conditions using 45 to 47C for annealing temperature and 2.0 to 2.5 mM final concentrations of MgCl2. The cowpea microsatellites were amplified with 52 C annealing temperature and 2.5 MgCl2. Both sets of markers were analyzed on agarose (2.0%) gels with ethidium bromide staining. The cowpea microsatellites were analyzed on polyacrylamide (6%) gels with silver staining.

Results and Discussion

Microsatellites have been successfully transferred among related animal species such as birds, tortoises, primates (eg. gorillas/apes), ungulates (eg. horses/cattle) and rodents (eg. rats/mice) more than among plant species. Ultimately it is the genetic distance between species and genera that determines the ability of SSR primers to amplify in different genomes and the ability to transfer microsatellites between species must be determined empirically.

Soybean and common bean appeared to be especially divergent in regard to their microsatellite loci. The soybean genomic ATTn microsatellites generally do not amplify well in common bean. At low annealing temperature and lax conditions required to amplify PCR products with these primers in common bean, these markers amplify multiple bands from common bean, tepary bean, lima bean, cowpea or mung bean DNA that is completely different in size compared to the soybean allele. The likelihood that

these represent homoelogous microsatellite loci was deemed low and these microsatellites were not investigated further. Therefore, it seems that soybean primers may be less useful in common bean than we thought because of the evolutionary distance separating these species.

It seems that in plants, unlike animals, microsatellites loci are not well conserved over large genetic distance between species. Why some related lineages maintain intact microsatellites over evolutionary time or why microsatellites in certain location are more conserved is not known. However it seems that the gene-derived microsatellites may be more conserved than the microsatellites from non-coding sequences. Especially promisisng are any gene sequences in *Vigna* species which have microsatellites in them. VM21 represents a gene from *Vigna radiata* for ACC oxidase that contains an ATn repeat in the 3' untranslated region which amplifies well in a range of legumes. We will investigate whether the Phaseolus homologues of this clone, one of which has been sequenced, contains this microsatellite.

Future studies

In the future we plan to clone the alleles that amplify across legume species and sequence them in order to determine if the simple sequence repeat is conserved. We also plan to analyze more GAn, CTn and gene-derived microsatellites from soybean to determine if they are better conserved than the ATTn microsatellites. We are collaborating with a group in Canada that is looking at another set of soybean microsatellites and will be comparing our results with theirs.

It will also be interesting to determine whether common bean microsatellites amplify most member of the Phaseolus genus and whether this extends to *Vigna* species as well. At IITA, a project has begun to look at the amplification in cowpea of gene-derived microsatellites developed at CIAT from common bean. The overall gene order along the chromosomes, or synteny, of many legume genera is thought to be conserved, however this has been less well studied than in the grass family. Soybean, common bean and mung bean have been shown to have regions of macrosynteny across much of their genomes, however there has been no analysis of microsynteny or sequence conservation at homoelogous loci in the genomes of these related legumes.

If we can obtain a set of microsatellites that can amplify consistently across some legume crops, we may use them for comparative mapping - it will important therefore to determine if the microsatellites amplify homoelogous locations in the genome.

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Table 1. Panel of economically valuable tropical legumes used to test cross-species amplification.

Crop	Species	Subtribe	1N Chrom.	Genotype
Common Bean - Andean	*Phaseolus vulgaris*	Phaseolinae	11	G19833
Common Bean - Mesoamerican	*Phaseolus vulgaris*	Phaseolinae	11	DOR364
Tepary Bean	*Phaseolus acutifolius*	Phaseolinae	11	--
Lima Bean	*Phaseolus lunatus*	Phaseolinae	11	Mezcla
Mung bean	*Vigna radiata*	Phaseolinae	11	--
Cowpea	*Vigna unguiculata*	Phaseolinae	11	La Molina
Soybean	*Glycine max*	Glycininae	20	Williams
Pidgeon Pea	*Cajanus cajan*	Cajaninae	11	IS-10
Chacha fruit	*Erythrina edulis*	Erythrininae	na	--

1.3.5 Microsatellite map developed for common bean

M.W. Blair, F. Pedraza, E. Gaitán, J. Tohme

Introduction

Microsatellites are polymerase chain reaction (PCR) based markers that detect length polymorphisms at loci with simple sequence repeats. They are also single-locus markers that are specific to a given place in the genome. Microsatellites are advantageous because they are readily amenable to relatively high throughput marker assisted selection strategies. The specificity of microsatellite marker for use in MAS selection depends on tight genetic linkage of the marker with a gene that produces a reliable phenotype. Genetic maps are needed to determine where microsatellites are located in the genome and what genes they may be linked to.

Microsatellites have been found to be distributed densely throughout the genomes of higher plants, making them very appropriate for genetic mapping. Nearly-saturated microsatellite maps are now available in several crop plants including soybeans, rice, wheat, barley, etc. It would be tremendously useful to have a genetic map for common bean consisting entirely of microsatellites. These second-generation markers would be easy to assay and would enable a large number of segregating individuals to be analyzed in gene and QTL tagging studies. For now, we have implemented a set of forty microsatellite markers in genetic mapping studies for common bean at CIAT. These microsatellites come from genomic sequences, gene or cDNA sequences and database

searches mostly of common beans but also of other legumes as described in other sections of this annual report.

Materials and Methods

The parents of the DOR364 x G19833 RIL population were surveyed for polymorphism with 60 microsatellites that could be reliably PCR amplified. The polymorphic microsatellites were used to amplify DNA from the 87 recombinant inbred line progeny and the parents of the population. PCR product were run on silver-stained polyacrylamide gels and scored for the parental allele that they represented. Segregation data was used to place the microsatellites on a genetic map constructed with RFLP, RAPD, AFLP and SCAR markers (CIAT annual Report, 1998) using the software application Mapmaker.

Results and Discussion

Forty-three microsatellites (71.6%) were polymorphic for the parents of the DOR364 x G19833 population. Figure 1 shows the amplification pattern of 10 of the polymorphic microsatellites on a subset of 24 recombinant inbred line progenies. No difference in band intensity between the cDNA and genomic derived microsatellites was observed. Heterozygous loci were rarely found and the majority of RILs were homozygous for all markers.

A total of 38 microsatellites could be located on the genetic map of common bean. An additional 5 microsatellites from Yu et al (1999) could be placed by comparative mapping between the DOR364 x G19833 and BAT93 x JaloEEP58 (Freyre et al., 1998) maps. Each chromosome was tagged with at least one microsatellite. Two chromosomes, B02D and B04B had a relatively greater number of microsatellites, 11 and 9 markers respectively, placed on them. Considering that the total genetic distance of the entire map was 2,157 cM, the average distance between microsatellites was 51.4 cM. However many large gaps remain and the range in distance between microsatellites ranged from 17.8 (on B02C) to 242 cM (on B11J), depending on the chromosome.

The markers BMy-11 and BMd-28, both from cDNA sequences consistently amplified two polymorphic bands per reaction, which are suspected to represent duplicate loci. In another mapping population, the two BMy-11 loci co-segregate and are presumed to be very tightly linked (under 1 cM). Several microsatellites designed for related phytohemagglutinin gene sequences amplified single bands but were found to co-segregate, indicating that this gene family clusters at a single location in the genome. The microsatellites mapped during the course of this research will be invaluable for marker assisted selection because they are simple to analyze, specific for single genes of interest and diagnostic in most crosses due to their high level of polymorphism.

Future Activities

We will be studying the potential of specific microsatellites to be used in MAS selection for specific genes with which they are linked. The mapped microsatellites will provide a good set from which to chose markers for diversity studies and future QTL analysis. We plan to develop a set of fluorescent microsatellites for accurate allele calling and high-throughput mapping. A full set of anchor markers for the bean genome will probably require 300 or more working microsatellites, therefore the work of developing and mapping new microsatellites will continue.

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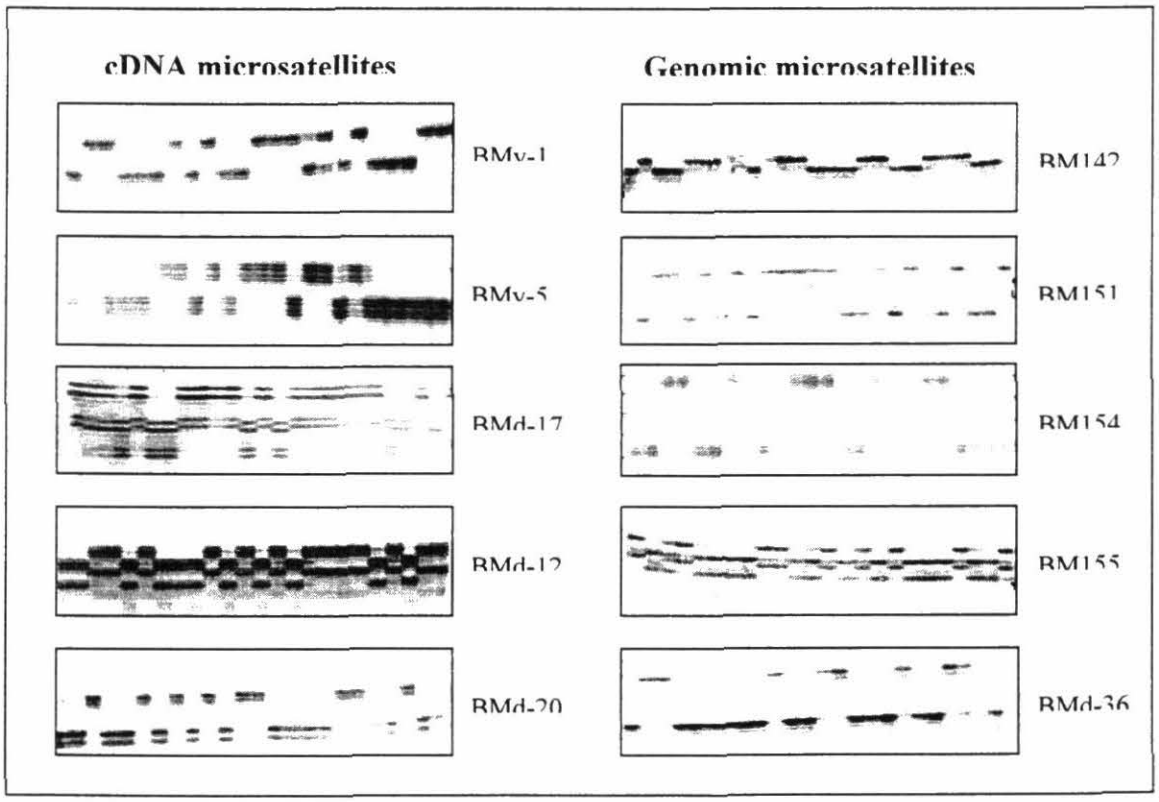

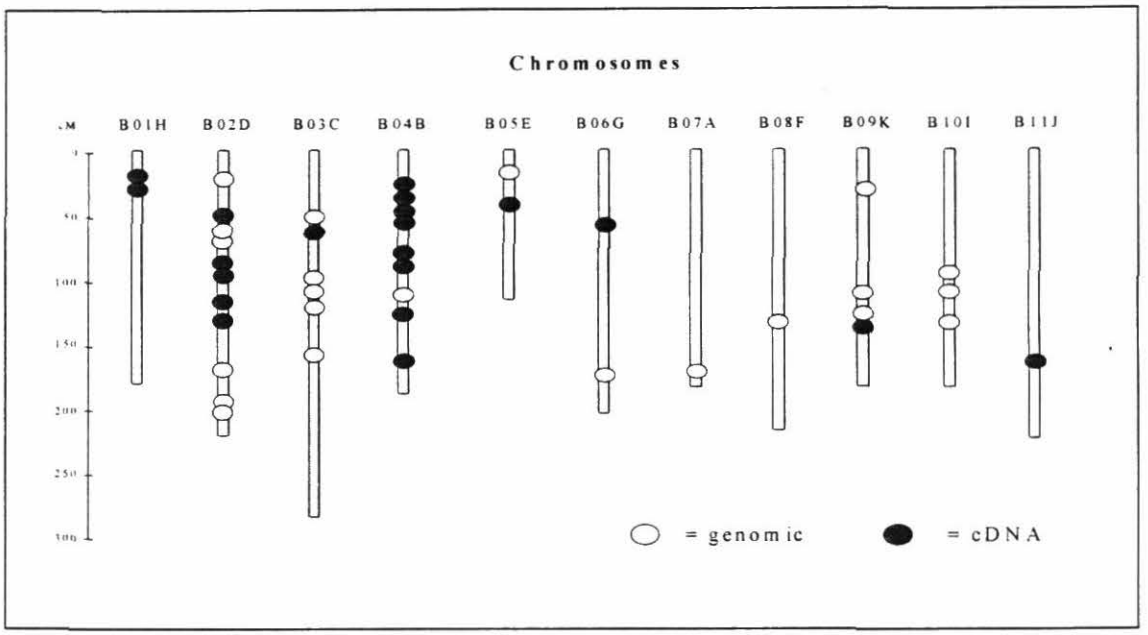

Figure 1. Bean Microsatellites (BM) from both cDNA and genomic sequences amplified and analyzed on silver stained polyacrylamide gels (A) and mapped on a framework genetic map (B).

1.3.6 Development of microsatellite markers for the Cassava molecular genetic map

R.E.C. Mba, M. Fregene and J. Tohme

Introduction

Over the past few years, CIAT has been developing molecular markers for use in cassava genetic studies and improvement. A predominantly RFLP-framework map (Fregene et al., 1997) had been developed, but it had the drawback of being inherently difficult to transfer to the NARS in Africa, Asia and Latin America, where most of the cassava improvement activities are carried out. In order to overcome this, a project, which is still ongoing, was initiated to develop PCR-based molecular markers. A total of 186 microsatellite or simple sequence repeat (SSR) markers have been developed: 14 described in Chavariaga-Aguirre et al., (1998; 1999) and 172 described in Mba et al., (2000). The latter also describe the initial mapping of 36 of these SSR markers. In order to achieve a target of placing at least 200 SSRs, corresponding to approximately 1 SSR marker per 10cM of the cassava molecular genetic map, further SSR-discovery efforts from a cassava root and leaf cDNA library were initiated. More SSR loci were also mapped on the cassava genome.

Materials and Methods

Mapping of SSR markers. SSR markers with a unique allele in either or both parents of the mapping population were used to screen the 150 progenies making up the F_1 mapping population. The segregation data of the markers that fitted the expected ratio of 1:1, presence:absence of the unique parental allele were used to place the markers on the framework map, using the linkage analysis computer package MAPMAKER 2.0. (Lander et al.,1987). The data analyses followed the same procedures as described for the mapping of the initial 36 SSR markers (Mba et al., 2000).

SSR discovery from cassava cDNA library. A cassava cDNA library was constructed commercially by Life Technologies (MD) from RNA extracted from leaf and root tissue. Approximately 80,000 clones were picked from this library and arrayed onto 6 single high-density filters using the QBOT robot (Genetix PLC, UK) of the Clemson University Genome Institute (CUGI), Clemson, SC. The filters were screened with the appropriate di-, tri- or tetranucleotide and end-labeled with $\alpha[^{32}$ P]dATP (Maniatis et al., 1987). A total of 587 putative positive clones were isolated. The sequencing and initial primer designs and syntheses were as described for the whole genomic SSR clones (Mba et al., 2000).

Results and discussion

Genome location of SSR markers. Figure 1 shows the map positions of 77 SSR loci from the 172 SSR markers analyzed to date on the male- and female-derived molecular genetic maps. Linkage groups nomenclatures are as described for the molecular genetic map of cassava by Fregene et al. (1997), except for groups O and P, which have now been merged with other groups. The SSR markers reveal an almost complete spread over the cassava genome—at least one marker being placed on all but one of the 18 linkage groups. The existence of markers with unique alleles in both of the parents or "allelic bridges" will assist in the construction of a consensus map of analogous male- and female-derived linkage groups for the cassava genome.

SSR discovery from cDNA library. The sequencing of the 587 putative SSR-containing cDNA clones is ongoing. From the approximately 400 putative SSR-containing cDNA clones sequenced thus far, 167 contain SSR loci in good enough positions for primer design. Less than 10% redundancy has been observed. Many of these clones contained more than one repeat motif, at times in different loci. However, a great majority of the repeat loci were the CT/GA repeat, which accounted for over 81% of the SSR-containing clones suitable for PCR primer design.

Pilot parental survey of SSR markers from cDNA. A batch of 32 primer pairs was designed and synthesized from the SSR markers obtained from the cDNA library. All these primer pairs amplified the corresponding locus in the cassava genome in the parents of the cassava F_1 mapping population. Interestingly, over 60% polymorphism was obtained with these markers. Moreover, a number of them show allelic bridges, which

indicates that the saturation of the cassava molecular genetic map with SSR from both whole genomic DNA and cDNA libraries will be feasible in the very near future.

Ongoing activities and future perspectives

- Completion of the sequencing of the cDNA clones
- Primer design and syntheses for suitable clones
- Genome location of the polymorphic loci
- Commencement of a high-throughput DNA microarray diversity study of the cassava genome

Other outputs

Important outputs have been the application of these SSR markers in several ongoing cassava genetic studies, both within and outside CIAT:

- Application in the tagging of the ACMD-resistance locus in CIAT. This has revealed two SSR markers that are closely linked to the ACMD-resistance locus, one of which has been mapped.

- Application in the tagging of ACMD resistance in IITA, using a different population

- Application in the CIAT–University of Uppsala (Sweden) cassava genetic diversity studies in Africa

- Application in the CIAT–IRD collaborative effort to tag the CBB-resistance locus

Publications

- A manuscript, "Simple sequence repeat (SSR) markers of the cassava (*Manihot esculenta* Crantz) genome: Towards an SSR-based molecular genetic map of cassava"

- A poster describing the mapping of 77 SSR markers was presented at the Plant and Animal Genome VIII (Jan.) conference in San Diego, CA.

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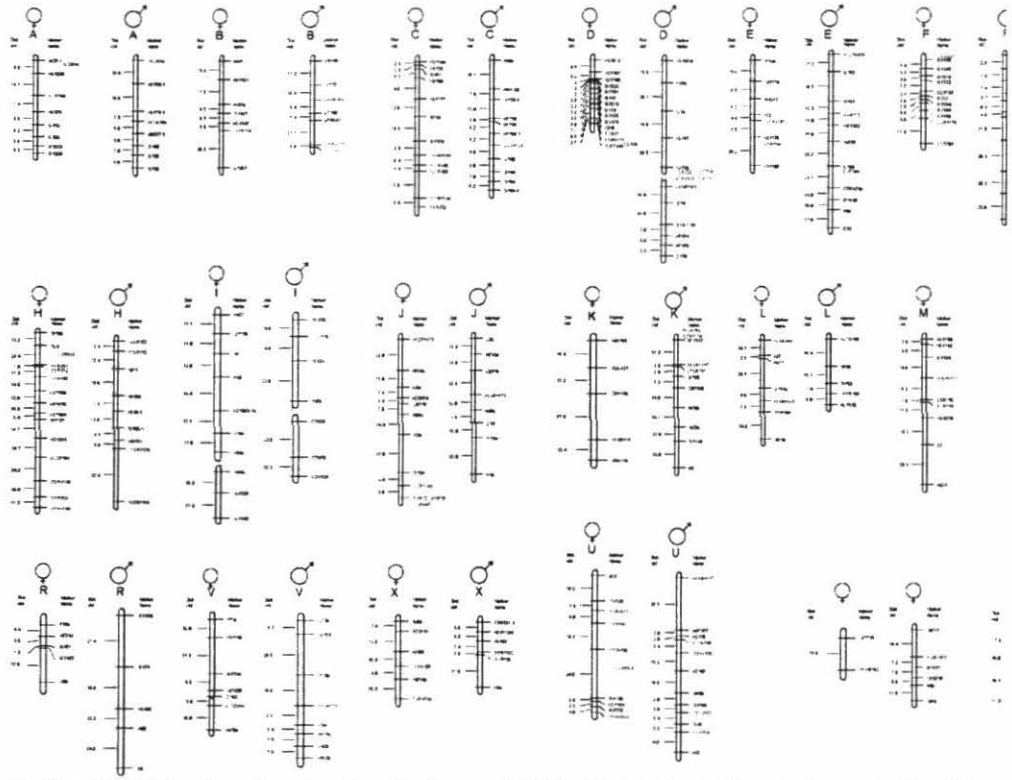
Figure 1.



Position of 77 SSR markers, shown in red, on the frame work (LOD>2.0) molecular genetic map of cassava. Map distance Map units. Groups at the lower right hand corner are yet to be merged with the analogous male- and female-derived linkag

1.3.7 Progress towards a PCR-marker based map of cassava and its in cassava breeding

J. P. Gutierrez, M.C. Suarez, E. Barrera, M. Fregene

Introduction

The application of the molecular genetic map of cassava in crop improvement is becoming more and more of a reality in light of successes in discovering associations between mapped markers and genes controlling traits of agronomic traits. However one draw back remains and that is the majority of the markers on the map do not lend themselves easily to the large-scale high throughput genotyping required for MAS. An effort to remedy the situation was initiated two years ago and has currently yielded more than 200 SSR markers of which at least 90 have been placed on the male- and female-derived maps. However many more SSR markers are required to provide wider coverage, and there is a need to convert existing mapped RFLP markers to PCR-based markers. The need for a PCR-based map of cassava is all the more urgent considering that an advanced back cross QTL (ABC-QTL) mapping project has been initiated to introgress enhanced root quality traits from wild relatives of cassava. The success of any ABC-QTL relies heavily on a reliable frame-work map amongst other criteria.

Materials and Methods

The SSR enriched library from variety CMC40 enriched for $(CT)_8$, $(GT)_8$, developed by Keith Edwards, Bristol University, UK, and cloned in pJV1, has been used to identify more than 150 SSR markers. It was decided to plate and screen many more clones from the library again with the oligos $(CT)_{20}$, and $(GT)_{20}$. The dot blot screening of the library is as described earlier (Mba et. al. 2000). Plasmid DNA was isolated from a total of 1000 positive clones by the QIAprep plasmid isolation kit, and 1-3ul of plasmid preparation was sequenced on an ABI377 automated sequencer, using the Universal M13 primers at the Cornell University Biotechnology Resource Center. Primer design was by the web based software Primer 3.0 found at http://waldo.wi.mit.edu/cgi-bin/primer/primer3.cgi.

The genetic map of cassava comprises of at least 250 RFLP markers on the male- and female-derived maps. It is an important resource and if they can be converted into co-dominant PCR-based markers sequencing and primer construction they will go a long way to ensure a quick completion of a PCR-based map of cassava. Plasmid DNA was prepared from overnight mini-prep cultures of *E.coli* stocks containing the appropriate RFLP probe using the QIAprep plasmid isolation kit. The clones are ready and will be sequenced shortly from both ends using the universal and reverse M13 primers (for PUC18 plasmids), and T3 and T7 primers (for pBlueScript plasmids).

Results

A total of 2400 putative clones were identified from screening 10,000 clones. A sub-set of 1000 clones were selected for sequencing. Of this about 450 clones contained unique

SSR markers and primers, 20-mers long, could be designed for 300 clones from regions flanking the repeats. Other clones had the SSR too close to the end of the fragment, or had very short, <4 di- or tri-nucleotide repeats. The primers will be synthesized and tested in the parents of the mapping population and polymorphic ones scored in the progeny.

Highlights of Outputs

- Sequencing of 1000 positive SSR clones and the design of primer pairs for 300 new SSR markers.
- Initiation of the conversion of RFLP markers on the genetic map of cassava to PCR based markers such as sequence tagged sites (STS) of cleaved amplified polymorhic sites (CAPs)

Future Activities

- Survey of 300 new SSR primer pairs in the parents of the cassava map population and evaluation of polymorphic markers in the progeny

- Primer design for 250 RFLP and evaluation of their ability to detect polymorphisms as PCR products (STSs) or after digestion with restriction enzymes (CAPs)

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1.3.8 Serial analysis of gene expression of resistance to CMD: candidate genes for the positional cloning of the CMD resistance gene

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Introduction

The objectives of the second phase of the RF funded CIAT-IITA CMD resistance mapping project is to identify molecular markers tightly linked to different sources of CMD resistance and to clone the resistance gene(s) for faster deployment of resistance genotypes and as tightly linked (zero cM) markers for MAS. Having achieved the first objective a strategy to identify candidate R genes, differentially expressed in CMD resistant genotypes, for map-based cloning was outlined based upon the serial analysis of

gene expression (SAGE). The SAGE technique is a powerful sequence-based method that is routinely used in human genetics research, especially cancer research, to identify novel genes differentially expressed in cancerous cells as target for new medicines. The SAGE profiling of genes expressed in plants was first described by Matsumura et. al., of the paddy-field rice research project, Iwate Biotech Research Center (IBRC) to reveal the gene expression pattern of rice plants under different germination and disease conditions. At CIAT, the bulked segregant analysis (BSA) approach had earlier been used to identify a simple sequence repeat (SSR) marker linked to a dominant gene that confers resistance to the devastating cassava mosaic disease (CMD). The SAGE and BSA techniques were combined to obtain the gene expression pattern in CMD resistant and susceptible plants as a first step to identifying candidate genes for map-based cloning of the CMD resistance gene.

Materials and Methods

Leaf tissue for SAGE analysis came from progeny of the TME3 X TMS30555 CMD resistance mapping progeny. About 1g of young leaf shoots were harvested from each of the 40 resistant plants and 40 susceptible plants used earlier to identify a CMD resistance marker by BSA. The plants have been kept in the field under very high disease pressure at the IITA high rain forest Station, Onne, Nigeria for more than two years and were ratooned at 12 months after planting. Ratooning cassava plants greatly increases virus disease pressure. The leaf samples were bulked for resistant and susceptible plants, ground to a fine powder in liquid nitrogen and dissolved in 5 volumes of "RNA later" (Ambion Inc.) and shipped directly to Japan.

Leaf tissue in "RNA later" was recovered by centrifugation in microfuge tubes, 2min room temp, and total RNA isolated using the Qiagen (Gmbh) RNA mini or midi prep kit. Typical yields were about 800ug total RNA/g of leaf tissue. Messenger RNA isolation was by the Amersham-Pharmacia Biotech (PLC) mRNA isolation kit. Construction of the SAGE library was with 4.5ug and 6ug mRNA for the resistant and susceptible samples respectively, according to the SAGE protocol, as modified by Dr H. Matsumura. Additional modifications made include:

- Optimization of ditag PCR, by performing a $MgCl_2$ concentration curve of 1.5mM, 2mM, 2.5mM, 3mM, 3.5mM, 4mM, 6mM, and 8mM using ditag template dilutions of 1:5, 1:10, and 1:20, maximizes efficiency of ditag generation. To prevent contamination of ditag PCR with previously amplified ditag, the required PCR reactions, 600 in all, were performed the same day prior to running ditag PCR PAGE gels.

- Treatment of NlaIII digested ditag with 400ul of streptavidin beads before and after PAGE electrophoresis, to reduce the risk of contamination by linker molecule, and thus increase the purity of ditag molecules which greatly enhances the efficiency of concatemerization of ditags.

- Replacing the pZERO plasmid vector of the SAGE protocol with another vector that permits the blue/white screening of recombinant plasmids; in this case phosphorylated (RT Inc.) pGEM-3Z (Promega inc.) plasmid was used. Nine nanograms of vector is the optimum amount for the ligation reaction with a 3:1 insert:vector ratio in a 10ul reaction volume, and 4 hours incubation time at 16^0C. Knowing the exact the concentration of inserts, for example by ethidium bromide dot quantitation, is therefore required.

- Electroporation into 40ul of ElectroMax DH10B cells (GibcoBRL) of the ethanol precipitated ligation, using the BIO-RAD Electro-pulser and the following conditions: 2.5kV, 25uF, 100ohms, 0.1cm cuvette. Electroporated cells are recovered in 1ml SOC and 100ul plated on 10cm ampicillin (100ug/ul), IPTG/X-gal LB plates.

White colonies were picked into 10ul of sterile water and 10ul of a PCR-mix added; PCR thermal cycling was according to the SAGE protocol. About 3ul of the PCR reaction were run on a 1.2% agarose gel, stained with ethidium bromide, and visualized on an UV trans-illuminator. Inserts above 586bp, approximately 29 tags, were selected for PCR clean up, with the QIAGEN kit, for sequencing. Sequencing was done with the T7 primer on a Perkin Elmer 377 Automated sequencer, using 1.5ul from the approximately 50ul volume obtained after PCR product purification. Sequence data was edited and saved as several text files, no larger than 9kb, then inputed into the SAGE bioinformatics program.

Results

Two SAGE libraries, of more than 100,000 clones with an average insert size of 620bp , were constructed from mRNA isolated from the resistant and susceptible progeny bulks. About 288 clones were sequenced from each library and a total of 5733 and 7053 tags, total of 12,786 tags, were obtained from the resistant and susceptible SAGE libraries. About 1,700 unique genes were expressed at equal levels in both samples. Of these, 10 abundantly expressed genes accounted for about 5% of all expressed transcripts. One hundred and eight transcripts were differentially expressed in the resistant bulk, at >5% probability level, including several transcripts that were completely absent in the susceptible bulk; twenty eight transcripts were at least 5 times more abundant in the resistant bulk (Fig 1). Primers were synthesized from these 28 tags for further analysis, namely tag annotation and genetic mapping. Transcripts that are found to map to the same region as the CMD resistant gene, in other words, that are strongly associated with the CMD resistance phenotype, will be used for fine-mapping, to screen BAC libraries for contig mapping and finally complementation. Partial length cDNAs for more than10 of the 28 tags have been recovered by PCR, using the tags as sense primers and an anti-sence primer from the plasmid vector, from a cDNA library constructed from mRNA of the resistant bulk in the vector pYES (Pharmacia biotech). Sequencing of the partial cDNA revealed down stream known to be expressed in response to pathogen attack in plants, including a peroxidase gene. Annotation of other tags is still ongoing.

Highlights of Outputs

- Implementation of the SAGE protocol for cassava
- Generation of 5,733 and 7,053 tags respectively for 2 mRNA samples obtained from
- CMD resistant and susceptible bulks, a total of 12,786 tags.
- Annotation of 10 of the 28 differentially expressed tags in the two bulks.

Problems Encountered

The lack of ESTs in cassava, especially in CMD resistant genotypes has considerable slowed down the annotation of the tags.

Plans for Next Year

- Genetic mapping of the 28 tags
- Generation of ESTs from the resistant bulk project to hasten tag annotation, in collaboration with the Iwate Biotech Center, Kitakami

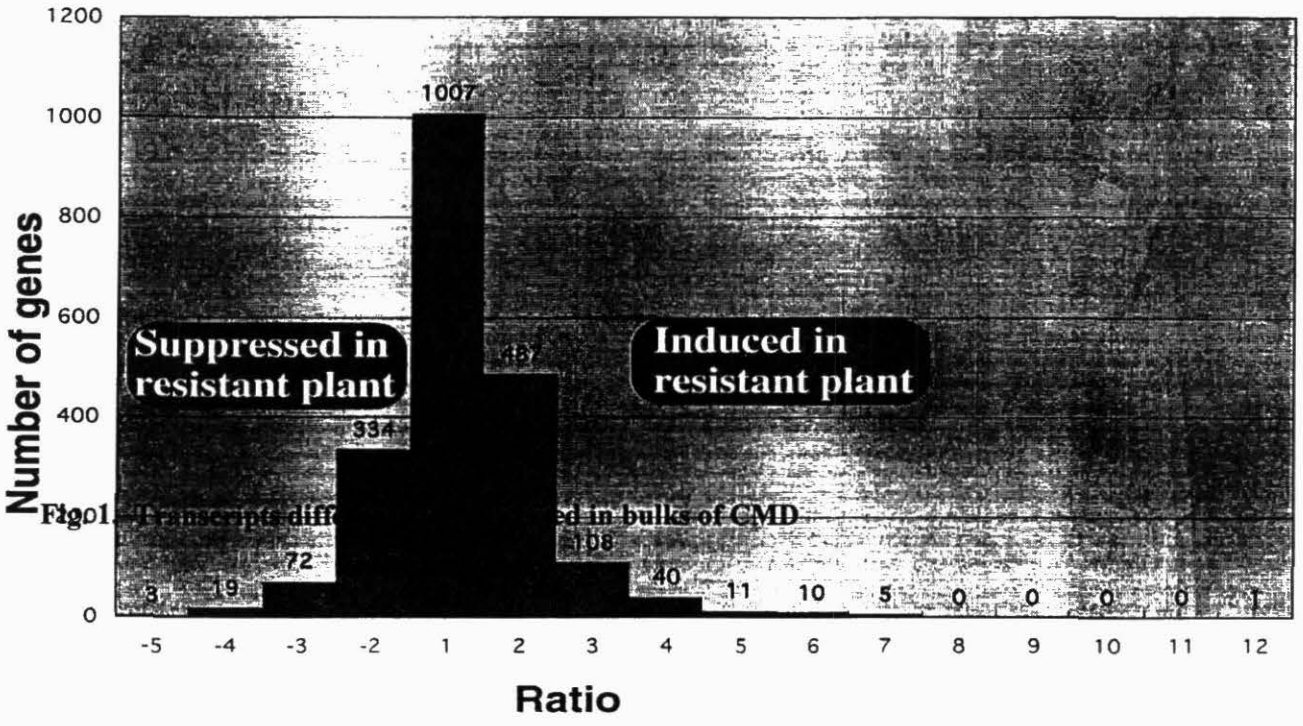

Figure 1. Transcripts differentially expressed in bulks of CMD resistant and susceptible genotypes.

1.3.9 Positional cloning of a CMD resistance R gene

A. Akano, E. Barrera, M. Fregene, A.Dixon

Introduction

The heterozygous nature of cassava implies that any attempt to introduce any trait, even when those controlled by a single gene leads to the loss of a favorite variety. A more efficient way to introduce single gene controlled traits, such as CMD resistance, is through genetic engineering. Following the discovery of genetic markers linked to the gene controlling the new source of resistance to CMD and current efforts to identify candidate genes by the serial analysis of gene expression of CMD resistance, the stage is set for positional cloning of the CMD resistance gene. Three important criteria for positional cloning are a fine map of the appropriate genome region, based on a large mapping population, a bacterial artificial chromosome (BAC) library, and an efficient transformation protocol for complementation analysis. An appropriate cross for fine mapping was identified at IITA and permission was sought and obtained to isolate DNA from the cross. A cassava BAC library has been constructed by CIAT in collaboration with the Clemson University Genome Institute (CUGI), however the BAC library was in a cassava genotype other than TME3, donor parent of the new source of CMD resistance, plans are underway to construct another library from TME3. Finally the BIBAC agro-bacterium based transformation kit for the introduction of large DNA fragment into plants was obtained from Cornell University (Dr Carol Hamilton) and is being tested out on cassava at CIAT.

Materials and Methods

The fine mapping population consists of two crosses between TME 3, the new source of CMD resistance, and TME117 or TMS30555, a susceptible land race and a susceptible improved line with a total of 789 progeny. The population was phenotyped for resistance to CMD under high natural field infection pressure in June 2000. DNA was isolated from very young fresh leaves according to Dellaporta et al. (1983).. Total DNA obtained was dissolved gently overnight at 4 °C in TE (10mM Tris-HCL 1mM EDTA) and quantified by fluorimetry (TKO 100 Hoefer). The CMD phenotyping and DNA isolation of the mapping population was done at IITA during a visit by the post-doc CIAT scientist, and the DNA was shipped to CIAT for genotyping with candidate genes that will soon be identified.

In preparation for transformation of candidate BAC clones with the BIBAC vector, friable embryogenic callus (FEC) were induced in a CMD susceptible lines CM2177-2 using a protocol described by Taylor et al. (1996. Embryos were obtained by direct embryogenesis, culturingsomatic embryos on Greshoff and Doy salts supplemented with vitamins and 10 mg/l picloram. Several selection rounds were carried out in this medium to obtain pure friable embryogenic cultures. The FECs are intended for use in the BIBAC agrobacterium-mediated transformation system; in preparation for this four

different *A. tumefaciens* strains containing BIBAC2.H150 have been cultivated in medium AB and the bacteria concentration measured.

Results

About 200-500microgram of good quality DNA was obtained for each of the 537 genotypes, sufficient for both RFLP and PCR-based analysis of the population. CMD resistance valuation revealed the expected ratio 1:1. FECs were successfully induced in CM 2177-2 although the frequency was very low, they are currently being proliferated in liquid medium supplemented with Schenk and Hildebrand salts and vitamins and 10mg/l Picloram. Also the cultivation of BIBAC constructs in medium AB is succesfull.

Highlights of Outputs

- DNA isolation of 537 genotypes from population derived from a cross between CMD resistant and susceptible African landraces
- Evaluation of CMD resistance under high disease pressure of the population.
- FECs developed in cultivars CM 22I7-2 and TMS60444
- BIBAC transformation system received from Connell University confirmed to be still viable

Future Activities

- Genotype the 537 progeny with candidate genes identified in the cassava SAGE experiment
- Make a BAC library from TME 3 and build a contig around genome region carrying the CMD resistant gene
- Transform the FECs with candidate BAC clones carrying the CMD gene using the BIBAC system

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1.3.10 Characterization of cassava proteins that interact with the *pthB* gene from *Xanthomonas axonopodis* pv. *manihotis* using the two-hybrid system.

C. Gonzalez , M. Chavez and V.Verdier
CIAT-IRD

Introduction

The two hybrid system or interaction trap, uses yeast and transcriptional activation of a reporter system to identify proteins that specifically interact with a target protein of interest. In this method, one plasmid is used to express the probe or "bait"protein as a fusion to the heterologous DNA-binding protein LexA. The plasmid expressing the LexA-fused bait protein is used to transform yeast possessing a dual reporter system responsive to transcriptional activation through the LexA operator (1).

The purpose of this study is to develop the two-hybrid system to characterize cassava proteins involved in the interaction with the PTHB protein of *X. axonopodis pv. manihotis* (2).

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 (His3), to clone the PTHB protein; pGAD (Trp1), to clone the cDNA library; the reporter plasmids pSH18-34 (Ura - LacZ) and pJK101 (Ura - LacZ) and the control plasmids pSH17-4 (His3) y pRFHM1 (His3). 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 glucose whitout histidine and uracil). Transformant strains *E. Coli* DH5α-with plasmid pLEXA and pGAD were grown in Luria Bertani (LB) with ampicilin 50ng/ml.

Transformation of yeast. Yeast strain EGY48 was transformed by electroporation method with the plasmids pLexA (pLexA/*pth*B) + pSH1834 and with the plasmids pSH17-4+pSH18-34 and pRFHM1 +pSH1834 as positive and negative transformation control respectively.

Characterization of the bait protein expression and activity. The transcriptional and repressor activity of LacZ gene by β-Galactosidase activity were tested on plates containing X-Gal (40mg) and 100ml of 1M potassium phosphate. The activation of Leu was assayed in selective medium with and without leucine.
Expression of the PTHB/PLEXA fusion protein was conducted by ELISA, using the EGY48 yeast lysed cells transformed with the plasmids pSH17-4 + pSH18-34 as a test and *E. coli* DH5α lysed cells transformed with the clone A315 (pGEX/*pth*B) as positive control. The antibody anti-PTHB (dilution1/1000), negative rabit antibody (not inmunized) and the antibody anti-rabit marked with peroxidase were used.

Construction of a cDNA. For the "prey" library, we synthetized cDNA from mRNA isolated from stems of CBB resistant variety MBRA685, inoculated with *Xam* strain CIO90. Plant materials were collected 7days after inoculation and stored to - 80°C until its extraction. The mRNA was isolated using magnetic beads (DYNAL) and the cDNA was synthesized using the Stratagene Kit. The cDNA inserts were cloned in the down stream site of the acidic transcriptional activation domain of pGAD. This plasmid was previously digested with *Xho*I and *Eco*RI enzymes. Then, the yeast strain (transformed with pLexA+pSH1834) was transformed with the plasmid pGAD+cDNA library by lithium acetate method and plated directly onto X-Gal SC omission medium lacking Histidine, Uracile, Leucine and Tryptophan (CM/ Glu, His-, Ura-, Leu-, Trp-).

Results and Discussion

The entire open reading frame of *pth*B gene was cloned to the C-terminus of the DNA-binding domain of LEXA in the "bait" plasmid pLEXA, this clone was sequenced to verify correct insertion 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-*pth*B fusion protein entered the yeast nucleus. The test for Leu requirement showed that the bait protein alone did not activate transcription of the LexA operator-LEU2 gene, observing no growth on medium lacking Leu. The ELISA test demonstrated the expression of the PTHB protein.
From the library constructed, only 300 clones were obtained, which were cloned in pGAD and transformed into EGY48 containing the plasmids pLexA and pSH1834. Results obtained suggested that the EGY48 lacks some markers and possibly that a mutant was selected.

Ongoing Work

- Implementing the work with a new yeast strain
- To obtain other two cDNA libraries from resistant CBB variety MBRA685 inoculated 48 hours with *Xam* strain CIO151and from cassava leaves that show HR to *Xam.*
- Transformation of the cDNA library into yeast containing pLEXA+pSH1834 using heat shock method.
- Screen and selection of interacting proteins
- Transformation into *E.coli.* and sequencing positive isolates.

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Donors
Colciencias and IRD.

1.3.11 Developing the cDNA-AFLP technique to study changes of plant gene exprssion in the *cassava-Xam* interaction

M. Santaella, C. Gonzalez [1], A. Badillo[1], M. Fregene

Introduction

In previous molecular studies, Jorge et al. (1999) suggested that several genes are probably required for resistance to CBB, based on the location of 12 QTL's in the framework map of cassava, which explained 9-27% of the phenotypic variance. Recently, a cDNA-AFLP strategy was developed toward studies in differential expression in *Solanum* and has been used to generate 70 transcript-derived fragments (TDF's) specific for the tuberization process in potato (Bachem et al., 1996). This is a robust and reliable method of RNA fingerprinting that allows the characterization of gene expression in a wide range of biological processes (Bachem et al., 1996) and has been applied in tomato (Durrant et al., 2000), cassava (Suarez et al., 2000), and Arabidopsis (van der Biezen et al., 2000).

In order to identify genes in cassava involved in the defense reaction against the pathogen *Xanthomonas axonopodis* pv. *manihotis,* we are analyzing the changes occurring in the transcription process with an incompatible reaction. For this purpose, we are implementing the cDNA-AFLP technique, comparing the expression patterns between two cassava varieties (resistant and susceptible to *Xam*).

Materials and methods

Stem inoculation: Cassava plants resistant (MBRA685 and SG107-35) and susceptible (MCOL1522) to CBB, were inoculated with *Xam* strain CIO151 by stem puncture. Plants were collected in the greenhouse in liquid nitrogen and stored immediately at -80°C. Plants were collected as follow: 1) non inoculated plants 2) 24 hours, 3) 48 hours, 4) 72 hours, 5) seven days after inoculation and 6) 24 hours after stem puncture with sterile water.

RNA extraction: Plant tissue was ground to a fine powder using liquid nitrogen and 3g were used for RNA extraction. The proteinase K method, described by Hall et al. (in

Rocha, 1995) was used with some modifications. The purity and quantity of the RNA was calculated according to the absorbance at 260nnm/A280nm measured in a spectrophotometer.

cDNA synthesis: mRNA was obtained from total RNA using magnetic poly-A DYNAbeads according to the manufacturer instructions (DYNAL). The synthesis of the first strand of cDNA was carried out following different protocols. It was finally standardized using an oligodT primer (PROMEGA) and the Superscript II reverse transcriptase (Gibco BRL), removing previously the poly-A DYNAbeads. Second strand cDNA synthesis followed the protocol from Bachem (http://www.spg.wau.nl/pv/staff/aflp.htm).

cDNA-AFLP: The cDNA was digested with a pair of restriction enzymes *Eco*RI/*Mse*I (a rare and frequent cutter, respectively). Adapters corresponding to each restriction site were ligated according to AFLP protocol described by Vos et al (1995). A PCR preamplification was performed using one of the adapter's strands as primer without selective bases (PCR +0). The PCR profile consist on 35 cycles of 94°C for 30s, 52°C for 30s and 1 min extension at 72°C. The second amplification was performed using two selective bases for each primer, with all possible combinations (256). The PCR product was electrophoresed for 2h and 30min on a 5% denaturing polyacrylamide gel [5M Urea] in 1X TBE at 100W and 50°C. Afterwards, the gel is processed with the silver staining technique, in which silver nitrate binds to DNA and makes it visible as bands.

Results and Discussion

During this year, all the methods have been under standardization, solving the problems that have arisen during the development of the project.

RNA of the different cassava varieties was extracted at the different times established, except for the resistant variety SG107-35, from which RNA was extracted at 0 and 24 hours after inoculation. Samples with high purity (ratio A260/280 around of 1.8-2.0) were selected for the reverse transcription process, in order to avoid enzyme inhibition problems by the presence of proteins or phenolic residues. For optimal cDNA synthesis, we found that the DYNAbeads have to be eluted first, and then use oligodT sequences to prime the synthesis of the first strand instead of the oligo dT attached to the DYNAbeads. Once the second strand is synthesized, the cDNA should be cleaned with phenol-water, chloroform-isoamil (24:1), and precipitated using ammonium acetate and ethanol (95%).

For the standardization of the AFLP reaction, we performed dilution series of the cDNA, ranging from 1/20 to 1/100, and of the preamplification reaction (1/30 to 1/60) prior to selective amplification (PCR+2). The pattern of bands observed in the final electrophoresis depends specially on the amount of template used for the reactions. If there is a lot of template, a black smear would probably hide important bands in the pattern. This process is still under standardization.

Ongoing work

- Evaluation of 256 AFLP *Eco*RI/*Mse*I primer combinations
- Analysis of the expression pattern and identification of molecular markers
- DNA sequencing of polymorphic bands and search for similitude's
- Hybridization of RNA from resistant and susceptible varieties with specific probes

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1.3.12 Characterization and expression of resistance gene analogs in cassava.

P. Zuluaga and V. Verdier

Introduction

In a previous work, we characterized three classes of RGAs isolated from cassava by using degenerate primers designed from the conserved domain Nucleotide Binding Site (NBS) of the resistance proteins L6 (Flax), RPS2 (*Arabidopsis*) and N (Tobacco) ((BRU Annual Report, 1999). In this this work we used a set of primers that enable us to differentially isolate RGAs from the two major groups of the NBS domains. Group I NBS domains contain group-specific motifs that are linked to the TIR sequence in the N terminus, and which are significantly associated with dicot species (Qilin, P. et al. 1999). The MOTIF1 primer (Lopez, C and Acosta, I.. pers comm.) corresponds to the Group I NBS domain. Group II NBS domains are always associated with putative coiled-coil domains in their N terminus and appear to be present throughout the angiosperms (Qilin, P. et al. 1999). The PRS3 primer was designed from the Group II NBS domain of the R-genes (Peñuela, S. pers. comm.).

Analysis of the resistance-gene analogs (RGAs) showed high homology with the nucleotide-binding site (NBS) of previously cloned resistance genes. However, NBS domains are not unique to R-genes and identification of other motifs would provide more compelling evidence of the relationship between RGAs and plant R-genes. While this technique is useful for identifying disease-resistance loci, identification of functional resistance gene within a cluster of paralogs is much more difficult. Some members of a resistance gene cluster are nonfunctional.

Identification of RGA sequences from expressed gene messages (mRNA) will provide evidences for function of these sequences. In this study, we used class-specific RGAs probes to screen a cassava's cDNA library in order to determine the functionality of RGAs isolated.
The objectives were to i) to isolate new RGAs classes and map them in the F1 population used to develop the cassava molecular map ii) identify expressed gene sequences in a leaf cDNA library corresponding to functional RGAs and iii) identify additional R-gene conserved motifs based on cDNA sequences.

Materials and Methods

DNA Extraction and RGA Isolation

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). 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.

Table 1. Degenerate oligonucleotide primers based on the amino acid sequences of the highly conserved domains NBS and TIR

Domain	Primer name	Sequence peptide/nucleotide	Reference
NBS	S2	G G V G K T T GGI GGI GTI GGI AAI ACI AC	Leister et al., 1996
TIR	TIR1S	D/E V F L/P S F R/S G GAI GTN TTY TTI TCI TTY AGI GG	Lopez, C., Acosta, I. (pers.comm.)
TIR	TIR5AS	P/V F Y M/D V D P IGG GTC IAC GTC GTA GAA IAC IGG	Lopez, C., Acosta, I (pers.comm.)
MOTIF1	MOTIF1AS	F L/K D/C I A C F GAA GCA IGC GAT GTC IAG GAA	Lopez, C., Acosta, I. (pers.comm.)
PRS3	PRS3	**GGR AAI ARI SHR CAR TAI VIR AAR C**	S.Penuela (pers.comm.)

RGAs Mapping

RGAs were used as probes to detect RFLPs in cassava genomic DNA (CM2177-2, MNGA2, SG107-35, MCol1522) digested with a set of eleven different restriction enzymes (*Alu*I, *BamH*I, *Eco*RI, *Eco*RV, *Dra*I, *Hind*III, *Hae*III, *Mse*I, *Pst*I, *Sau*3A and *Xba*I). We also determine the correlation between the phenotype showed by the 150 F1 individuals CM6744 (SG107-35 x MCol1522) and RGA segregation. These plants were inoculated with five strains of *Xanthomonas axonopodis* pv *manihotis* (*Xam*) (cio1, cio84, cio136, cio295 and orstom 27).

cDNA library screening

Young leaves and roots from NGA2 were used for RNA extraction using the proteinase K method (Suarez, MC 1998 tesis). GIBCO BRL manufactured the cDNA library. To study the RGA expression , 89000 clones were screened from this library with 12 class-specific RGA probes. Hybridization were performed at 65°C in 0.5M sodium phosphate, 7% SDS, 1mM EDTA and 10 mg/ml denatured salmon sperm DNA hybridization buffer. Washing solutions were 2X SCC, 0.1% SDS for 15 min and if necessary followed by washes with 1X SCC, 0.1% SDS solution for 5 min.

cDNA Sequencing and Motif Identification

A total of 16 cDNA clones were detected by the RGAs probes and characterized by a restriction enzyme analysis using a set of restriction endonuclease (*Alu*I, *Cfo*I, *Hae*III, *Sau*3A).

Nucleotide sequences of the clones were determined using the ABI PRISM Dye Terminator kit (Perkin-Elmer) with primers T7 and SP6 and an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer) and edited with Sequencer (Genecodes, Ann Arbor, MI). The cDNA sequences were analyzed and conserved motifs were identified using BLAST algorithm (Altschul et al. 1997).

Results

Characterization of new RGAs

With primer combinations S2-MOTIF1 and S2-PRS3 a single band 680bp was amplified for each combination. Clones from these bands show high homology with R-genes and RGAs from Arabidopsis. With primers from the TIR domain we amplify a 280 bp product that shows the expected size and homology with the corresponding domain of R-genes. In total, we characterized 12 classes of RGAs. Two classes are from the TIR domain primers. The other 10 classes are divided in two groups. Classes 1 to 5 corresponds to NBS Group I and are linked to the TIR sequence in the N terminus and classes 6 to 10 corresponds to NBS Group II and are associated with putative coiled-coil domains in their N terminus.

Hybridization of the different RGAs use a s a probe did not revealed any polymorphism within the parents with the set of enzymes used.

As an alternative, RGAs detect RFLPs between SG107-35 and MCOL1522, which are the parentals of the 150 F1 population CM6744. We wanted to test if there is any correlation between RGA segregation and the phenotype of the progeny after inoculation with 5 different strains of XAM tested. Until now, we have no correlation between the molecular data and the phenotype showed by the inoculated plants.

cDNA library screening

12 RGAs representing the 12 classes were used as specific probes to screen cassava leaf and root cDNA libraries to determine their expression. Sixteen cDNA clones ranging in length from 1 to 2-kb were identified.

Characterization of the cDNA clones

A total of 16 clones were detected by the RGA probes from the cDNA library (A-3, A-7, A-16, A-17, C-20, F-2, F-13, F-14, I-10, J-8, K-10, L-16, M-10, N-5, O-11, P-6) and digested with restriction enzymes to verify if they were unique. Nucleotide sequences of the clones were determined using the ABI PRISM Dye Terminator kit (Perkin-Elmer) and an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer) and edited with Sequencher (Genecodes, Ann Arbor, MI). The cDNA sequences were analyzed and conserved motifs were identified using the BLAST algorithm (Altschul et al. 1997).

Sequence analysis

Sequence analysis of the unique clones showed that only one (L16) 1690 kb, from the RGA class 10-probe shows homology with R-genes. Additional to the NBS, this sequence revealed an LRR domain, characteristic of plant R-genes. The highest homology was with a NBS/LRR disease resistance protein from *Arabidopsis thaliana* (score=134 and e-value=2e-34). We did not detect other motifs like TIR or LZ. The other 15 clones sequenced presented homology with putative ubiquitin like proteins and ribosomal proteins.

Conclusions

Our results permitted to characterize 12 classes of RGA and one cDNA clone related to R gene. Presence of the NBS and LRR domains and absence of the TIR or LZ domains confirm that this clone belongs to the NBS Group II. In conclusion, we report the constitutively expression of a protein that shares characteristics with the resistance proteins previously reported and that the RGA L-16 is a member of the NBS-LRR resistance gene family.

On going activities

- Use of a BAC library to do physical mapping of RGAs.
- Use the L-16 clone in the two hybrid assay to detect possible interaction with the PTHB protein of *Xanthomonas axonopodis* pv *manihotis*.
- Screening a cDNA library (CBB resistance).
- Patterns of L-16 expression during infection process

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1.3.13 Use of resistance gene analogs to initiate dissection of resistance gene clusters in common beans

I.F. Acosta ,E. Gaitán and J. Tohme

Introduction

The study of disease resistance in common beans (*Phaseolus vulgaris*) is important because this major staple crop is one of the most susceptible to diseases such as anthracnose, angular leaf spot (ALS) and bean golden mosaic virus (BGMV) (Schwartz and Pastor-Corrales, 1994). Recent efforts to understand bean disease resistance better led to the identification of a set of Resistance Gene Analogs (RGAs), employing PCR with degenerate primers (BRU Annual Report, 1999). Quantitative trait loci (QTLs) detected for anthracnose, BGMV and ALS resistance were co-localized with RGA classes 1, 2 and 9, respectively. A manuscript reporting these data is in preparation.

Resistance (R) genes are clustered in the genome of several species as shown by genetic and molecular studies (Richter and Ronald, 1999). They display an apparent multiallelic structure or group as genetically separable loci. Examples of such complex resistance loci are found in maize (Richter et al., 1995), flax (Ellis et al., 1995), lettuce (Witsenboer et al., 1995) and barley (Jorgensen, 1992). Furthermore, different genes within the same cluster determining resistance to diverse pathogens have been reported in tomatoes (Dickinson et al., 1993; Jorgensen, 1992; Kaloshian et al., 1995; Vanderbeek et al., 1994; Zamir et al., 1994). R-genes are thought to be functionally and evolutionary related. The sequences of several R-gene clusters from rice (Song et al., 1997), tomatoes (Parniske et al., 1997) and lettuce (Meyers et al., 1998) have now shed light on the molecular mechanisms leading to their evolution. Interestingly, RGA classes 1, 2 and 9 from beans display a complex hybridization pattern, characteristic of a multigene family. It was concluded that these RGAs are part of R-gene clusters that contain determinants of anthracnose, BGMV and ALS resistance; therefore, RGAs could be used to initiate the dissection of these R-gene clusters from beans. Two main objectives are being pursued:

- Identify expressed gene sequences in bean cDNA libraries corresponding to RGAs
- Use and construct contigs of bacterial artificial chromosome (BAC) clones containing the RGA families

cDNA library screening and cDNA sequencing

To examine RGA expression, a leaf cDNA library with RGA classes 1, 2 and 9 was screened. The library was custom-made (Gibco-BRL) from RNA collected from uninfected young bean leaves. About 90000 cDNA clones were screened by hybridization, using RGAs as probes. Positive clones were sequenced using the Dye

Terminator Cycle Sequencing Kit and an Applied Biosystems Prism 377 DNA sequencer; and sequences were edited with Sequencher (Genecodes, Ann Arbor, MI). Database searches were performed with the BLASTX algorithm (Altschul et al., 1997). Each RGA class detected a corresponding cDNA clone. They were <1500 bp long and could encode partial polypeptides. cDNAs from classes 1 and 2 contain the amino-terminal region of the coding sequence because they show homology with the corresponding region of R-genes from tomato (*I2*) and lettuce (*Dm3*), which are of the non-TIR type. Analysis of these clones is currently under way to identify de coiled-coil domain characteristic of this kind of R-genes. However, the clones are truncated in their carboxy-terminal region so that the other characteristic domain of R-genes, leucine rich repeats (LRRs) were not identified. Expression of truncated R-gene sequences could have evolutionary and functional implications that are object of discussion.

Isolation of BAC clones

RGA classes 1, 2 and 9 and marker Bng160 were used as probes to screen filters containing 18000 gridded clones of a genomic BAC library of beans. They represent 4.5 genome equivalents. DNA gel blot hybridizations were performed according to standard protocols (Sambrook et al., 1989). From this point, the work has concentrated on 42 BAC clones detected by RGA class 2, which is located in chromosome B04, associated with a QTL for partial resistance to BGMV. The R-gene cluster detected by this RGA also contains an anthracnose R-gene reported by a French group (Geffroy et al., 1999). Bng160 is a molecular marker located in close association with the QTL for BGMV partial resistance but distant 6 centimorgans (cm) from RGA 2. However, Bng160 also shows a complex hybridization pattern, and it was reasoned that it could detect some of the clones identified by RGA2. Of the 42 BAC clones containing RGA2, 13 were also detected by Bng160.

Analysis of BAC clones

The 42 BAC clones were mini-prepped using a modified alkaline lysis protocol (Sanchez et al., 1999) that guarantees a cleaner product. HindIII fingerprints were obtained for individual BAC clones by digestion of BAC DNA and analysis of the resulting fragment sizes through separation on 1% agarose gels. Fragments will be compared between BACs to identify duplicated bands as potentially overlapping fragments. In the meantime, overlap has been confirmed by DNA gel blot hybridization, using RGA 2 and Bng160 as probes.

BAC-end sequencing

BAC clones (13) from RGA2 and Bng160 span an interesting region, with an R-gene cluster containing the QTL for BGMV partial resistance. Additional markers positioned there include the SCAR ANO17G, which is homologous to the LRR domain of R-genes and the microsatellite J845555 (F. Pedraza, pers. comm.). These markers will be

employed to establish possible overlapping between BAC clones. The development of informative PCR-based markers for the ends of the BACs is also required to identify genuine overlaps (Meyers et al., 1998). In order to obtain BAC end sequences in a low-cost, less time-consuming manner, a sequencing strategy that uses BAC clones as a direct template was developed. Clear sequences ranging from 600-700 bp have been obtained. Initially, only the T7 ends of the 13 BAC clones of interest have been sequenced. BLAST searches using end-clone sequences found some significant similarity to sequences in the databases (Table 1). The presence of an RGA class 2 in one of the BAC end sequences confirmed the presence of this genic family in BAC clone 10 and revealed some genomic structure characters of its amino-terminal region (Table 1). Other homologies are indirectly related to resistance (BAC clones 5 and 9) as potential components of responses to pathogens or stress, events that have been correlated before. All sequences are potentially suitable for new PCR-based markers; e.g., the BAC 7 T7 end contains a. microsatellite.

Table 1. Genomic structure characters in the amino-terminal region of BAC clones

BAC Clone	1.3.13.1 BAC End Sequence Features
4	Weak similarity with nitrogen-fixation protein
5	Homologous to putative N-acetylglucosaminyltransferase and to pathogen-induced ESTs
7	Homologous to the promoter of the bean malic enzyme gene; contains a microsatellite not isolated before
9	Homologous to salt-inducible proteins
10	Contains one member of the RGA class 2 family: Part of its amino-terminal coding region and a probable first intron

Isolation of regions flanking RGAs

Clone BAC 34-C8, which is known to contain RGA 2, was digested with EcoRI. Fragments were ligated to an EcoRI adapter and PCR-amplified using the primer ECOR1ADAPTER1 in combination with RGA 2 specific inverse primers designed to isolate regions 5' and 3' of the NBS. Only the region flanking the 5' end of RGA 2 produced an amplification product (1 kb long) that was cloned and sequenced. As cDNA clones and T7 end from BAC 10, it was homologous to non-TIR R-genes *I2* from tomatoes and *Dm3* from lettuce. However, it has a putative intron at 5' end. The genomic structure of this and the other sequences is shown in Figure 1. It was concluded that they represent different members of the genic family represented by RGA2, characterized by a non-TIR amino-terminal domain, an NBS and a predictable LRR that is expected to be isolated soon.

Ongoing work

- Screening of 90000 filter-gridded clones from a cDNA library with an average insert size >1600 bp to isolate full-length sequences of RGAs.

- Sequencing of SP6 end from BAC clones containing RGA 2 and Bng160.
- Design of PCR-based markers from BAC end sequences.
- Analysis of HindIII fingerprintings of the 42 RGA2 BAC clones to complement contig construction
- Analysis of BAC clones identified by RGA classes 1 and 9.
- Isolation of RGAs from *Brachiaria* in order to locate a putative R-gene cluster that could contain an NBS-LRR R-gene conferring resistance to spittelbug (currently, we have isolated 11 RGA classes).

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1.3.14 Towards positional cloning of rice blast-resistance genes

G. Gallego, I. Acosta, M. C. Duque and J. Tohme

Introduction

CIAT is dissecting the genetics of rice blast, using crosses between the varieties Fanny and Irat13, which are susceptible and resistant, respectively, to different isolates of *P. grisea* (SRL-1 to SRL-6). The project focuses on fine mapping genes on chromosome 6 of rice: Pi-i, Pi-z (pi-2), Pi-8, Pi-9(t), Pi-13(t), Pi-22(t),* which have been reported by several authors.

The DNA of 104 lines (from cross Fanny x Irat13) is being used to construct a fine map of chromosome 6, using probes for RFLPs (Cornell University, probes RG and CDO). MapMaker, with an LOD of 6.0 and θ of 0.4, was used to map new markers such as microsatellites, RAPDs, SCARs, AFLPs and RGA (CIAT Annual Report, 1999). Resistance to a blast isolate from lineage 1 (cica 9-31-4) was mapped using phenotypic data from greenhouse plants, falling between the markers RG648 and RG456. The interval between these two markers and the "potential gene" was saturated with more markers as described by Giovannoni (1991).

Giovannoni's methodology comprises the formation of two DNA pools from individuals homozygous for opposing alleles for a targeted chromosomal interval, defined by two or more linked RFLP markers. The DNA pools are then screened for polymorphism. Polymorphic DNA bands should represent DNA sequences within or adjacent to the selected interval. It is expected to find markers linked to the potential resistance gene(s) within 1 cM, which will allow a more accurate screening of BAC libraries to construct contigs.In addition, marker SCAR B10, which is close to RG456, was PCR-amplified in the parentals. Resistant and susceptible parents and lines showed bands of different molecular size. The DNA bands were then cloned and sequenced.

Results and discussion

A fine map of chromosome 6 was constructed with several markers (Fig. 1). Resistance to isolate cica 9-31-4 was located within 1.5 cM from RG648, and 2.3 cM from RG456.

Six hundred RAPD primers with resistant and susceptible DNA bulks were PCR-tested for interval RG648/RG456. Of these, 16 were polymorphic and evaluated in the progeny. Ten mapped to chromosome 6, adjacent (outside) to the interval; none was inside the interval.

64 combinations AFLPs (Gibco-BRL kit) were tested with the same two DNA bulks. Thirty-two bands were polymorphic and co-segregated with the resistant bulk. All 32 bands were eluted from gels and cloned. They were also evaluated as RFLPs, with the parentals showing some polymorphism with the enzymes EcoRV and HindIII. The evaluation of the progeny with these probes is still ongoing.

Misclassification of susceptible and/or resistant individuals may have contributed to the partial success in saturating interval RG648/RG456. It is important to reclassify individuals by measuring the percentage of affected leaf area (ALA). Individuals classified as resistant should have from 0-3% ALA, while susceptible individuals would have more than 70% ALA.

The marker SCAR B10 shows a 1-kb band in resistant individuals and a 703-bp band in susceptible ones. When these bands were cloned and sequenced, an $(AT)_{115}$ microsatellite was found in the resistant parent only (Irat13). A null allele (absence of AT_{115}) was present in the susceptible parent Fanny. A similar situation has been reported for molecular markers for rice blast resistance genes (Zheng et al., 1995).

The AFLP1 marker showed a close linkage for resistance to isolate cica 9-31-4. It will be possible to develop a PCR-based marker from AFLP1 (SCAR) for large screenings in marker-assisted selection (MAS).

Ongoing work

- Test more markers to saturate the interval RG648/RG456
- Screen BAC libraries with markers in close linkage to rice blast-resistance genes
- Assemble contigs with positive BACs
- Use ALA data for QTL analysis.

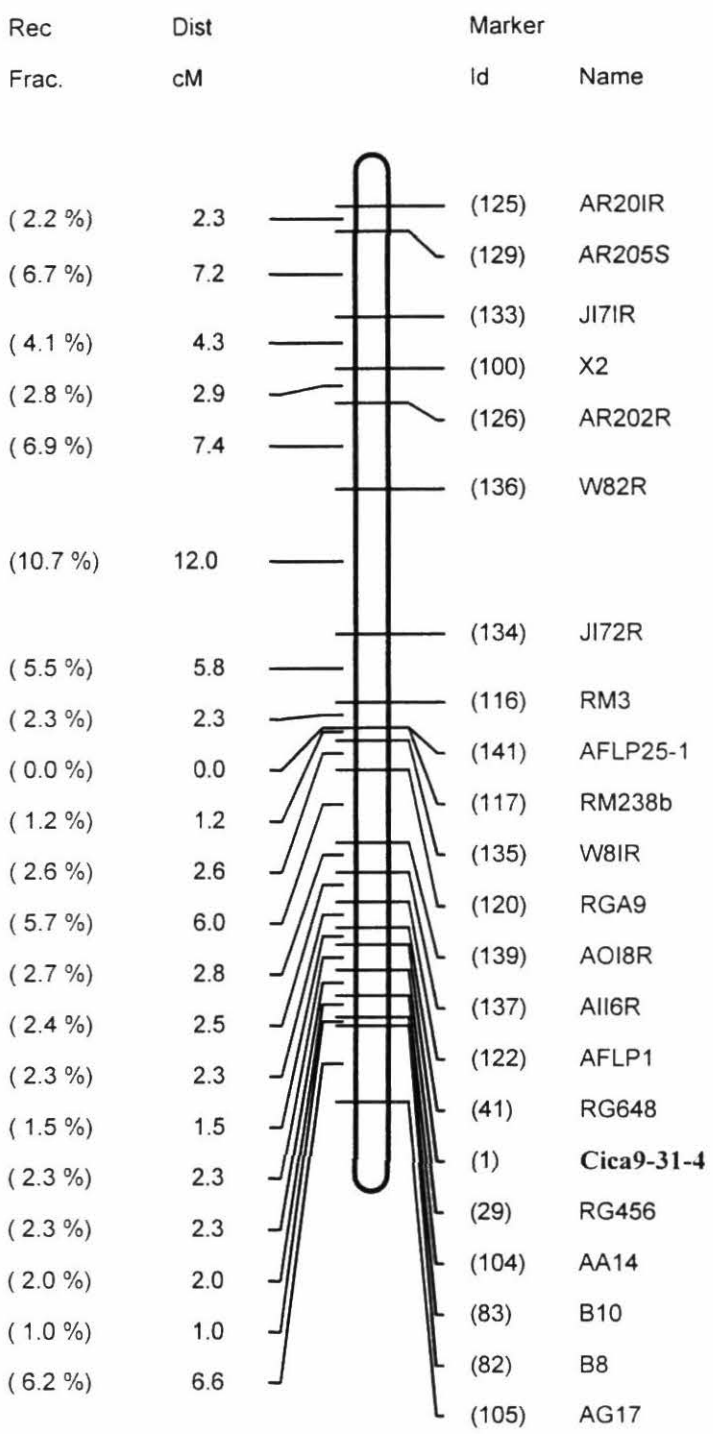

Figure 1. Molecular map of rice chromosome 6 (cross Fanny X Irat13).MapMaker, LOD 6, $\theta = 0.4$.

1.3.15 Development and use of SSR and AFLP markers for constructing a molecular genetic map for *Brachiaria*

E. Gaitán, O.X. Giraldo, J. Miles and J. Tohme

Introduction

A comparative molecular genetic map of *Brachiaria* has been constructed using RFLP markers from rice, pearl millet and sorghum as a first step for genetic analysis of traits of agronomic importance. In an attempt to make marker technology available in *Brachiaria*, a project was embarked upon to place simple sequence repeat (SSR) and AFLP markers on a *Brachiaria* map. The purpose was to develop and map at least 96 SSR markers and two combinations of AFLP markers.

As PCR-based markers, microsatellites and AFLPs will facilitate the application of markers in a breeding program. With microsatellites locus-specific primers were designed and used to analyze crosses between apomictic, sexual and spittlebug-resistant parents to increase the saturation of the *Brachiaria* map (unpublished data). Such primers will provide codominant, PCR-based and locus-specific markers that could be used in gene tagging and marker-assisted selection for a spittlebug-resistance gene and an apomixis gene. With AFLP markers two combinations of primers were used to analyze the same populations.

Methodology

Microsatellites: PCR amplification and evaluation of polymorphism. The PRIMER computer program (version 3.0) was used to design 97 locus-specific primer pairs. These primers were then tested for PCR amplification and polymorphism, using DNA from the parents of both mapping populations (CIAT 606 x Clone 44-3 and CIAT 6294 x Clone 44-3). The PCR reaction was carried out in a 20-µl final vol. containing 20 ng of genomic DNA, 0.1 µM of each forward and reverse primer, 10 mM Tris-HCl (pH 7.2), 50 mM KCl, 1.0-2.5 mM MgCl2, 250 mM of total dNTPs and 0.5 units of Taq DNA polymerase. The temperature-cycling profile was an initial denaturation step for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing from 45-65°C for 15 sec, and primer extension at 72°C for 15 sec. Three microliters of the PCR reaction was resolved on 6% polyacrylamide-sequencing gels for 2 h at 100 W, and DNA visualized by silver staining according to the manufacturer's instructions (Promega Inc., USA), with some modifications. Polymorphic primers were used to evaluate 316 individuals belonging to CIAT 6294 x Clone 44-3 populations, using the PCR conditions standardized for each one.

AFLP methodology. AFLP fingerprinting of 215 individuals belonging to CIAT 6294 x Clone 44-3 populations was carried out using the AFLP Analysis System I kit from Gibco-BRL, following their instructions with some modifications. All individuals were evaluated using four combinations of primers.

Results

Microsatellite amplification. Ninety-seven primer pairs were designed and used to amplify in all three parentals (apomictic parental - CIAT 606, sexual clone 44-3 and CIAT 6294) of two mapping populations. When evaluated in all three parentals, 35 polymorphic primer pairs were obtained (Table 1). Polymorphism was observed in 22 primer pairs between two parentals, and only 5 primer pairs amplified in the -606 accession. The polymorphic amplification and the mobility in the parentals using several primers can be observed (Fig 1). The range of the amplification was from 120-330 bp.

Table 1. Summary of PCR amplification using 3 parentals (CIAT-606, Clone 44-3, CIAT 6294)

Primers designed	**97**
polymorphic (3 parentals)	**35**
Polymorphic (2 parentals)	**22**
Only 1 parental (CIAT606)	**5**
Monomorphic	**11**
Nonspecific	**11**
No amplification	**14**

Figure 1. Amplification using 22 primer pairs with DNA from CIAT-606, CIAT6294 and Clone 44-3.

In the entire F1 progeny of 215 individuals, 54 primer pairs were amplified: CIAT6294 x Clone 44-3. Some of them amplified in only one parental (CIAT-6294), while others amplified more than one allele. For this study one locus could be screened on a single polyacrylamide gel loading. A typical wide-sequencing gel with 96 well combs, loaded sequentially three times, was used to analyze the genotypes of 215 individuals simultaneously per locus combination, using silver-staining detection (Fig. 2). Silver staining provides a valuable alternative to the use of radioisotopes. It is rapid, sensitive and inexpensive, and can be used routinely and economically to detect a variety of PCR-based amplification products.

AFLP Results

An analysis was made of 64 combinations of primers with both parentals CIAT6294 and Clone 44-3 and both bulk of 20 resistant DNA and 20 susceptible DNA to spittlebug. Some fingerprintings showed low numbers of polymorphic bands between parentals, while others showed a good number. The combinations with polymorphic bands between both parentals and bulks were chosen, and four combinations were used (E-AAG/M-CTG, E-AGG/M-CTT, E-AAC/M-CAT and E-AGC/M-CTT) to amplify 20 individuals from F1 progeny because they showed a sufficient level of polymorphism and a high number of bands (Fig 3). In two combinations more than 30 polymorphic bands were observed. All 215 individuals were analyzed with these four combinations, and a total of 130 polymorphic bands were observed.

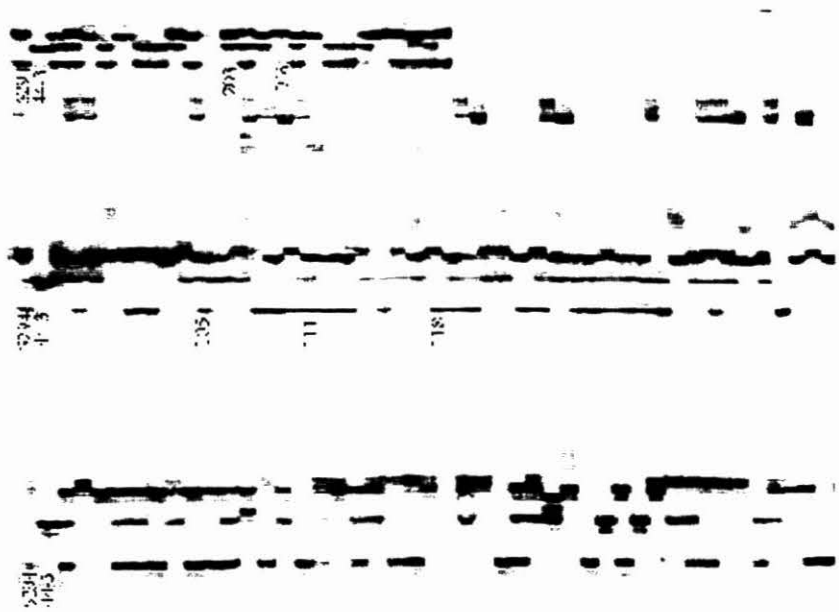

Figure 2. Panel of a silver-stained polyacrylamide gel showing analysis of F1 individuals at the GM90 microsatellite locus. Parental alleles (CIAT6494 and Clone 44-3) are identified at the beginning of each row.

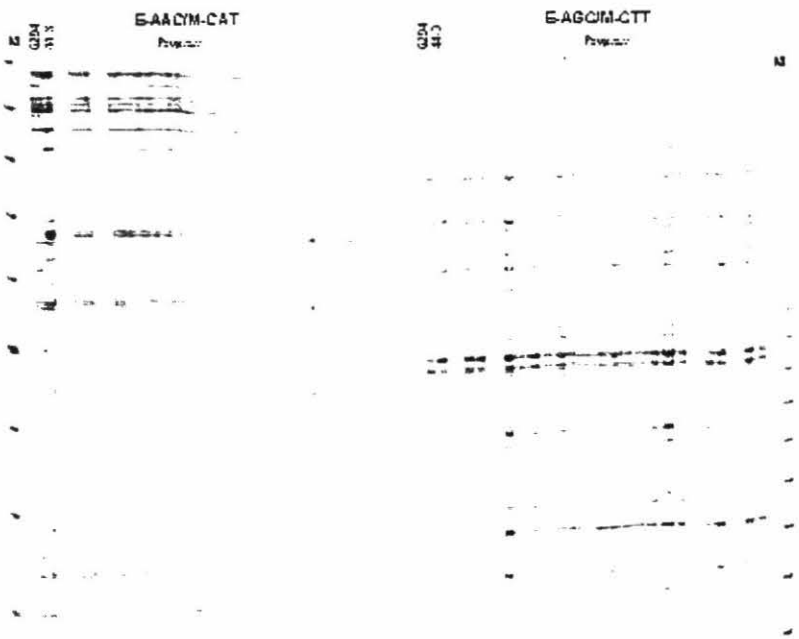

Figure 3. AFLP profiles of both parentals and 20 F1 individuals amplified with two different combinations. Line M is a 30-330 bp molecular marker.

Ongoing activities

- Construction of a molecular map in *Brachiaria* using microsatellites and AFLP markers anchored with RFLP clones from other grasses (Fig.4)

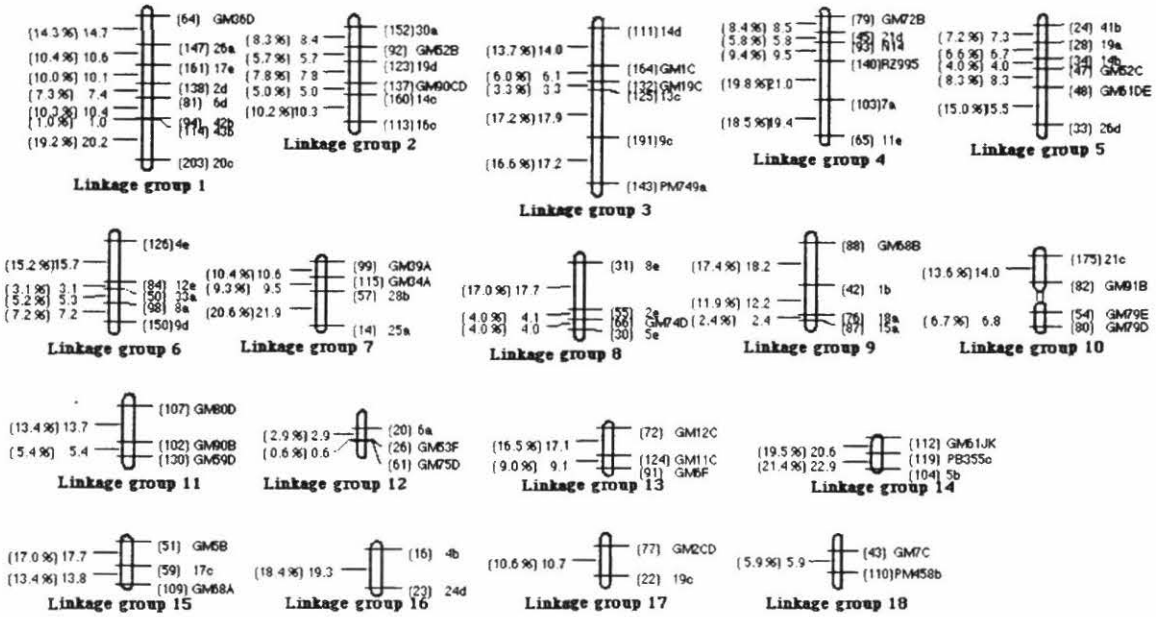

Figure 4. Preliminary *Brachiaria* framework map consisting of SSR, AFLP, SCAR and RFLP. (Lod = 6 and theta= .20)

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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

- A methodology for the reproducible genetic transformation of tepary bean was developed.
- Production of Friable Embryogenic Callus (FEC) in MCol2215 (Venezolana) and regeneration of whole plants. Efficient expression, in FEC of Mcol2215, of constructs containing an insect resistant gene through *Agrobacterium*-mediated transformation. Stable expression of same constructs in FEC and embryos of cultivar TMS 60444
- A colony of *Chilomima clarkei* established in Nataima (CORPOICA), Tolima, for permanent supply of eggs to perform bioassays at CIAT. Artificial diets developed and tested to rear *Chilomima clarkei*, under contained conditions, at CIAT
- Transgenic rice plants carrying the anti-fungal protein gene PAP were generated, plants with different levels of expression were selected, and will be evaluated for sheath blight resistance under greenhouse conditions.
- As part of the Science Park collaboration, CIAT assisted in the generation of transgenic sugar cane carrying viral resistance gene for ScYLV, and transgenic tomato targeting resistance to tomato fruitworm and budworm.
- CIAT obtained registration from the Colombian Biosafety Commission for the generation and importation of transgenic plants for germplasm development, as well as approval to conduct field trials with transgenic rice plants at CIAT experimental Station. A selected number of transgenic lines with resistance to RHBV, and F2 populations derived from crosses with different varieties will be planted in the field on November 2000. This trial is the first official field test of transgenic material in Colombia. .

2.1.1 Genetic transformation of *Phaseolus* beans

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Introduction

The common bean (*Phaseolus vulgaris* L.), has been recalcitrant to conventional *in vitro* plant regeneration methodologies, as has been the case with other grain legumes. This has delayed the application of recombinant DNA technologies in the breeding of this crop. Transgenic plants have been obtained only by the direct introduction of foreign DNA into the meristems through particle bombardment, thus overcoming the necessity of an *in vitro* regeneration protocol. Although transgenic plants carrying agronomically important genes have been produced severally using this methodology (Russell et al., 1993; Aragao et al., 1996), there is to date no transgenic common bean plant in the market worldwide. The reason for this may be that:

(i) Transformation methodologies available are still too inefficient; and/or
(ii) Stable expression of the transgenes in the obtained plants has not been as desired.

Agrobacterium mediated transformation has not been achieved in common bean as has been the case in one wild genotype of tepary bean (Dillen et al. 1997).

The main objective of our research is the development of efficient and reproducible genetic transformation protocols. For achieving this, both *Agrobacterium* and particle gun mediated transformation are being explored.

Methodology

Mature but not dry pods were used as sources of sterile explants (whole mature seeds without one cotyledon) for transformation experiments. Particle bombardment was carried out with the common bean genotype, Bayo Madero. The plasmid used was the pGV1040 which carries the NPTII, BAR and GUS genes. After bombardment, transformed tissue was selected in a culture medium containing 10 mg/l of phosphinothricin (PPT), the active ingredient of the herbicide Basta™. *Agrobacterium* transformation was carried out with the hypervirulent strains LBA4404 pTOK233 (Hiei et al. 1994) and C58C1 harboring different binary plasmids. Meristematic calli induction and plant regeneration were performed as described earlier (Mejía-Jiménez et al. 1998).

Results: Genetic transformation of tepary bean through Agrobacterium

A methodology for the reproducible genetic transformation of tepary bean has been developed. This includes the following steps:

(i) Sonication of the explants for wounding;
(ii) inoculation with hypervirulent Agrobacterium strains;
(iii) coculture at 22°C for 3 days;
(iv) induction of a meristematic callus for 1 month;
(v) selection of transformed meristematic callus tissue in a culture medium with hygromycin;
(vi) induction of bud differentiation of the selected calli; and
(vii) whole plant regeneration through micrografting.

This protocol is different in many ways from that developed by Dillen et al. (1997). The most important difference is the use of embryo axes attached to one cotyledon instead of pedicels of greenhouse growing plants as explants. The former is much easier to produce, sterilize and to handle in general.

Three transgenic plants from independent experiments have been produced. Although no southern blot has yet been done, the segregation of GUS + and GUS – progeny plants and the transfer of the GUS transgene by sexual crosses, confirms their transgenic character. Hygromycin resistant, GUS expressing meristematic calli and transgenic plants were produced only from the tissue of intraspecific hybrids of tepary bean: G40065xNI576 and G40022xNI576 (table 1). These hybrids involve the genotype NI576, the same that could be transformed by Dillen et al. (1997). G40065 and G40022 are two cultivated tepary bean accessions that we chose as the best meristematic callus forming genotypes from the screening of more than 20 different accessions of tepary bean.

Table 1. Stable GUS expressing meristematic callus tissue recovered after inoculation of tepary bean embryo axes with the Agrobacterium strain LBA4404 pTOK233 and transgenic plants obtained.

Genotype*	Inoculated Embryo Axes	Selected GUS + Clones**	Transgenic Plants Regenerated
G40025	80	0	
G40035	70	0	
G40022 x NI576	35	1	1
G40065 x NI576	43	0	
G40022 x NI576	48	4	
G40025	34	0	
G40022 x NI576	28	0	
G40025	80	0	
G40022	365	0	
G40065 x NI576	164	0	
G40065 x NI576	63	1	1
G40025	45	0	
G40022 x NI576	120	1	1
G40065	88	0	
G40065	392	0	
G40065	198	0	

* All are cultivated genotypes with the exception of NI576

** After a six-week culture on culture medium containing 30 mg/l hygromycin.

Although many wild common bean accessions (G10013, G10024, G12875, G12922, G12947, G23429, G23490, G23511A, G23652, G23653 and G27893) and cultivated (La Victoire, G3807, G18253, A295, G04090, G01853, Tamazulapa, BAT 93, G6413, G18255, G05706, G04449, G11071, G02997, Vax1, Vax2, Vax3, Vax4, Vax5, Vax6, C20, Jalo EEP 558, Olathe Pinto, Carioca, Dor 364, Bayo Madero, BAT477 and Ica Pijao) have been tested with this transformation methodology, no GUS expressing regenerable tissue has been obtained.

It is noteworthy that stable transformed GUS expressing meristematic callus could be obtained only from hybrids which included the genotype NI576 as male parent and not from other tepary genotypes (which also show high transient expression and form a m-calli of good quality). This would suggest that some nuclear genes of NI576 could be involved in the *Agrobacterium* transformation competence of this genotype.

Biolistic transformation of common bean

A methodology for the genetic transformation of common bean through particle bombardment of mature seed meristems is also being developed. This methodology involves the following sequential steps:

(i) Explant preparation for exposing the meristematic regions of the embryo axis to the microprojectiles;
(ii) bombardment with gold particles coated with DNA;
(iii) induction of a meristematic callus out from the bombarded explants;
(iv) selection of meristematic callus resistant to 10 mg/l PPT during 6 weeks;
(v) bud induction from the selected tissue; and
(vi) whole plant regeneration through micrografting of the induced buds.

Meristematic calli of the genotype Bayo Madero that resist concentrations as high as 10 mg/l PPT for 6 weeks (which are lethal to control explants), are being obtained from these experiments. These calli also show a weak blue coloration in response to the GUS test, whereas control explants remain white. Plants from these calli will be regenerated and analyzed for the presence of the transgenes in the next few weeks.

Transfer of transgenes from tepary to common bean through interspecific crosses

Another possibility for obtaining transgenic common bean plants is to initially transform tepary bean through *Agrobacterium*, and then to transfer the transgenes to common bean through interspecific hybridization. The use of selectable (antibiotic resistance) or screenable (GUS) marker genes could facilitate and speed up this process, by making it easy to select the plants which contain the transgenes in the progeny of the crosses.

The hybridization as well as the introgression of genes from tepary to common bean through interspecific crosses has been difficult. The use of advanced congruity backcross (CBC) hybrid lines of both species, which we developed in the past years (Mejía-Jiménez et al. 1994), could facilitate the hybridization process. These advanced CBC lines were produced by the method of Haghighi and Ascher (1988), by backcrossing the hybrids obtained several times alternately with genotypes of both species.

Seventeen hybrids of the cross CBC7 x (G40022xNI576)-T2+ (GUS positive T2 transgenic plant) were produced. However they were totally sterile and could not be backcrossed with common bean genotypes or CBC hybrids, to produce viable fertile hybrids. It was necessary to make the following several complex crosses before viable CBC hybrids with a *P. vulgaris* cytoplasm could be obtained and which express the GUS transgene:

(i) first develop advanced CBC lines with a P. acutifolius cytoplasm;
(ii) cross the GUS+ transgenic tepary bean plant as male parent to them; and finally
(iii) cross the GUS+ CBC hybrid to an advanced CBC hybrid with P. vulgaris cytoplasm.

More than 40 GUS-positive CBC hybrids having the cytoplasm of P. vulgaris have been obtained, many of them are expected to be fertile.

In previous crossing experiments we have been able to establish that fertile CBC hybrids with a *P. vulgaris* cytoplasm can be crossed (as male or female parent) to every common bean genotype. Thus, it will be possible to transfer the transgene to any common bean variety by conventional breeding methodologies.

Transfer of traits involved in competence to *Agrobacterium* transformation from tepary to common bean trough interspecific hybridization

Most common bean genotypes of agronomic importance tested so far show little or no transient GUS expression after inoculation with hypervirulent *Agrobacterium* strains, like the LBA4404 pTOK233. This is in spite of the fact that many mono- and dicotyledons plant species have been transformed with this strain. This shows the lack or reduced competence of the regenerable tissues of these cultivars to *Agrobacterium* transformation Although our experiments have shown that the use of tepary bean to introgress transgenes into common bean is possible, it can take more than three years to develop a transgenic common bean variety by this way. It would be faster if the Agrobacterium-transformation competence (ATC) genes could be transferred through interspecific hybridization to important cultivars of common bean to produce ATC common bean genotypes. Several interspecific CBC hybrid lines have been produced, which include ATC tepary bean genotypes in their pedigrees. From these, several F_3 lines are being tested for increased competence to *Agrobacterium* transformation. Once developed, the ATC common bean line can be used to transfer the ATC genes to elite common bean genotypes through conventional breeding.

Conclusions

Advances have been achieved in both *Agrobacterium*-mediated and biolistic transformation of tepary and common bean. Both methodologies should be further developed since they offer different advantages. While biolistics is a more genotype-independent methodology, *Agrobacterium* allows the achievement of higher transformation efficiencies. The latter also permits the introduction of DNA fragments of the desired size to the plant cells (which in some cases can be more than 100 kb) and also

the insertion of one or few copies of the transgenes. This can lead to more predictable patterns of expression of the transgenes.

Future plans

- To regenerate and analyze plants from the GUS positive, PPT resistant calli of Bayo Madero, obtained from the transformation with the particle gun.
- To further optimize the genetic transformation of competent genotypes through Agrobacterium
- To select hybrid lines with an increased competence to *Agrobacterium* transformation from the interspecific crosses which include the tepary bean genotype NI576
- To further develop the biolistic transformation methodology in order to obtain a more genotype-independent transformation methodology
- To initiate the transformation with other important agronomic genes

Training conducted during 2000

Nathalie Colpaert, Institute of Plant Biotechnology for Developing Countries, University of Gent, Belgium. Training in genetic transformation in Phaseolus Beans. 6 weeks

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2.1.2 New Genetic Constructs for Cassava Transformation

L.I Mancilla, Y.J Ladino, P Chavarriaga and W.M Roca

Introduction

The use of antibiotic resistance genes in transgenic plants, suitable for human consumption, is currently a major public concern, even though the safety of these markers has been tested and proven for several years. Non-antibiotic based selection of transgenic plants is now widely accepted (Joersbo *et al.*, 1998). We assembled genetic cassettes with the *man*A gen(mannose 6-phosphate isomerase, from *E. coli*) to use it as a selection marker for cassava transformation. Additionally, we cloned the *cry*1A(b) in front of the cassava-specific promoter CsVMV (from the Cassava Vein Mosaic Virus; Verdaguer *et al.*, 1996; ILTAB, 1997) to enhance expression of this gene in cassava. CsVMV directs a strong and constitutive expression in a large variety of plants like tobacco, cassava, rice and tomato.

Methodology

Using standard cloning techniques, the *man*A gene was placed between the 35S promoter and termination signals. The gene was then placed next to the *gus*-intron and *cry*1A(b) genes (see Figure 1). Similarly, in a different plasmid, the *cry*1A(b) gene was placed in front of the CsVMV promoter and terminated by the NOS3' signal (see Figure 2).

Results and Discussion

A new plasmid, pSGMC (Figure 1), containing three genes (*gus*-intron, *cry*1A(b) and *man*A) within the left and right borders (T-DNA) was constructed. pSGMC is 12.49 kbp long, and contains the spectinomycin-streptomycin resistance gene for selection in bacteria. pSGMC was introduced into *Agl*-1 and C58C1 *Agrobacterium* strains by electroporation for transient gene expression assays in cassava. The assays showed that the *gus*-intron gene is expressed in the embryogenic cells, which confirms that the T-DNA is properly transferred to them. This plasmid will be used in larger transformation experiments for stable transformation.

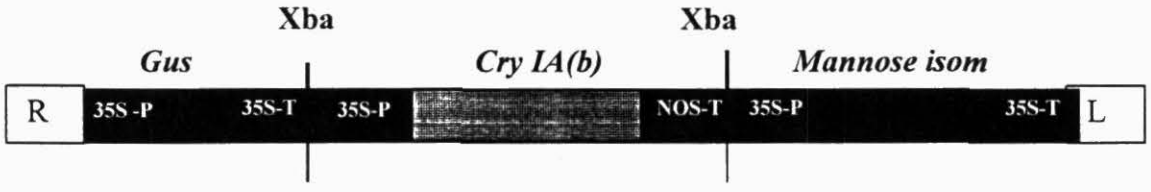

Figure 1. Diagram of plasmid pSGMC containing *gus*-intron, *cry*1A(b) and *man*A.

The cry1A(b) gene was placed in front of the CsVMV promoter in the new plasmid pILCry18, which is 15067 bp long and contains the *npt* II gene under the NOS regulator sequences (Figure 2). pILCry18 actually serves as an intermediary plasmid from which the *cry* gene, regulated by the cassava-specific promoter, may be moved to other cassettes containing the marker genes *man*A and *gus*-intron.

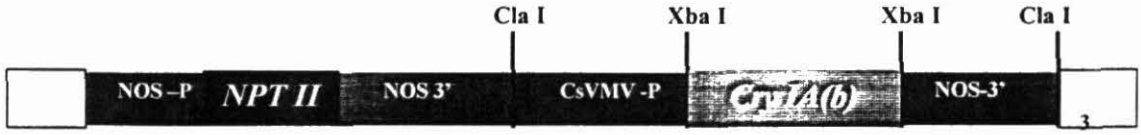

Figure 2. Diagram of the pILCRY18 plasmid containing the *cry*I A (b) gene under regulation of the CsVMV promoter.

Conclusions

- Two new gene cassettes, containing new selectable marker genes and cassava-specific promoters, were developed for cassava transformation.
- The T-DNA of pSGMC is transferred and expressed (the *gus*-intron gene) in cassava embryogenic cells.

Future Activities

- Set up large transformation experiments using pSGMC and cassava embryogenic tissues
- Move the *cry*1A(b) gene, with its regulatory sequences, from pILCRY18 to other cassettes containing the *man*A and *gus*-intron genes. Set up transformation experiments with the new construct.

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2.1.3 Expression of recombinant *CRY* 1A(b) protein and developing artificial diets to rear the cassava stem borer *Chilomima clarkei.*

L.I. Mancilla, C. Ramirez, C.J. Herrera, P. Chavarriaga and W.M. Roca

Introduction

To evaluate the effect of the *CRY* recombinant toxin (from the gene *cry*1A(b)) on cassava stem borer (*Chilomima clarkei*) larvae, we cloned the *cry*1A(b) gene into a vector (pGEX-5X-1) for expression of recombinant proteins in *E. coli*. The protein obtained from the bacterial cultures will be used to evaluate its toxic effect on stem borer larvae using synthetic diets that are being tested. To guarantee enough supply of stem borer larvae, an artificial colony is being established at Nataima (Tolima) in collaboration with CORPOICA.

Methodology

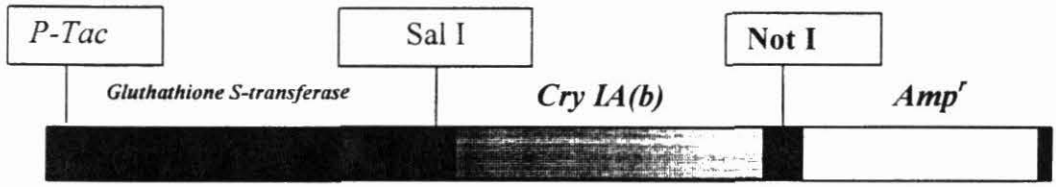
The *cry*1A (b) gene was cloned into pGEX-5X-1 expression vector using standard cloning techniques. The new plasmid pGEXCry has the entire coding sequence of *cry*1A(b) attached to the 3'-end of the Glutathione S-transferase gene sequence. The recombinant protein will then be expressed as a fusion protein (Figure 1). pGEX-5X-1 was introduced in *E. coli* via electroporation, and the cells grown in liquid medium, after induction with 1mM IPTG, to produce the fusion protein. The level of protein expression was evaluated in 12 % polyacrilamide electrophoresis gels

For the methods to establish an artificial colony, and to rear *Chilomima* under laboratory conditions, please refer to IP-3 report.

To develop the diets, the ingredients described in Table 1 were mixed and, after sterilizing by autoclaving, approximately 25 ml were poured in 50 ml plastic recipients. Two or three larvae were placed in each recipient and stayed in darkness in the laboratory. Evaluations were done daily to check survival of larvae.

Results and Discussion

Recombinant CRY Protein Expression
No differences between induced and non-induced bacteria were observed. More induction time (beyond 4 hours) may be necessary to produce larger amounts of the fusion protein in *E.coli*. Additionally, assays for the expression of the Glutathione S-transferase gene will serve as indicators of expression of the recombinant protein.



pGEXCry (7.72 Kbp)

Figure 1. Diagram of construction with *cry*1A(b) gene in pGEX-5X-1 expression vector for obtain recombinant protein.

Artificial Diets to Rear C. clarkei.

A colony to rear the stem borer under laboratory and greenhouse conditions was established in Nataima, Tolima, in CORPOICA's experimental station. *C. clarkei* larvae and adults were collected from cassava cultivars in the region. The whole cycle of development was completed under greenhouse conditions (see report of IP-3 project). Eggs laid by adults under laboratory conditions, on paper or nylon screens in glass jars, are being collected and sent to CIAT to test two artificial diets. These experiments are still running, although preliminary results showed better results with Miskimen-Pan diet (Table 1).

Table 1. Composition of two diets to rear *Chilomima clarkei*.

2.1.3.1.1 Component	**Diet 1** g/100 ml (CIAT)	**Diet 2** g/100ml (Miskimen-Pan)
Steam Cassava flour	8.0	12.0
Wheat germ	4.0	None
Bacto-agar	4.0	3.5
Choline chloride	0.1	None

Conclusions

- The cry1A(b) gene has been cloned in an expression vector to produce protein for bioassays (toxicity tests with *C. clarkei*).
- Artificial diets are being tested to rear *C. clarkei*, under contained conditions, at CIAT.
- A colony of *C. clarkei* has been established in Nataima, Tolima, for permanent supply of eggs to perform experiments at CIAT.

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2.1.4 Effect of mannose on the growth of friable embryogenic callus (FEC) of cassava (cv. Venezolana)

Y.J. Ladino, P.Chavarriaga and W.M. Roca

Introduction

The sugar mannose is being used for selection of transgenic plants instead of antibiotics (Joersbo and Okkels, 1996) due to the fact that the latter is currently a major public concern. The gene *man*A, from *E. coli*, encodes the enzyme mannose-6-phosphate isomerase, which allow cells to grow on media containing mannose as a carbon source. Plants do not normally contain the *man*A gen, so if *man*A is introduced through transgenesis in plant cells, and expressed correctly, mannose can be used as a selective agent to recover transgenic plants. We tested the effect of mannose on the growth of FEC of Cassava to estimate the levels at which mannose could be used for selection of transgenic cassava.

Methodology

We grew FEC on GD medium (Gresshof and Doy, 1974) containing variable concentrations of mannose (0 to 2%), sucrose (0 and 2%), or both (2% sucrose and 0,5% mannose). The medium was supplemented with 50 uM Picloram as growth regulator. Initial and final fresh weight of FEC was measured in each treatment. Each treatment had two replicas, with six repetitions per replica (six FEC clusters per plate with two plates per treatment).

Results and Discussion

The results are summarized in Figure 1. There was an apparent effect of mannose on the growth of FEC compared with controls containing sucrose (C1, 2% sucrose). Controls almost doubled the difference between final and initial fresh weight, which suggests that mannose, at concentrations below 2% in the medium, may reduce the growth rate of FEC, although not halt it completely. No mannose and no sucrose (T1) in the medium had apparently the same effect as 2% mannose (T5), suggesting that concentrations above 2% mannose may be detrimental for cell growth. Adding a little sucrose (0,5%) to a medium containing 2% mannose (C2) seemed to have the same effect as 0,5 to 1% mannose alone

in the medium (T2 to T4). For selection of cassava transgenic cells, mannose at concentrations between 1 and <2%, or 2% mannose plus 0,5% sucrose, may be desirable.

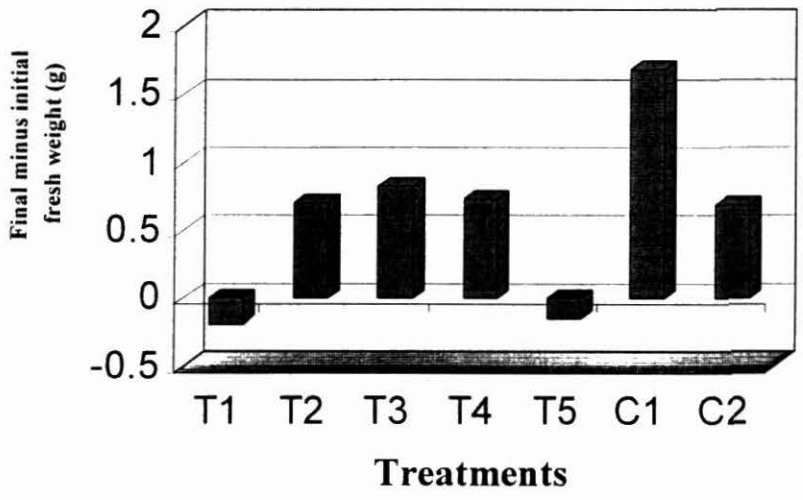
Conclusions

- Mannose, as the sole carbon source in the medium, slows the growth of FEC
- Concentrations larger than 2% mannose in the medium may halt growth of FEC
- Mannose plus sucrose (2% and 0,5% respectively) also seem to slow the growth of
- FEC
- Concentrations of mannose between 1 and <2% may be used to select transgenic cells of cassava expressing *man*A

Future activities

- Fine-tune the concentration of mannose for selection of transgenic cells
- Determine viability of cells after exposure to mannose

Figure 1. Effect of mannose on the growth (fresh weight difference) of FEC from cassava cultivar Venezolana. T1= no mannose and no sucrose; T2= 0,5% mannose; T3= 0,7% mannose; T4= 1% mannose; T5= 2% mannose; C1= 2% sucrose; C2= 2% mannose plus 0,5% sucrose.



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2.1.5 Transformation of cassava (cvs. Mcol2215 and TMS60444) with a gene for insect resistance

Y.J Ladino, V. Segovia, P. Chavarriaga and W.M. Roca

Introduction

The stem borer (*Chilomima clarke*; Lepidoptera) has become one of the major pests affecting cassava yields in Colombia. Until recently it was assumed that the stem borer was restricted mainly to Northern (Atlantic Coast) and Eastern (Llanos) Colombia. However, more recent field evaluations have shown that the stem borer has reached several cassava fields in the Department of Tolima (Magdalena Valley; see IP-3 report). The fast spreading of the stem borer is threatening other plantations where it has not been reported yet (i.e. in Valle del Cauca and Quindio). We are trying to introduce, through transgenesis, insect resistance genes into two cassava varieties: Mcol2215 and TMS60444. The former is a variety selected by farmers of the Northern Coast, and the latter is a model variety for which efficient transformation has been achieved.

Methodology

We used Friable Embryogenic Callus (FEC) of both varieties to introduce the plasmid pBIGCRY (see Annual Report 1999), carrying a gene from *Bacillus thuringiensis* (*cry*1A(b)), one selectable marker (*npt*II), and one scorable marker (*gus*-intron). The *cry*1A(b) gene confers resistance to Lepidoptera, and stem borers, in several crops. *Agrobacterium tumefaciens* was used as the vector to transfer the genes into cassava. Selection with the antibiotic Paramomycin is being applied to potentially transformed FEC. Although the target variety is Mcol2215, we used TMS60444 to speed up the process of introducing the gene, checking its expression and determining if it confers resistance to *C. clarkei*.

Results and Discussion

We obtained FEC from TMS60444 expressing the gus-intron gene after 15 to 20 days of selection with Paramomycin. These calli were freed of selection to produced somatic embryos at cotyledon stage. Some of these embryos still showed gus expression, although less intense than with FEC, after over 20 days of no selection. More than 100 potentially transformed embryos (at cotyledon stage) are on regeneration media, without selection, to produced whole plants.

Transformation of Mcol2215 has proven more difficult. Although several experiments have been performed, and transient expression of the gus-intron gene is quite efficient in FEC from this variety, no stable-transformed calli have been obtained. FEC from Mcol2215 grows slower than that of TMS60444, under non-selective conditions. On selection medium (with Paramomycin), FEC proliferation seems to be halted. Viability

tests of FEC that has been freed of selection (for more than a month) indicated that cell survival after antibiotic treatment is minimal for Mcol2215.

Conclusions

- Stable transformation of TMS60444 using the plasmid pBIGCRY may be achievable, judging by the stable expression of the gus-intron gene in FEC and cotyledon-stage embryos of this variety

- Transformation of Mcol2215 with pBIGCRY has been more elusive. Sensitivity to treatment with antibiotics may be an obstacle to obtain stable transformed FEC with this variety

- The media may need to be enriched (nitrogen sources) to help weak, slow-dividing,potentially transgenic cells grow faster and survive after selection

Future Activities

- Test lower concentrations of antibiotics (paramomycin, geneticin) to select transformed FEC of Mcol2215
- Use positive selection, with Mannose (*man*A gene), as an alternative to antibiotics for selection of transgenic FEC of Mcol2215
- Enrich media with caseine hydrolysate or aminoacids (asparagine, tryptophan) to help FEC from Mcol2215 to develop further after selection

2.1.6 Control of RHBV (Rice Hoja Blanca Virus) through nucleoprotein mediated cross protection in transgenic rice

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[1] IP4 Project,

Introduction

The main goal of this project is to provide new source(s) of resistance to complement the single breeding resistance source present in most of the commercial varieties grown in Latin America nowadays. This breeding source is conferred by one or two genes, and does not protect plants at ages younger than 25 day-old. The project aims to transform rice with novel gene(s) for RHBV resistance, and to incorporate these genes into Latin American commercial varieties or into genotypes to be used as parents in breeding. Previous reports described the generation using particle bombardment and selection of Cica 8 transgenic plants carrying the nucleoprotein (RHBV-N) viral gene. 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. Advanced generations

of transgenic lines with stable RHBV resistance has been selected. In contrast, the Cica 8 non-transgenic control is highly susceptible throughout the whole life cycle, showing severe disease development and most plants die at 60 days after inoculation. Last year, results also suggested that the resistance conferred by the N transgene towards RHBV disease is expressed independently of the genotype background. The transgenic resistance 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). Results showed that the non-transgenic F1s control plants were susceptible, whereas the transgenic F1s were resistant even when inoculated at 10-day-old. These results suggested that the protection conferred by the RHBV-N transgene is inherited and expresses independently of the genotype background, and that the transgene could be used to complement the natural resistance source. A total of 421 selected transgenic lines representing various generations, and F2 populations derived from crosses with FB007, Oryzyca 1, Iniap 12, and Cica 8 will be planted in the field on November 2000. These lines will be evaluated for RHBV resistance and agronomic traits following International as well as the Colombian environmental biosafety regulations at Palmira experimental station. The approval for field testing by the Colombian Biosafety Committee was issued on September 2000. Following it is reported the characterization of mode expression of the transgenic resistance conferred by RHBV-N, and the progress generating transgenic rice containing the RHBV non-structural 4 (NS4) gene from the RNA 4.

Characterization of RHBV-N resistance in transgenic plants

The level of resistance conferred by the RHBV-N viral gene was evaluated by scoring the percentage of leaf area diseased, severity of symptoms, and vigor. Evaluations were conducted once a week starting 5 days after removal of viruliferous insect vectors, up to 54 days after inoculation. To conduct this study, preliminary 25 transgenic lines represented by 8 plants each were evaluated at 15 days after germination. Of these, eight lines were selected showing high vigor. These lines were used to study the effect of the plant age on the level of transgenic resistance. Two disease pressures were used, intermediate disease pressure (colony of 65% of virulence) and high disease pressure (colony of 70% of virulence), and each plant was inoculated with four insects per plant.

Line A3-49-60-12-3-3 showed the highest level of resistance throughout the whole life cycle. Between 74% to 81% of the plants did not show any disease symptoms when inoculated either at 14 days or 28 days of age, and only a 22% of the plants showed more than 25% of the leaf area affected when inoculated at 14-day-old (Table 1). In contrast, Cica 8 control showed between 70% of the plants with severe disease symptoms at 14-day-old (Table 1). Line A3-49-60-4-5-8 showed intermediate level of resistance at 14-day-old and 71% of the plants without symptoms at 28-day-old (Table 1). About 70% of the plants of line A3-49-60-19 had less than 25% of leaf area affected at 14-day-old, whereas Cica 8 showed 100% of plants highly diseased (Table 2). In general, the level of transgenic resistance increased at 28-day-old when yet Cica 8 control is still highly susceptible (Table 1 and 2). Sister lines A3-60-12-3-1 (susceptible) y A3-60-12-3-3 (resistant) showed different disease reaction indicating that the resistant phenotype is still

segregating at the T4 generation or gene silencing maybe affecting the expression of the RHBV-N gene in some of the plants. A comparative Southern blot analysis using methylation sensitive and methylation insensitive restriction enzymes will be conducted to elucidate if gene silencing is involved in the lost of gene expression.

Analysis was conducted to compare the mode of phenotypic expression of RHBV-N transgenic resistance respect to the standard breeding resistance source derived from Colombia 1. Fifteen day-old plants from the varieties IR8, Cica 8, Oryzica 1, Oryzica Llanos 5, Colombia 1, Fedearroz 50 and Fedearroz 2000 were inoculated in the field using plots of four rows per variety, and a colony of 70% of virulence. All the seven varieties, including the highly resistant variety Fedearroz 2000, showed disease symptoms. Although Fedearroz 2000, had the lowest percentage of plants with symptoms and a reduced leaf area diseased. When symptoms developed, including on those of highly resistant varieties (i.e. Fedearroz 2000), symptoms appeared as the typical white leaf strip or white spots throughout the leaf similarly to the highly susceptible varieties IR8 and Cica 8. In contrast, when diseased symptoms developed in resistant transgenic plants they sometimes appeared as necrotic spots resembling hypersensitive reaction. Often times these symptoms were mainly observed on the original leaves were the insects fed onto, but new leaves appeared free of symptoms giving a recovery phenotype to the plant. This type of resistant phenotype suggest that the mode of action RHBV-N resistance might be restraining the virus replication or mobility throughout the leaf once the plant cells are infected.

Table 1.- Disease resistance on T4 transgenic plants inoculated at 14 day-old and 28 day-old

Line	Age at inoculation	Leaf area affected (% plants) 0	>0-25	>25-100
A3-49-60-12-3-3	14	74	4	22
A3-49-60-4-5-8	14	54	0	46
Cica 8 (control)	14	22	9	70
A3-49-60-12-3-3	28	81	19	0
A3-49-60-4-5-8	28	71	12	16
Cica 8 (control)	28	33	0	66

Between 22 to 24 plants were evaluated per line per total of three Replications

Table 2.- Disease resistance on T2 transgenic plants *inoculated* with high pressure at 14 day-old and 28 day-old

Line	Age at inoculation	Leaf area affected (% plants) 0	>0-25	>25-50	>50
A3-49-60-10	14	47	0	0	53
A3-49-60-13	14	25	4	12	58
A3-49-60-19	14	53	12	18	18
A3-49-56-15	14	9	13	17	61
A3-49-60-12-3-1	14	7	0	0	93
A3-49-101-18-19-2	14	15	0	8	77
Cica 8	14	0	0	0	100
A3-49-60-13	28	44	26	17	13
A3-49-56-15	28	29	21	17	33
Cica 8	28	0	0	0	100

Between 16 to 24 plants were evaluated per line per total of two replications

2.1.7 Generation of transgenic rice containing the RHBV non-structural 4 (NS4) gene from the RNA 4

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Earlier studied conducted by Lee Calvert and coworkers at CIAT using specific antiserum had indicated that the major NS4 protein is expressed in rice plants, but not in any of the instars or adult plant-hoppers (*Tagosodes orizicolus*). There also appears to be a control mechanism that allows expression in the plant but not in the planthopper, since the complete RHBV genome could be isolated from viruliferous vectors. In contrast the N protein is expressed in both the plant and the insect vector. It is inferred from the differential expression of these proteins that the major NS4 protein may has a function that is needed in the plant but not in the plant-hopper. The differential plant-insect NS4 expression, and the similarity of NS4 sequence with well characterized helper proteins described for other insect transmitted viruses, suggest that NS4 might be involved in the RHBV transmission from the plant to the plant-hopper, or in the virus movement from cell to cell. The strategy for the expression of the RNA4 in transgenic rice is to determine the function of the major NS4 protein and study the potential for a novel and different method of producing viral resistant plants.

The RHBV NS4 gene in sense and anti-sense orientations 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 was also cloned into vectors carrying the hygromycin-cat 1 intron gene from Peter Waterhouse's laboratory at CSIRO, Australia (Table 3).

The *indica* rice varieties Cica 8 (control for transformation efficiency), Palmar, and Cimarrón are being used as targets. Palmar (high grain/ milling quality) and Cimarrón (high yielding variety) are commercial varieties from Venezuela highly susceptible to RHBV. Part of the project is funded by the Centro Tecnológico Polar, Venezuela. A total of 24 transgenic plants carrying the NS4 sense orientation, and 25 plants carrying NS4 anti-sense orientation driven by the 35S CaMV promoter were produced from independent events by *Agrobacterium* mediated transformation using the Agl1 strain. Southern analyses using *Bam HI* or *Eco RI* which excise the complete NS4 gene in sense or antisense orientation, or using *Sal I* which does not cut the gene cassette within the right and left borders were used. Results indicate that 100% of the plants had integrated single non-rearranged copy of the gene (Figure 1). Northern and Western analyses will be conducted and RHBV resistance evaluations will proceed next season.

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

Name	Gene	Promoter	Vector/orientation	Other genes
pIC001	NS4	35S CaMV	PC1300/sense	hpt, Kan
pIC003	NS4	35S CaMV	PC1300/asense	hpt, Kan
pIC002	NS4	35S CaMV	PC1301/sense	hpt, Kan, GUS-intron
pIC004	NS4	35S CaMV	PC1301/asense	hpt, Kan, GUS-intron
pIC005	NS4	Ubiquitin	NT168/sense	--
pIC006	NS4	Ubiquitin	NT168/asense	--
pIC007	NS4	Ubiquitin	PWBVec8/sense	hpt-cat intron
pIC008	NS4	Ubiquitin	PWBVec8/asense	hpt-cat intron
pIC009	NS4	35S CaMV	PWBVec8/sense	hpt-cat intron
pIC010	NS4	35S CaMV	PWBVec8/asense	hpt-cat intron

2.1.8 Foreign genes as novel sources of resistance for fungal resistance

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Introduction

The fungal complex composed *Rhizoctonia solani* (sheath blight), *Helmithosporium, Rhincosporium*, and *Sarocladium* is already causing important rice yield losses in the Southern cone of South America and increasing spreads had been reported in Colombia, Mexico and Venezuela. All rice varieties are susceptible and there are not known sources of stable genetic resistance for these diseases in rice. In the case of sheath blight, IRRI had placed a major effort in developing biological control strategies for this disease without success either. At present, the control of this complex mainly depends on use of fungicides (Dr. Fernando Correa, CIAT Rice Pathologist, Cali, Colombia, personal

communication). Recently, FLAR suggested CIAT (Dr. Peter Jennings, personal communication), to develop molecular strategies for incorporating resistance to this fungal complex. However, very little is known about the interaction between the rice and these pathogens in order to direct specific resistance strategies for each of these fungi. Of the four, the plant-pathogen interaction with *Rhizoctonia solani* is the better known.

Work conducted by another principal investigator of this project (Dr. Nilgun Tumer, Biotechnology Center at Rutgers University, USA) showed that a pokeweed antiviral protein (PAP), a 29-kDa protein isolated from *Phytolacca americana* (a weed naturally found from USA to Argentina), has a ribosome-inactivating ability. Mutated versions of PAP gene has potent antifungal activity (Zoubenko et al., 1997). Homozygous progeny of transgenic tobacco plants expressing these PAP genes displayed resistance to the fungal pathogen *Rhizoctonia solani*. Transgenic PAP potato showed protection against *Phytophtora infestans*, and transgenic PAP turfgrass are resistant to various fungal pathogens. These results suggest the possibility of designing molecular strategies for incorporating fungal resistance by introgression of mutant PAP gene(s) in transgenic rice plants. Here we report the progress made during the first year of this project.

Production of rice plants carrying various versions of PAP gene

Indica rice varieties Cica 8 (control for transformation efficiency), Palmar, Cimarrón, and Fundarroz PN1 are used as targets. Palmar and Cimarrón shows high and moderate tolerance to sheath blight, whereas Fundarroz PN1 and Cica 8 are highly susceptible to sheath blight. The strategy includes to evaluate the mode of action of PAP in highly susceptible as well as in tolerant sheath blight genotypes, to determine if PAP could increase the level of protection. This project is supported by the Centro Tecnológico Polar, Venezuela. To generate new point mutations in the PAP gene, a rapid change site directed mutagenesis kit from Stratagene was used. Gene constructs carrying various mutant versions of the PAP gene, were placed in vectors driven by 35S CaMV promoter or maize ubiquitin promoter, and using hygromycin resistance as gene selection.

Eight new PAP mutations were generated directed to change the aminoacids 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 are being transformed into tobacco first to check the gene expression and toxicity before using them for rice transformation.

Two mutated versions of PAP (I deleted and II) already tested for no toxicity in turfgrass (another monocot species) are being 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 plasmids had been used successfully by Waterhouse to transform 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.

A total of 35 independent transgenic events carrying the PAPI deletion mutant gene, and 50 independent transgenic events carrying the PAPII gene had been generated up to now using the *Agrobacterium* strain Agl1. A first set of plant tissue was sent to Rutgers this summer for analysis and plants with PAP gene expression were identified based on Western analysis (Figure 1). PAP expressing plants will be evaluated for sheath blight resistance under greenhouse conditions, while detailed molecular analyses are being conducted to determine the number of gene copy and patterns of integration into the rice genome.

Table 1.- New mutations generated in PAP gene.

Name in Plant Vector	Name in Yeast Vector	Mutation	AA Change
NT296	NT299	PAPI Del	I4M
NT298	NT300	PAPI Del	T18M
NT317	NT311	PAPI Del	I13M
NT319	NT312	PAPI Del	V8M
NT	NT	PAPI Del	Y16M
NT	NT	PAPI Point	Y16A
NT	NT	PAPI Point	Y16S
NT	NT	PAPI Point	Y16Phe

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

Name	GEN	Promoter	Vector/orientation	Other genes
NT178	PAPId	Ubiquitin	NT168	--
NT301	PAPId	Ubiquitin	NT294	GFP, hpt-cat intron
NT303	PAPId	Ubiquitin	PWBVec10a	GUS, hpt-cat intron
NT306	PAPId	Ubiquitin	PWBVec8	hpt-cat
RT126	PAPII	Ubiquitin	NT168	---
NT302	PAPII	Ubiquitin	NT294	GFP, hpt-cat intron
NT304	PAPIl	Ubiquitin	PWBVec10a	GUS, hpt-cat intron
NT305	PAPII	Ubiquitin	PWBVec8	hpt-cat

Figure 1.- Western blot analysis of transgenic Palmar carrying the PAPI deletion mutant gene. Analysis showed plants with various levels of PAP expression

2.1.9 Genetic transformation efficiency of rice using particle bombardment or various Agrobacterium strains

E. Tabares, L.O. Duque, G. Delgado, L.F. Fory , Z. Lentini

Introduction

Previous year, we reported the results on the optimization of *Agrobacterium* mediated transformation of local rice varieties using the LBA 4404 (pTOK233) strain-plasmid combinations. It included *indica* rice (CICA 8, IR-72, INIAP 12) adapted to irrigated (flooded) conditions, and *japonica* rice (CT 6241, CT 10069, and Lastisday-Fofifa) adapted to acid soils (savanna) and highland (hillside) environments. 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). Following is reported the phenotypic characterization of T0 and T1 plants, a comparison of transformation efficiency between LBA 4404 and AGL1 strains, particle bombardment. Currently the optimized protocol using strain Agl1 is being use to incorporate NS4 sense/anti-sense genes for RHBV and PAP genes for fungal resistance in *indica* rice reported in section 2.1.6 and 2.1.7.

Agronomic characteristics of T0 and T1 *Agrobacterium* derived transgenic plants

Agronomic traits of T0 and T1 transgenic plants generated with LBA4404 (pTOK233) were evaluated in the greenhouse. Results indicated that differences were not seen neither on flowering, nor on plant height between the transgenic lines and the corresponding control (Table 1). Variation respect to control were noted on the number of tillers, fertility and total grains weight per plant. Some plants showed sterility at T0, but T1 plants derived from selected T0 showed no difference on fertility and grain weight respect to control (Table 1).

Table 1.- Agronomic traits of CT10069, CT6241, and CICA 8 transgenic plants generated by *Agrobacterium* mediated transformation.

Genotype	Generation[1]	Flowering (days)	Height (cm)	Tillers (cm)	Fertility (%)	Total grain weight per plant (g)
CT10069	T0 (1-18)	59 - 69	101 - 130	3 - 18	2.2 - 94	0.6 - 18
	Control	60 - 67	107 - 128	5 - 15	90 – 95	19– 20
CT6241	T0 (1-24)	51 - 69	80 - 104	4 - 15	0.4 - 92	0.7 - 15
	Control	53 - 70	85 - 100	5 - 12	91 – 93	15 – 17
Cica 8	T0 (1-3)	61-85	60-66	11-15	0-2	2-4
	T0 (4-43)	62-96	65-80	5-17	63-87	4-16
	Control	90-93	70-80	7-10	79-89	13-19
	T1 (7-41)	ND	68-75	10-18	87-97	18-39
	Control	ND	70-75	14-16	94-95	30-36

Genetic transformation efficiency using particle bombardment or various Agrobacterium strains

Transgenic plants from the *indica* varieties Cica 8, Palmar, Cimarrón, and Fundarroz PN1 were generated using the *Agrobacterium* strains LBA4404 (Hei et al., 1997) or Agl1 (Wang et al., 1997) carrying either of the plasmids pTOK233 (hpt-gus intron genes, Hei et al., 1997), pCambia 1301 (hpt- gus intron-NS4 genes), or pWBVec 8 (Wang et al., 1997) (hpt cat intron-NS4 or PAP genes). No major differences were noted in the percentage of hygromycin resistant plants regenerated per initial callus agro-infected (Table 2). Between 50% to 100% of the plants regenerated had integrated the transgenes as indicated by the Southern blot analysis, giving a transformation efficiency

of 13% to 26% (Table 2). In contrast, about 6 to 9 fold increase in the regeneration of hygromycin resistant plants per explant was noted, and the transformation efficiency was 14 to 17 times higher when using *Agrobacterium* respect to biolistic for *indica* types (Table 2). The transformation efficiency of *indica* with *Agrobacterium* is similar to that of *japonica* with particle bombardment (Table 2). In general, plants generated via *Agrobacterium* showed simple-non rearranged gene introgression whereas most plants recovered from particle bombardment contain multiple rearranged insertions (Figure 1, and Table 2). Currently, evaluations to determine the stability of gene inheritance and gene expression throughout T2 and T3 generations are in progress.

Table 2.- Transformation efficiency of rice using Agrobacterium tumesfaciens or particle bombardment

Method / genotype	Strain / plasmid	Callus Hyg^r (%)	Plants/ Callus Hyg^r (%)	Plants/ explant (%)	Plants Southern$^+$ (%)	Efficiency (%)	Gene Integration Pattern
Agrobacterium							
indica	LBA4404 pTOK233	60.4 (32.9)	43.0 (17.1)	26	50-100	13-26	Simple Non-rearranged
indica	Agl1 pCambia 1301	38.5 (4.9)	57.0 (17.6)	22	80-100	18-22	Simple Non-rearranged
indica	Agl1 pWBVec8	59.9 (19.8)	31.6 (23.9)	19	ND	ND	ND
Biolistic							
indica	NA	57.8 (9.3)	5.2 (0.8)	3	30-60	0.9-1.8	Multiple rearranged
japonica	NA	74.0 (12.0)	67.6 (11.0)	50	30-60	15-30	Multiple rearranged

Numbers in parenthesis refer to the range of plant numbers analyzed. (NA) not applicable. (ND) Not determined. (Hyg^r) Hygromycin resistant.

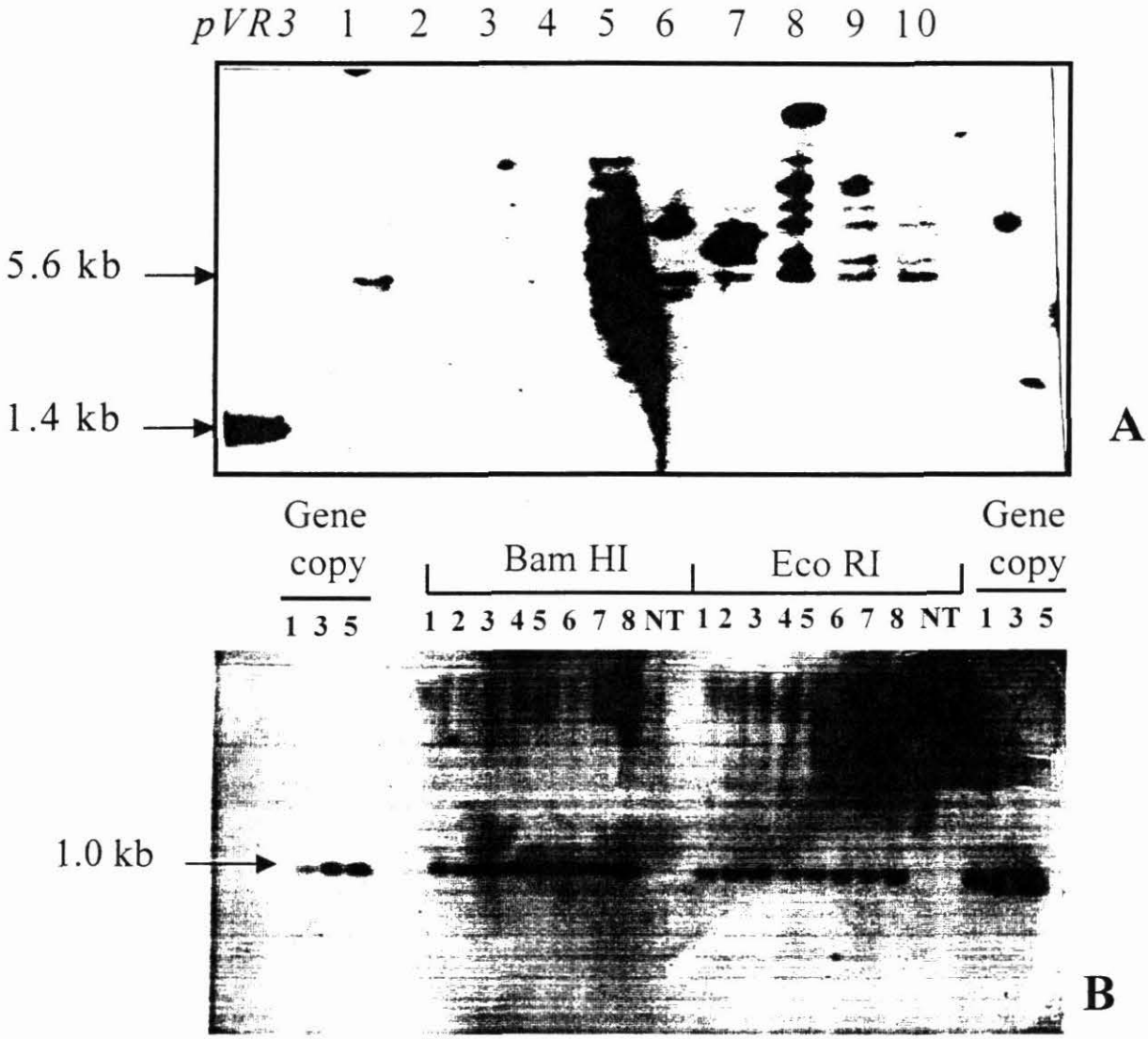

Figure 1.- Southern blot analysis of plants of Cica 8 generated using (A) particle bombardment carrying the pVR3 plasmid containing the RHBV-N gene, and (B) Agrobacterium Agl1 carrying the pCambia 1301 containing the RHBV-NS4 gene, respectively.

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2.1.10 Development of selection systems for the generation of transgenic rice according to current food biosafety requirements

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[1] IP4 Project

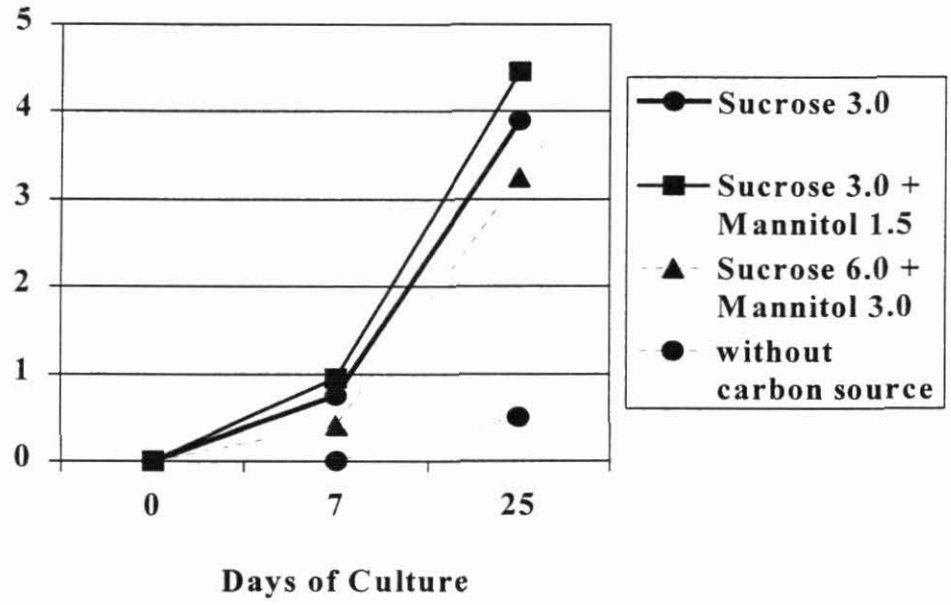
Introduction

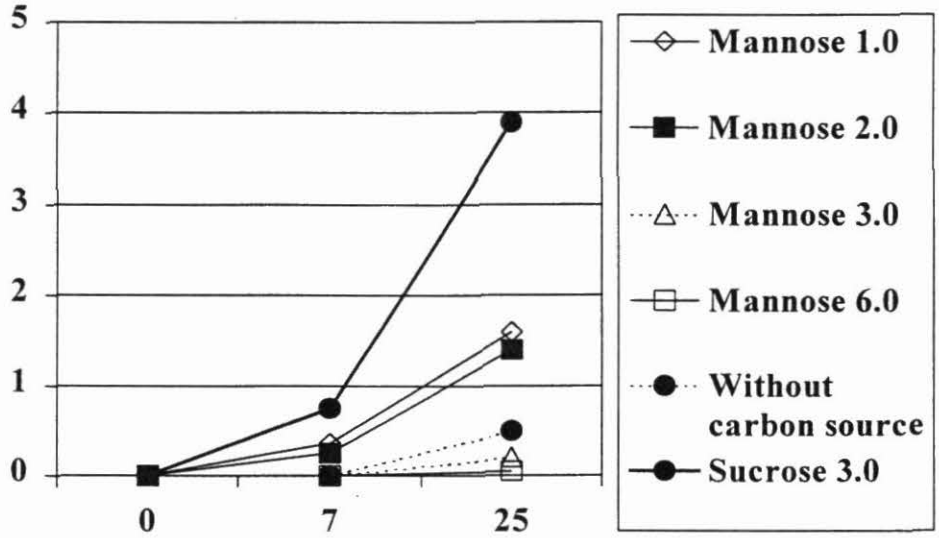
Most selection systems commonly used for the generation of transgenic rice using either biolistic or *Agrobacterium* mediated transformation, relies on the use of the *hpt* gene conferring antibiotic resistance to hygromycin. Although the selection using hygromycin resistance gene has been highly efficient for the production of transgenic rice, and hpt gene has been de-regulated for transgenic crop directed to human consumption, it has not yet been approved for animal feed since hygromycin is sometimes used for veterinary applications. As a response to public perception concerns, recent international food biosafety recommendations suggest to use non-antibiotic selection markers genes. From the year 2005 it is most likely that food or feed crop containing antibiotic resistance will not be approved for commercialization. Recent developments using selection systems different from either antibiotic or herbicide resistance suggest that the phosphomannose isomerase (*pmi*) is an efficient selection gene for production of transgenic plants from maize, sugar beet, and cassava (Wang, et al. 2000; Negrotto et al., 2000). When non-transgenic cell tissue are cultured on mannose, after uptake mannose is phosphorylated by a hexokinase yielding mannose-6-phosphate which accumulates in plant cells and causes severe growth inhibition. Mannose isomerase gene converts mannose-6-phosphate to fructose-6-phosphate, thus giving to the plant cells the capacity of metabolizing mannose as a carbon source. Following is described the progress made towards the development of a selection system using mannose isomerase gene as a positive selection in rice. Embryogenic callus derived from mature embryos of *indica* rice Cica 8 was cultured on callus medium containing 1% mannose (168 mOsmol/kg), 2% mannose (212 mOsmol/kg); 3% mannose (261 mOsmol/kg), and 6% mannose (429 mOsmol/kg), to determine the concentration of mannose which totally inhibits the callus growth of rice. Controls consisted of callus medium containing 3% sucrose (200 mOsmol/kg), or without carbon source (100 mOsmol/kg). In order to determine if the growth inhibition was due to the effect of mannose and not by an increase in osmotic potential by the mannose, medium containing a combination of sucrose and mannitol resembling the osmotic potential of mannose 3% and 6% were also included. These treatments consisted of 3% sucrose + 1.5% mannitol (261 mOsmol/kg), and 6% sucrose + 3% mannitol (429 mOsmol/kg), respectively.

Callus showed a significant reduction in growth when cultured on mannose containing medium respect to sucrose. An inhibition of 59%, 64%, 95% and 98% were noted when callus were cultured on mannose 1%, 2%, 3%, and 6%, respectively (Figure 2). The level of this inhibition was even higher than that observed on medium without carbon source which shows a 87% of growth inhibition respect to 3% sucrose (Figure 2). The inhibition

of growth was due to the effect of mannose and not due to an increase of osmotic potential, since callus on 3% sucrose + 1.5% mannitol or 6% sucrose + 3% mannitol develop alike to those on 3% sucrose (Figure 2). Based on these results in the case of Cica 8 a concentration of 3% mannose is considered sub lethal, and 6% mannose a lethal dose. Work is now in progress targeting various *indica* rice genotypes using *Agrobacterium* mediated transformation and *pmy* as a selection marker gene.

Figure 2.- Callus growth inhibition of rice cultured on medium containing mannose as a carbon source.





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

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Tomato (*Lycopersicon esculentum* Mill) is one of the most important crops in the fresh vegetable market as well as in the food processing industry (Rick and Yoder, 1988). Tomato is the major consumed vegetable crop in Colombia, with a planted area of 15.000 hectares yielding 450.000 tons per year (UNAL, 1997). In Colombia, this crop is highly affected by several pests and diseases, and abiotic stresses such as drought, high and low temperatures, and salinity. Since 1985, the vegetable breeding program at the Universidad Nacional de Colombia, Palmira Campus, has as main objective the development of varieties with resistance or tolerance to some of these traits. In 1997, this program released the tomato variety UNAPAL Arreboles, which has several traits attractive to tomato growers such as fruit firmness and good adaptability specially to the Valle del Cauca region. But this variety is susceptible to 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 difficult 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 inter-specific 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 successfully to obtain resistance against Lepidoptera pests in various economical important crops (i.e. maize, cotton).

In previous reports it was described the evaluation of three protocols commonly used for tomato callus induction and plant regeneration (Fillatti et al., 1987, Narvaez, 1993, and Ultzen et al ., 1995). Results indicated that the highest response for callus induction and plant regeneration is noted on M3 medium sequence (Ultzen et al ., 1995). An increase in response of about 2-fold and 4-fold on callus induction and plant regeneration was noted

on M3 media respect to the other media tested. The lowest response was obtained on M2 medium (Fillatti et al., 1987).

Last year, two *Agrobacterium* mediated transformation protocols commonly used for tomato McCormick et al., 1986; Fillatti et al., 1987), were tested using the tomato variety UNAPAL-Arreboles. This year three Agrobacterium strains C58C1, Agl1 and LBA4404 containing the pBIGCry construct (L.I. Mancilla at CIAT) were used. This gene construct contains the *cry1Ab* gene driven by the 35s CaMV promoter, the nptII gene for kanamycin resistance as selection markers, and the gus-intron as a reporter gene. Gus transient expression analysis of the explants after 48 hours of co-cultivation with the three *Agrobacterium* strains showed that the strain LBA4404 presented a major efficiency of transformation on tomato UNAPAL-Arreboles.

A total of four hundred tomato explants (cotyledonary leaves of 7-10day-old plantlets) were infected with LBA4404/pBIGCry weekly. After co-cultivation for 48 hour, about 10% of the explants were analyzed for gus transient expression. Explants from cultures showing transient expression were transferred to selection media containing kanamycin for selection. After three weeks on selection media, regenerated plantlets were recovered. The number of transformed plantlets isolated varied among the different experiments. From 0 to ten plants were recovered per experiment.

A total of 51 putative transonic plants were produced from 8 experiments (400 explants by experiment). This shows an efficiency of 1.6 % for recovering kanamycin resistant plants from the initial agro-infected explant. Of these plants 15 were transferred successfully to the greenhouse and 6 plants had shown stable gus expression throughout the vegetative and reproductive life cycle. One of these clone (P35) has not flower yet after several months in the greenhouse, reason why not seeds are available from it. The other 5 clones (P2, P17, P28, P33, and P47) had set seeds, and currently being evaluated by Southern, Northern, and Western analysis to determine the introgression of the *cry1ab* gene into the Arreboles genome, and characterized the level of gene expression. Each cloned is being clonally propagated, and evaluations at T0 and T1 generations will proceed for morphological characterization and transgene inheritance studies, as well as for insect resistance bioassays.

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

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Wide spread of 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 seeds from clonal propagated infected plants, and transmission by the aphid vector *Melanaphis sacchari* widely present in the sugar cane region of the country. 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 infected with this virus 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, since transgenic sugar cane has already being generated in other laboratories using this gene transfer method.

Attempts to transform sugar cane were 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 was tested to select the most responsive genotype and use as target. Callus was 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. Last year results indicated that the routine protocol used for clonal propagation of sugar in CENICAÑA although efficient for the generation of plants, it was

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 tissue 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.

Callus bombarded using plasmids pGV1040, pCAMBIA 1201, and pCAMBIA 1301, following a two step-wise bombardment under 1,100 psi at 6 cm target distance, and by 1,300 psi at 9 cm indicated that between 20% and 49% of callus showed GUS transient expression 48 hours after bombardment. This year report include the optimization of bombardment conditions , the establishment of lethal and sub-lethal genetycin concentrations for selection of stable transformants at the embryogenic callus, plant regeneration, and plant rooting stages.; and the progress towards the recovery carrying the ScYLV coat protein gene.

Results using plasmid pAct-!D indicated that 51.3 % $\pm$ 27.8% of bombarded tissue showed gus transient expression 48 hr after bombardment (Table 1). As expected for biolistics, results varied from experiment to experiment. The conditions optimized were then used to generate transgenic sugar cane plants carrying the ScYLV coat protein gene.

Table 1.- GUS transient expression 48 hr after bombardment with plasmid pAct-1D

Experiment	Callus bombarded	GUS +	%	GUS -	%
1	22	18	81.8	4	18.2
2	15	7	46.7	8	53.3
3	35	18	51.4	17	48.6
4	25	17	68.0	8	32.0
5	34	21	61.8	13	38.2
6	53	35	66.0	18	34.0
7	41	39	95.0	2	5.0
8	43	39	90.7	4	9.3
9	47	43	91.5	4	8.5
10	51	38	74.5	13	25.5
11	62	35	56.5	27	43.6
12	43	27	62.8	16	37.2
13	59	22	37.3	37	62.7
14	38	12	31.6	26	68.4
15	36	8	22.2	28	77.8
15	40	0	0.0	40	100.0
16	36	3	8,3	33	91.7
17	29	12	41.3	17	58.6

Table 1 (cntd)

Experiment	Callus bombarded	GUS +	%	GUS -	%
18	27	7	25.9	20	74.1
19	26	6	23.0	20	76.9
20	30	6	20.0	24	80.0
21	30	22	73.3	8	26.7

Explants were cultured on medium containing genetycin concentrations of 0, 10, 20, 30, 40, 50, 60, and 70 mg/l. Forty days after culture, the percentage of dead tissue were assayed. Results indicated that genetycin 30 mg/l is a sub-lethal conferring about 50% of mortality, whereas genetycin 50 mg/l can be used as lethal antibiotic concentration for selection (Figure 1).

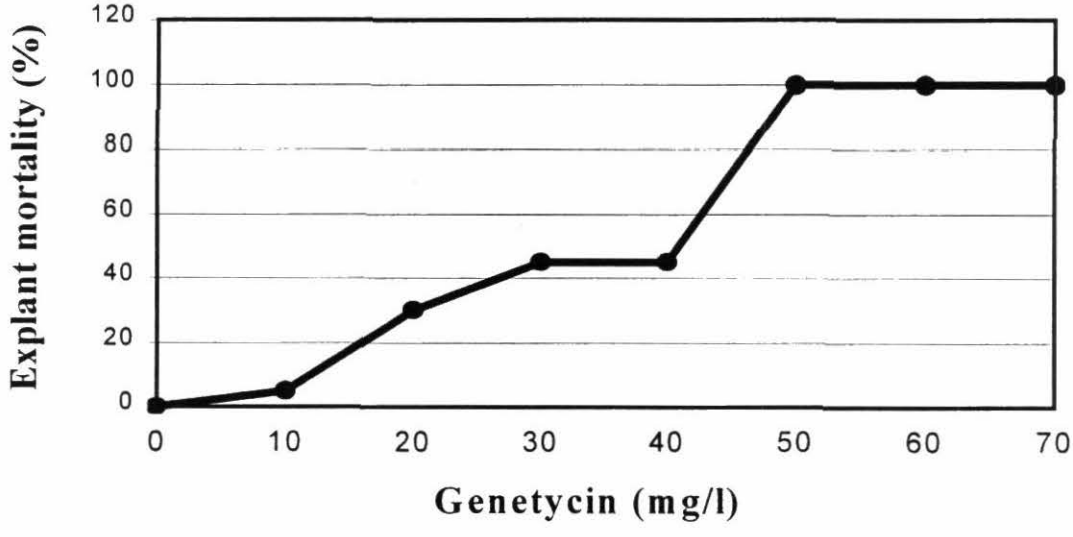

Figure 1.- Sensibility curve to the antibiotic genetycin of sugar cane explants use for production of transgenic plants

After a complete stepwise selection at genetycin 30 mg/l followed by genetycin 50 mg/l, a total of 68 petri plates with 20 to 25 explants each had been evaluated so far. About 50% $\pm$ 29 % of the bombarded callus were resistant to genetycin 50 mg/l. Of these resistant callus, about 4% $\pm$ 5% are regenerating plants. Plant regeneration is still in process. Preliminary data shows that most of the plants recovered are resistant to genetycin 50 mg/l when transferred to root induction medium. Future plans include the

complete molecular characterization of these putative transgenic plants to select those of which are going to be evaluated for ScYLV resistance.

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ACTIVITIY 2.2 Identification of points of genetic intervention and mechanism of plant-stress interactions

Main Achievements

- Progress in the study of the occurrence of secondary metabolites and enzyme activities related to wound responses that may play a determinant role during the postharvest physiological deterioration in cassava. Two main secondary metabolites, hydroxycoumarins and flavonoids were analyzed.
- The analysis of carotene content in yellow-seeded common bean varieties suggested that carotenoid levels are very low in both the dry bean embryo and in the seed coat and that the yellow color is probably not due to carotenoids, but rather to some other pigment, such as xanthaphylls
- A total of 500 accessions (not from the core collection) from the cassava world germplasm bank held at CIAT were evaluated for vitamin C and carotene content. For vitamin C in the roots, there were at least 120 genotypes with concentrations higher than the highest value found in the core collection For carotene content the values were very similar to those found in the core collection.

2.2.1. Identifying target points for controlling postharvest physiological deterioration in cassava

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Introduction

As a crop, cassava offers many advantages that have made it a suitable plant for poor farmers in marginal areas. Despite the foregoing, cassava suffers rapid postharvest deterioration, starting within 48 hours after harvest, rendering the root unpalatable and unmarketable. The postharvest deterioration process in cassava begins with physiological deterioration (PPD), followed by microbial deterioration from days 4 to 6 (Booth 1976).

PPD, the product of abiotic stress, is characterized by vascular streaking, a blue-black discoloration of the xylem parenchyma, followed by a general discoloration of the storage parenchyma. The tissue damage, produced during harvesting and handling of the

roots, results in a cascade of wound responses, which show many parallels to those in other plants (Wenham 1995).

Wound responses result in the defense of the injured tissue by the activation of lytic enzymes such as chitinases and β-1,3-glucanases; and oxidative enzymes such as peroxidases and phenol oxidases, which oxidase phenolic compounds produced by the induction of the phenylpropanoid pathway. The oxidation of phenols results in the production of compounds toxic to microorganisms and tissue browning (Beeching et al. 1998). These phenols also play a defensive role, acting as allelochemicals, antioxidants, metal chelators, UV protectants, phytoalexins, signaling molecules and polymers (lignin).

The goal of this project is to identify biochemical markers for PPD, studying the occurrence of secondary metabolites and enzyme activities related to wound responses that may play a determinant role during the PPD process. The identification of these PPD markers will facilitate the improvement of cassava germplasm with respect to PPD through the development of screening methods for use in germplasm evaluation by breeding programs.

Methodology

This strategy is based on the evaluation of cassava cultivars with different reactions to PPD (CM 2177-2 and M Col 22 as high response; M Nga2 and M Ven 77 as medium response; and M Bra 12, M Bra 337 and M Per 183 as low response), following a 7-day period of deterioration. After determining the key biochemical tests to be used, the progeny of a cross between two cultivars with different susceptibility to PPD, which are being used at CIAT to construct the genetic map of cassava, will be screened. The biochemical tests are the evaluation of secondary metabolites, such as the quantification of the soluble phenol content and specific compounds as coumarins and flavonoids by HPLC, and the activities of enzymes that can be related to PPD, such as phenylalanine ammonia-lyase (PAL), polyphenol oxydase (PPO), peroxidase (POX), catalase (CAT), chitinase and ß-1,3 glucanase.

Results

The quantification of the soluble phenol content in cassava root tissue undergoing PPD can be used as an indicator of phenylpropanoid metabolism induction. Results showed no clear differences among cultivars nor a marked tendency to increase or decrease concentrations over time.

As reported before, two main groups of secondary metabolites were identified in ethanol extracts of cassava roots undergoing PPD: hydroxycoumarins and flavonoids, specifically catechins. The hydroxycoumarins are scopoletin and esculetin and their respective glucosides scopolin and esculin. The catechins are (+)- catechin, (+)-gallocatechin and (+)-catechin gallate. Quantification of coumarins showed no significant changes in concentrations of esculin and esculetin during the PPD period, while scopoletin and

scopolin presented an increase in concentration over the same period. Scopoletin showed a more rapid accumulation than scopolin, which was then followed by a decrease in cv. M Col 22 and M Nga2 (Fig. 1a). A difference among cultivars is the late accumulation of scopoletin and scopolin in M Bra 337 on day 4. Scopoletin increases at day one, but the accumulation is not so obvious as that at day 4 (Fig. 1b).

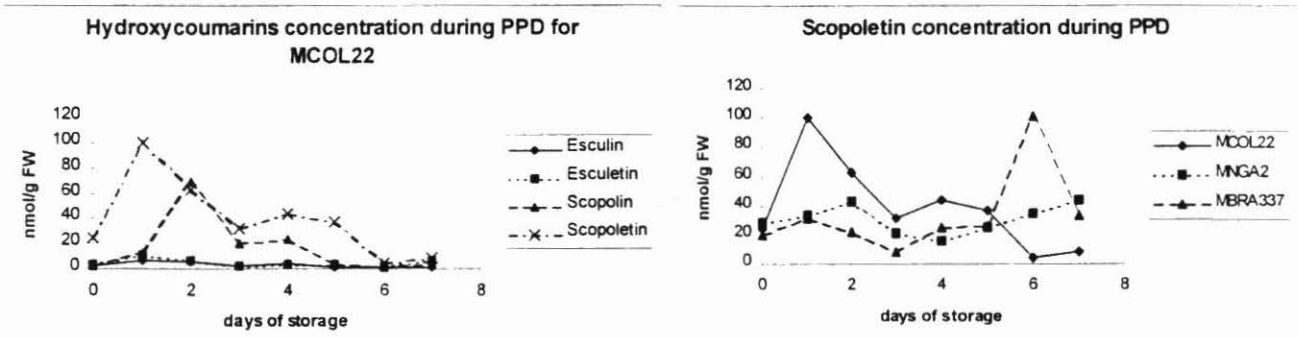

Figure 1. (a) Quantification of hydroxycoumarins in cassava root ethanolic extracts over 7 days for cv. M Col 22. (b) Quantification of scopoletin in cassava root ethanol extracts over 7 days for three different cv. (M Col 22, M Nga2 and M Bra 337).

Flavonoids were not synthesized *de novo* but were detectable in low concentrations during the first days of the storage period. The concentration of (+)-catechin gallate did not show significant changes over the PPD period, while (+)-catechin and (+)-gallocatechin started increasing after 2-4 days (Fig. 2a). There is a marked difference among cultivars in the accumulation of (+)-gallocatechin. Low-PPD cultivars accumulate higher quantities after 5 days in contrast with the other cultivars (Fig. 2b).

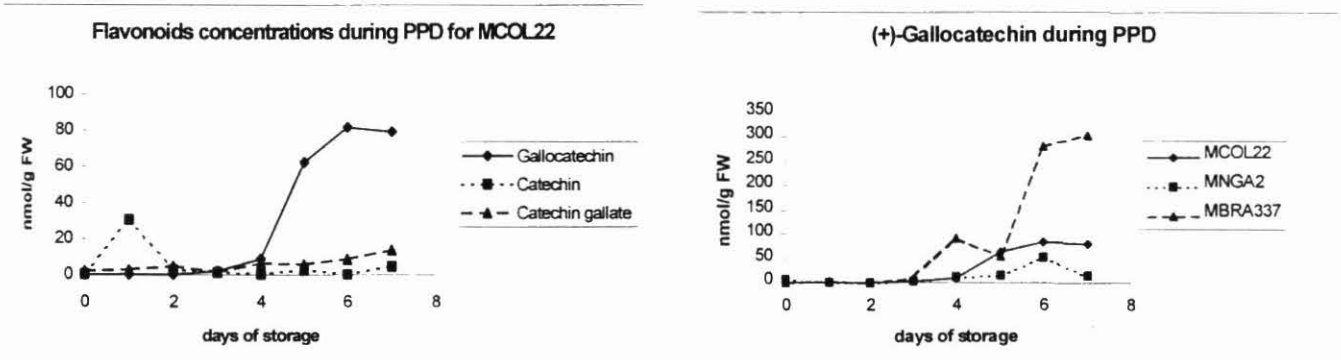

Figure 2. (a) Quantification of flavonoids in cassava root ethanol extracts over a period of 7 days for cv. M Col 22. (b) Quantification of (+)-catechin gallate in cassava root ethanol extracts over a period of 7 days for three different cultivars (M Col 22, M Nga2 and M Bra 337).

Although there is a significant difference in accumulation of flavonoids among cultivars, they cannot be considered as markers due to their late increase during the storage period. This late accumulation, could be a defense response to scopolin, due to their rapid increase in concentration during the PPD period.

The secondary metabolites present in extracts of cassava roots undergoing PPD proved to have antioxidative and antimicrobial properties. Flavonoids are very potent antioxidants, while scopoletin inhibits the growth of pathogens such as *Cladosporium cucumerimum* and *Fusarium avenaceum* (standard microorganisms for antimicrobial tests).

Regarding enzyme activities, PAL and CAT did not show any tendencies or differences among cultivars. In contrast, POX, PPO, β-1,3 glucanases and chitinases increased activity throughout the storage period. The lytic enzymes started to increase activity after 5 days, but did not show differences among cultivars. As for the accumulation of flavonoids, this fact can be related to a defense response to microbial decay. Both oxidative enzymes, POX and PPO, increase from the early stage of the deterioration, presenting higher values in the susceptible cultivars (Fig. 3). PPO shows a clearer difference among the cultivars, and the increase of activity in the most susceptible cultivar proved to have a direct correlation with tissue browning.

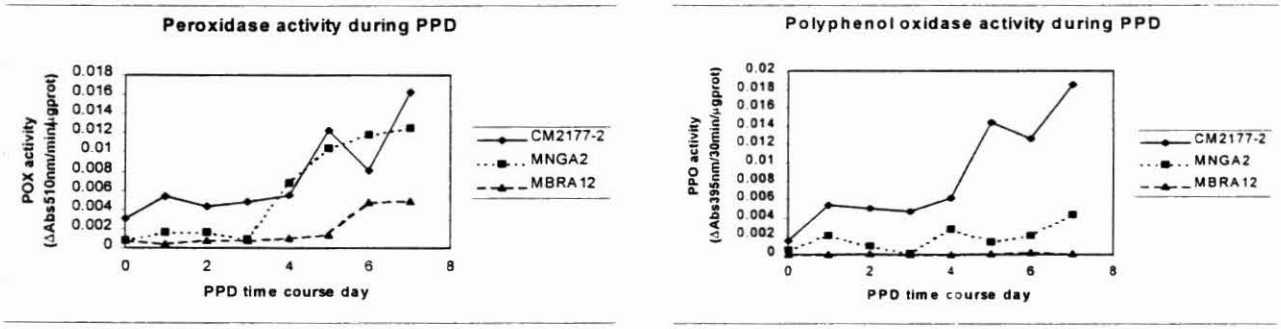

Figure 3. Enzyme activity quantification for peroxidase and polyphenol oxidase in cassava roots for three different cultivars (CM 2177-2, M Nga2 and M Bra 12) over a 7-day period.

The coumarins (scopoletin, esculetin and esculin) were tested as substrates for POX. The three phenols react with the enzyme root extract, but scopoletin presents the most interesting reaction. Scopoletin quickly oxidases, showing a dark bluish coloration. Then, after approx. 10 min., the solution starts to turn light in coloration and develops a black precipitate, like the precipitates observed in vascular streaking. Scopoletin is distributed throughout the storage parenchyma, but tissue prints for POX activity have shown that it is concentrated around the vascular parenchyma. This may reflect a positive correlation between vascular streaking and coumarin oxidation.

Ongoing activities

Present and future activities include the screening of a selected population of the genetic mapping cross progeny (M Nga2 x CM 2177-2). The biochemical tests are the quantification of coumarins and enzyme activities for POX, PPO and scopoletin-peroxidase.

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2.2.2. Determination of carotene content in yellow-seeded common bean varieties

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Introduction

Yellow-seeded beans are a minor commercial grain type from the Andean gene pool that are eaten in a subset of Latin American bean-producing countries, notably in Peru, Mexico and Brazil. Recently, they have become embroiled in a controversy in the United States where a patent was claimed on the marketing of a yellow bean variety. In the meantime, the scientific basis for the yellow color is not known. In this study we decided to analyze whether the yellow color was derived from Carotenoids or not. To do this we selected a group of accessions representing all the types of yellow beans that are found in primary and secondary centers of diversity to determine the vitamin A content of the seed and if there is genetic variability for the trait.

Materials and Methods

A total of 10 common bean genotypes with various tones of yellow-colored grain (from canary to sulphur yellow) were selected for this study (Table 1). Three parts of the grain were analyzed: 1) whole seed, 2) peeled seed (embryo) and 3) seed coat (both stored and fresh). The tissues were ground into a fine powder with a coffee mill and a tungsten ball bearing mill. Extraction began by resuspending 0.3 g (for the stored seed coat and embryo) or 1.0 g (for the whole seed) of ground tissue in 10 ml of petroleum ether and 5 ml of water at 35-60 °C. The mixture was homogenized before centrifugation at 3000 rpm for 5 min at 10 °C. The ether phase was removed to a new tube while the aqueous phase was re-extracted with an additional 10 ml of petroleum ether and combined with the previous ether phase. Both of these aliquots were dried down with sodium sulfate in a vacuum centrifuge. The extracts were then resuspended in 1 ml of petroleum ether and used immediately for the analysis. The samples were analyzed in a UV-visible spectrophotometer at 455 nm, using petroleum ether at 30-60 °C as a blank. A standardization curve was made from β-carotene in a petroleum ether solvent and used for the quantification of β-carotene in the sample. The linear regression of the calibration curve was C(ppm) = K x Abs +B, where K= 6.6707 and B= 0.2084. To confirm the presence of β-carotene in each group of samples, readings were made for the UV-visible spectra in the range from 200-600 nm and compared to the commercial standard (Sigma).

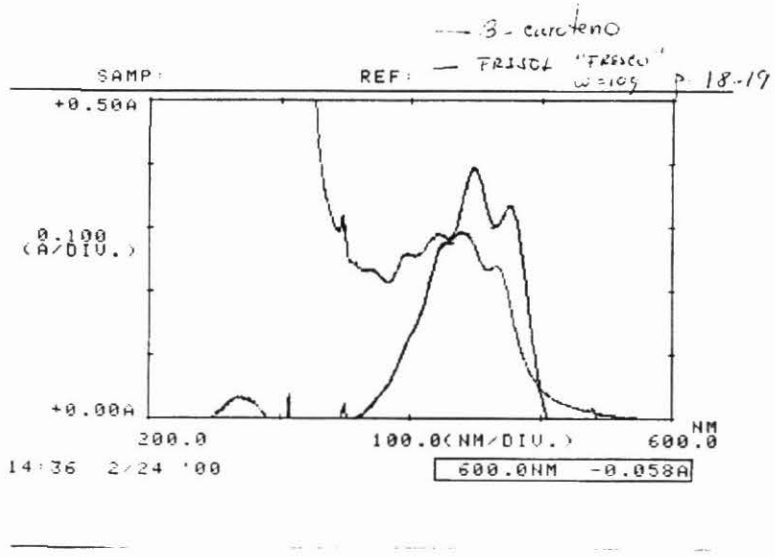
Results and Discussion

The amount of β-carotene found in the stored and freshly-ground seed coats was similar and relatively low, averaging 0.131 mg per 100 g of tissue. Fresh embryo had similar amounts of carotene as the seed coat, averaging 0.147 mg per 100 g of tissue. Whole seed contained even lower amounts of β-carotene, averaging 0.042 mg per 100 g of tissue. Since the absorbance levels at 455 nm were very low, no additional readings were taken at other wavelengths.

Since the first experiments were done with sample sizes (0.3 g and 1.0 g) that are at the lower limit of detection, a second trial was undertaken with a larger amount of freshly ground tissue. Ten grams of fresh tissue was extracted with a final volume of 1 ml and this was analyzed as described above. The spectra of the new sample, measured at wavelengths from 200 to 600 nm, was compared to that of the β-carotene standard. There generally was a good fit between the data for the sample and the standard, indicating that β-carotene was present in the fresh bean seeds (Figure 1).

These preliminary results show that carotenoid levels are very low in both the dry bean embryo and in the seed coat and that the yellow color is therefore probably not due to carotenoids, but rather to some other pigment, such as xanthaphylls.

Figure 1. UV spectra of β-carotene commercial standard (red line) as compared to fresh bean seed (black line)



Future Activities

For future β-carotene analysis, the amount of ground tissue will be increased to 10g. To avoid the interference by other pigments at 455 nm, the samples will be read at an absorbance wavelength of 490 nm. To confirm the presence of β-carotene, more sensitive tests using HPLC and Mass spectrophotometry will be undertaken. Mass spectrophotometry can determine the identity of xanthophylls that have been suggested as the major pigment in seed coat of yellow beans. The amount of B-carotene in various stages of bean seed maturation will be another area that we will pursue. Especially in the Andes and certain areas of the Caribbean, bean seeds are commonly consumed at physiological maturity but before they dry down. To do this the beans are shelled while the pods are still green or just starting to turn yellow but before they dry down to maturity. These beans are often referred to as green-shelled beans and command a premium price because of their fast cooking time. We will look at the changes in carotenoid levels during grain development. Another issue is the stability of the carotene upon cooking and whether this is different in mature dry versus green shelled beans.

Table 1. Analysis of carotene content of stored and fresh seed coat tissue from 18 common bean genotypes.

Genotype	Name	Origin	Weight (g)	mg carotene /100g tissue	
				Fresh seed coat	Stored seed coat
G-5703	Canario Corriente (LM2-57)	Peru	0.30	0	--
G-57	Swedish Brown	USA	0.30	0.10	0.19
G-2288	Maragwe Oga	Kenya	0.30	0.10	0.18
G-4547	Liborino de Mata	Colombia	0.30	0.09	0.16
G-11035	Bayo Regional	Mexico	0.30	0.12	0.20
G-13094	Mayocoba	Peru/Mexico	0.30	0.08	0.18
G-14253	Peru 13	Peru	0.30	0.09	--
G-19833	Caucha Chuga	Peru	0.30	0.07	0.20
G-21715	Dore de Kirundo	Burundi	0.30	0.09	0.31
G-22041	Garbancillo Zarco	Mexico	0.30	0.09	0.10

Table 2. Analysis of carotene content of whole seed and fresh embryo tissues in 10 common bean genotypes.

Genotype	Whole seed			Fresh Embryo		
	Weight (g)	Absorbance (λ 455nm)	mg carotene /100g tissue	Weight (g)	Absorbance (λ 455nm)	mg carotene /100g tissue
G-5703	1.0	0.013	0.03	0.30	0.017	0.10
G-57	1.0	0.019	0.03	0.30	0.028	0.13
G-2888	1.0	0.017	0.03	0.30	0.027	0.13
G-4547	1.0	0.024	0.04	0.30	0.037	0.15
G-11035	1.0	0.085	0.08	0.30	0.028	0.13
G-13094	1.0	0.029	0.04	0.30	0.047	0.17
G-14253	1.0	0.027	0.04	0.30	0.094	0.27
G-19833	1.0	0.041	0.05	0.30	0.043	0.15
G-21715	1.0	0.022	0.04	0.30	0.022	0.11
G-22041	1.0	0.022	0.04	0.30	0.026	0.13

2.2.3. Exploring the genetic potential for improving the micronutrient content of cassava

L. Chavez, H.Ceballos, J. Echeverry[1], F. Calle[1], T. Sanchez[1] and W. Roca
[1] IP-3 Project

Introduction

Improving the efficiency with which cassava acquires micronutrients and accumulates them in the roots and leaves can have enormous potential, not only in terms of human nutrition but also in terms of crop production. Results obtained at CIAT in terms of nutrient-use efficiency for P, K and Ca in cassava, combined with data from research programs in other crops, suggest that there is a potential for broad spectrum in micronutrients in this crop as well. In the case of cassava, woody stem cuttings are used

for propagation, and their quality influences early crop establishment as well as final root yield. It is expected that new cultivars with higher micronutrient contents will also have an agronomic advantage, which will ensure their competitiveness in the market place.

The short postharvest storage life of cassava is a characteristic that limits the marketability of the fresh root and necessitates either consumption or processing shortly after harvesting. Postharvest physiological deterioration (PPD) of cassava roots, which begins within 24 hours of harvest, results in crop and product-quality losses, high marketing margins and risks, and restricted management flexibility for farmers, traders and processors. Thus the reduction of PPD has been identified as a priority target for strategic research. In many respects, PPD resembles wound responses found in other better studied plant systems; but cassava appears to lack the wound-healing capacity that is normally associated with the inhibition of wounding responses. Normally, such defensive wound responses are inhibited by wound repair; however, this repair process does not occur in the harvested cassava storage root, leading to the hypothesis that unrestrained cascades of wound responses ultimately result in deterioration. An important component of these wound responses is the oxidation processes. Ascorbic acid and carotene are known to have antioxidant properties; therefore, PPD was measured in a sample of genotypes to evaluate the potential correlation between these two vitamins and PPD.

Objective

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 bioavailability of micronutrients and vitamins.

Methods

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

Ascorbic acid concentration measurements. The protocol for determining ascorbic acid by Fung and Luk (1985) was adjusted for cassava leaf and roots, taking as a base the procedure outlined in the 1998 annual report.

Correlation between vitamin content and PPD. Postharvest physiological deterioration was measured 6 days after harvest in 500 genotypes, whose ascorbic acid and carotene root concentrations were known. The correlation between vitamin concentration and PPD was then measured.

Results

A total of 500 accessions (not from the core collection) from the cassava world germplasm bank held at CIAT were evaluated.

Analysis of vitamin C content. Table 1 summarizes the ascorbic acid results for leaves and roots from the 500 accessions evaluated. As in the case of the genotypes from the core collection (see 1999 annual report), there was a strong skewness with long right tails. In general data from leaves were similar to those already reported for the core collection. In the case of vitamin C in the roots, there were at least 120 genotypes with concentrations higher than the highest value found in the core collection. In fact, the highest value was almost twice as high in this sample compared with that from the core collection (71.19 vs. 37.52 mg/100 g FW).

Analysis of carotene content. Carotene concentration in the leaves ranged from 12.05 to 96.42, with a mean of 43.59 mg/100 g FW (Table 2). These values are very similar to those found in the core collection. As in the case of vitamin C, carotene distribution was also skewed to the right, but to a lesser degree. Carotene concentration in roots showed a strongly skewed distribution with a long tail to the right (test = 2.11). Values ranged from 0.12 to 0.93 mg. Following the same trend observed for ascorbic acid, carotene concentrated significantly more in the leaves than in the roots, illustrating once again the excellent nutritional value of cassava leaves. There was no correlation between carotene and ascorbic acid concentrations in leaves and roots.

Correlations between vitamin contents and PPD. The correlation between PPD and vitamin C and carotene contents in roots were, respectively, +0.297 and –0.113. This positive correlation between vitamin C and PPD may be due to cumulative errors in measurements for both vitamin C and PPD or to genetic differences between the genotypes of the core collection and those reported herein.

The relationship between PPD and carotene content seems to be more consistent to the one reported previously (-0.169 vs –0.113). Therefore, the hypothesis that carotene content can help reduce PPD (through its antioxidant capacity) is supported again by these results. The graphs plotting the relationship between carotene in the roots and PPD (Fig. 1) suggest that >50 mg carotene/100 g FW, PPD does not exceed 30%. At lower carotene concentrations, the association is lost, finding a large variation in PPD that cannot be accounted for by carotene content. There is no association between vitamin C content in the roots and PPD (Fig. 2).

Table 1. Ascorbic acid concentration in leaves and roots of 500 noncore cassava accessions from CIAT's Germplasm Bank Collection.

Data from Leaves		Data from Roots	
Range (mg/100 g FW)	Frequency	Range (mg/100 g FW)	Frequency
0.0-45.0	39	0.0-7.0	4
45.1-90.0	110	7.1-14.0	39
90.1-135.0	139	14.1-21.0	105
135.1-180.0	101	21.1-28.0	126
180.1-225.0	56	28.1-35.0	88
225.1-270.0	36	35.1-42.0	67
270.1-315.0	9	42.1-49.0	32
315.1-360.0	6	49.1-56.0	26
360.1-405.0	1	56.1-63.0	7
405.0-450.0	2	63.0-70.0	5
>450	1	>70.0	1
Minimum	6.60	Minimum	4.88
Maximum	487.09	Maximum	71.19
Median	123.29	Median	26.47
Skewness†	1.02	Skewness†	0.67
Mean	133.76	Mean	28.79
SD	72.47	SD	12.34

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

Table 2. Carotene concentration in leaves and roots of 500 noncore cassava accessions from CIAT's Germplasm Bank Collection.

Data from Leaves		Data from Roots	
Range (mg/100 g FW)	Frequency	Range (mg/100 g FW)	Frequency
0.0-9.0	0	0.000-0.090	0
9.1-18.0	5	0.091-0.180	171
18.1-27.0	28	0.181-0.270	200
27.1-36.0	116	0.271-0.360	51
36.1-45.0	131	0.361-0.450	22
45.1-54.0	134	0.451-0.540	16
54.1-63.0	62	0.541-0.630	21
63.1-72.0	20	0.631-0.720	7
72.1-81.0	2	0.721-0.810	8
81.1-90.0	0	0.811-0.900	3
>90.0	2	>0.900	1
Minimum	12.05	Minimum	0.12
Maximum	96.42	Maximum	0.93
Median	43.30	Median	0.20
Skewness†	0.38	Skewness†	2.11
Mean	43.59	Mean	0.26
SD	11.64	SD	0.14

Segregation study to break the linkage between high carotene content and intense yellow coloration in the roots. In some crosses made between yellow- and white-rooted cassava varieties, it is desirable to break the apparent linkage between high carotene and intense yellow coloration in the roots (Graham et al., 1999). Table 3 summarizes the results from 397 progenies evaluated. Color intensity was estimated, using a 1-9 visual scale (1= white; 9 = intense yellow–orange roots). There was a strong correlation (0.81) between carotene content and color intensity. The correlation coefficients between these two variables for each individual family are listed in Table 3. The families CM 9733 and CM 9712, each with more than 15 plants, had low or negative correlations (–0.202 and 0.673, respectively). Other families had interesting results; but their performance was based on only a few plants (i.e. families CM 9149, CM 9150) or the segregation for color was reduced (family CM 9684), suggesting almost white roots in every plant). Based on these preliminary results, a few families were selected and from each plant making up the family, five stakes were taken and will be planted for future replicated evaluations to be carried out by January 2000.

Table 3. Families evaluated in the segregation study aiming at breaking or reducing the linkage between high carotene and yellow color in cassava roots.

Family	No. of Plants in Family	Carotene Content (mg/100g FW)	Color Intensity (1-9)	Carotene/Color Correlation
CM 7062	6	0.225	2.167	-0.486
CM 9149	2	0.296	2.500	--
CM 9150	1	0.296	2.500	--
CM 9153	10	0.291	2.600	0.851
CM 9249	40	0.486	4.450	0.841
CM 9629	17	0.330	3.824	0.886
CM 9679	44	0.387	3.818	0.844
CM 9680	50	0.307	3.220	0.860
CM 9681	29	0.304	3.897	0.960
CM 9683	56	0.257	3.036	0.825
CM 9684	5	0.251	1.800	-0.559
CM 9712	50	0.327	3.440	0.673
CM 9714	50	0.310	3.900	0.923
CM 9731	20	0.171	2.600	0.237
CM 9733	17	0.174	2.176	-0.202

Future activities

Finding high-vitamin cassava varieties is irrelevant if this beneficial trait does not benefit the farmers and end-users of the product. At this point it is clear that high-carotene content is a trait that can be exploited to reduce the problems related to its deficiency in human populations. Future objectives are as follows:

- To complete the evaluation of carotene content in the cassava collection held at CIAT
- To evaluate the potential correlation between carotene content in roots and PPD
- To evaluate the correlation between carotene content and color intensity in the progenies selected

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Graham R; Senadhira D; Beebe S; Iglesias C; and Monasterio I. 1999. Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. Field Crops Res. 60:57-80.

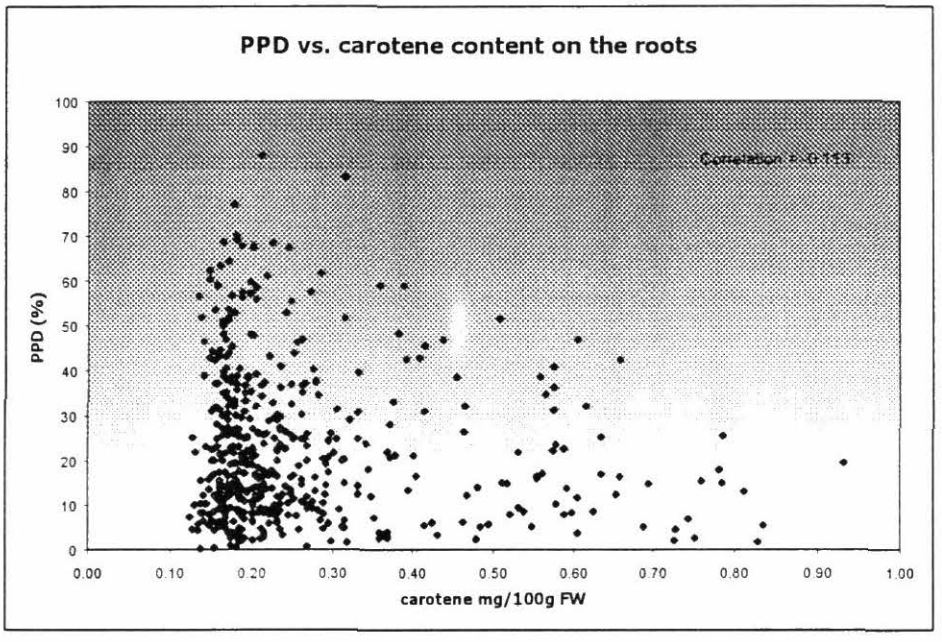

Figure 1. Relationship between PPD and carotene content in cassava roots.

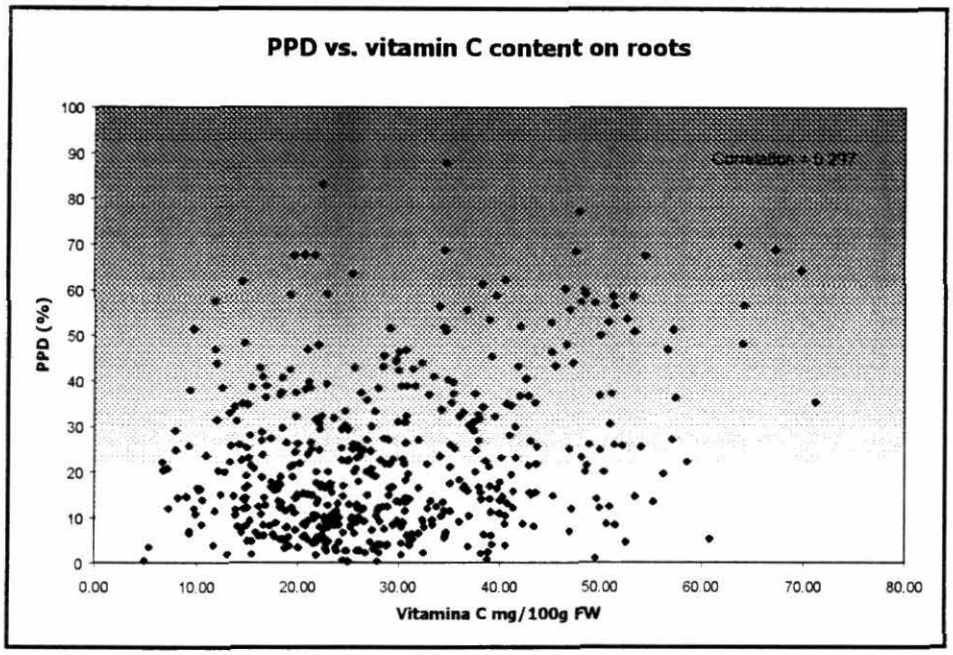

Figure 2. Relationship between PPD and vitamin C content in cassava roots.

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

Main Achievements

- Improvement of propagation rates of cassava cultivars Mcol 2215 and Mcol 1505 using RITA. We achieved substantial reduction in hyperhydricity of tissues.
- Sixty-six cassava accessions from the core collection cryopreserved and recovered after freezing in liquid nitrogen. Results were reproducible through time. Also, the first steps towards developing a methodology for cryospreservation of FEC were made.
- Validation of low-cost, cassava propagation methods with women (small farmers) from Santa Ana, Department of Cauca, Colombia. Expensive media components were replaced by cheaper, locally bought ingredients.
- The automated temporary immersion system RITA has shown to be an efficient system for the expedite generation of large number of embryogenic callus of *Brachiaria* and rice.
- A protocol for an increased rate of conversion from somatic embryos into plants suitable for genetic transformation was optimized for *Brachiaria*.
- This year the distribution of lines derived from rice anther culture represented about 26% of the lines distributed by FLAR to the member countries of the South.
- Optimization of an *in vitro* propagation methodology for selected Colombian varieties of lulo and development of an *in vitro* regeneration protocol for selected varieties through organogenesis

2.3.1 Development of friable embryogenic callus (FEC) and regeneration of plants of cassava cultivar MCol2215 (Venezolana)

D. López, P. Chavarriaga and W.M. Roca

Introduction

The main characteristic of FEC is the hundreds of globular embryos immersed in a semi-liquid matrix of hyaline cells. This type of callus was first developed for cassava (cv.TMS60444) by Taylor et al (1996). Since then, it has been successfully used for genetic transformation of the same cultivar several times (i.e., Raemakers, 1996, Schöpke, 1997). CEF has also been obtained with other cassava cultivars (i.e., Taylor et al 1997, Groll et al 1997). We induced CEF in the cultivar MCol2215, and regenerated plants from it. The FEC produced with MColl2215 is being used for transformation experiments to introduce insect resistance genes.

Methodology

The research was developed in three phases: 1) Inductin of somatic embryos; 2) Induction of FEC and 3) Plant regeneration. Young, immature leaves from in vitro-propagated plants were used as explants to induce somatic embryos on media with the fito-hormones 2,4-D or Picloram. Then, embryos were transferred to FEC induction medium with Picloram. The FEC was subcultured several times onto solid or liquid medium to increase the amount of material. Finally, the FEC was placed on regeneration medium to mature embryos and produce whole plants. The variety TMS60444 was used as a control throughout the experiments.

Results and Discussion

Varietal differences were observed in the induction of somatic embryos and FEC. Mcol2215 produced somatic embryos in up to 96% of the explants tested, while for TMS60444 the maximum percentage was 38%. Similarly, Mcol2215 performed better than TMS60444 for the induction of FEC. Up to 17.8 % of the somatic embryo clusters of Mcol2215 produced FEC. This variety also needed less time (about 40 days) to start producing FEC. TMS60444 needed about 80 days before the first FEC were observed. A summary of the main events concerning embryo production and conversion to plants is presented in Table 1.

Table 1: Number of somatic embryos (at cotyledon stage) and plantlets developed from FEC *on* medium containing BAP in two cassava cultivars (Mcol2215 and TMS60444). Each treatment had two replicas, composed of two plates with 10 FEC clusters each. The amount of tissue in each cluster was 0,2 ml.

Cultivar and hormone used to induced embryos	Total number of somatic embryos observed	Average number of somatic embryos per cluster	Total number of plantlets regenerated	Average number of plantlets regenerated per cluster	Percentage of embryos regenerating plants
MCol 2215 (2,4-D)	1	0.025	0	0	0
MCol 2215 (Picloram)	24	0.6	2	0.05	8.33
TMS 60444 (2,4-D; line <1 year old)	224	5.6	41	1.025	18.3
TMS 60444 OLD (2,4-D; line >4 years old)	564	14.1	156	3.9	27.65

Conclusions

- The variety Mcol2215 (Venezolana) produces CEF at frequencies higher than those of TMS60444
- Conversion of embryos (from FEC) to plants with Mcol2215 is less efficient than with TMS60444. In general, embryo-to-plant conversion seemed to depend on the genotype, the type of hormone used to induce embryos and on the age of the cell line.

Future activities

- The conversion of somatic embryos to plants, for cassava cultivar Mcol2215, should be improved by improving the purity of FEC, by manipulating hormone regimes, and by enriching the media with nitrogen sources (amino acids).

2.3.2 Induction of friable embryogenic callus (FEC) in cassava cultivars of commercial value

V. Segovia, Y.J. Ladino, D. Lopez, P. Chavarriaga and W.M.Roca

Introduction

The introduction of new genes into cassava *via transgenesis* is a reality. Transgenic cassava has been reported with at least two cultivars (Schopke et al., 1996; Sarria et al., 2000). However, the different laboratories that work on transformation of cassava should start focusing on commercial cultivars as targets to introduce desirable genes. The long breeding cycle of cassava makes it impractical to transform model cultivars and transfer the transgene(s) to commercial ones by classical breeding. We are analyzing the transformation potential of commercial varieties by: 1) developing FEC, an efficiently

transformed embryogenic tissue; and 2) testing FEC from these cultivars for their ability to be transformed using *Agrobacterium*. We present the results of the first phase.

Material and Methods

FEC was induced from young, immature leaves, or lateral buds of in vitro grown plants. Thirteen commercial genotypes were used as source of explants (see Table1). Explants were placed on embryo induction medium for 15 days. Then, when somatic embryos appeared, they were subcultured onto the same medium for at least 15 more days. Then, somatic embryos were transferred to FEC induction medium, and checked every week for the appearance of FEC. The experiment is still ongoing, although we present the results of induction of somatic embryos in these cultivars.

Results and Discussion

Table 1 summarizes the production of somatic embryos in 13 commercial cultivars of cassava. The first somatic embryos were observed after 25 days of placing the explants on embryo induction medium. Basically all genotypes produced somatic embryos at different frequencies from leaves or buds, or both. In all but one case, the induction of somatic embryos was more effective with buds than with leaves. The quality of the embryos produced from buds was also better since they had less non-embryogenic callus (not shown). So far only one cultivar, CM 3306-4, has produced FEC in two out of eight embryogenic clusters (groups of somatic embryos) on FEC-induction medium. The process of inducing FEC may take up to 6 months once the embryogenic callus is on FEC-induction medium. It is advisable to wait more time for a final evaluation.

Conclusions

- Its is possible to induce somatic embryos, at relatively high frequencies, in commercial cassava cultivars
- The induction of somatic embryos seems to be more effective from bud explants
- It is also possible to induce FEC in commercial cassava cultivars

Future Activities

- Continue doing weekly checks for the production of FEC in the rest of cultivars
- Proliferate FEC once it is produced
- Perform transformation experiments with *Agrobacterium*, using FEC as source of explants, for transient gene expression assessment

Table 1. Induction of somatice embryos in cassava cultivars of commercial value. (* already produced FEC)

Cassava Cultivar	Number of embryogenic calli per total number of explants induced. L= Leaf, B=Buds	Frequency (%)
MTAI 8	L = 4/27 B = 6/9	L = 15 B = 67
CM 3306-4	L = 27/29	93*
MDOM 2	L = 9/18	50
MCOL 1468	L = 3/18	17
CM 523-7	L = 18/44 B = 9/13	L = 41 B = 69
CM 4574-7	L = 7/15	47
MBRA 507	L = 10/18	55
MCUB 74	L = 12/32 B = 7/9	L = 37 B = 78
MBRA 12	L = 11/31	L = 35
CM 2177-2	L = 33/41	L = 80
SM 909-25	L = 7/8	L = 88
SM 1219-9	L = 8/11	L = 73
MECU 72	L = 6/10 B = 3/13	L = 60 B = 23

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

R.H. Escobar, N.C. Manrique and W.M. Roca.

Introduction

There are at least seven different procedures for vitrification: Encapsulation-dehydration, vitrification *per se*, encapsulation-vitrification, desiccation, pre-growth, pre-growth-desiccation and droplet freezing (Engelmann, 2000). In vitrification-based procedures, cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotection media and/or air desiccation. The encapsulation-dehydration technique (Palacio 1998) was established as an alternative to slow (Escobar *et al* 1997) and rapid freezing (Escobar *et al* 1997) for cassava. We adjusted the encapsulation-dehydration methodology, and used a wider range of genotypes to test the modifications.

Methodology

Adjustments were made on the growth conditions of plants that served as source of shoots. The reproducibility of the response after freezing across time (different freezing experiments) was tested. We tested 66 clones with the adjusted protocol.

Results and conclusions

It was determined that the conditions of in vitro plants that served as source of tissue could affect the response after freezing. Source material with the following conditions responded better:

- Plants with no previous cuttings
- Three to 3,5 months old plants
- Plants grown on 4E medium
- Fifteen to 20 plants per magenta

From 66 clones tested, ten (10/66 or 15%) showed shoot recovery at less than 30% after freezing (Table 1). Some cassava clones, e.g. MPer205 and MPar110, were recalcitrant to cryopreservation.

Supplementing the recovering medium with other growth regulators (Kinetin + IAA or Kinetin + IBA) improved the response after freezing.

The reproducibility of the methodology is summarized in Table 2.

Table 1. Response of 55 cassava clones from the core collection, cryopreserved in liquid nitrogen using encapsulation dehydration technique.

Cassava clone	Survival (%)	Shoot recovery (%)	Cassava clone	Survival (%)	Shoot recovery (%)
M Arg12	84.4	42.2	M Dom 2	68.8	68.8
M Arg 13	61.9	48.2	M Dom 3	92.3	76.9
M Arg2	100	88.8	M Dom 4	100	90
M Bra 124	69.6	42.2	M Dom 5	93.3	93.3
M Bra 125	77.4	71.5	M Ecu 165	46.9	9.5
M Bra 130	69.8	43.3	M Ecu 171	93.6	56
M Bra 132	88.8	60	M Ecu 31	35	15
M Bra 18	90	62.3	M Ecu 41	60.9	45
M Bra 258	73.8	73.8	M Ecu 82	53.3	30
M Bra 311	13.3	3.3	M Gua 15	92.3	83
M Bra 329	57.5	33.8	M Ind 8	65	50
Cassava	Survival	Shoot	Cassava clone	Survival	Shoot

clone	(%)	recovery (%)		(%)	recovery (%)
M Bra 404	100	46.7	M Mal 1	75	35
M Bra 542	91.9	56.7	M Mal 13	96.7	53.3
M Bra 691	82.9	37.7	M Mal 48	65	35
M Bra 698	95.8	90.1	M Mal 63	73.3	50
CM 4733-4	35.8	19.2	M Mex 8	81.7	54.2
M Col 185	81.8	58.1	M Mex 92	68.1	20.8
M Col 1438	93.3	28.1	M Nga 5	69.7	49.5
M Col 1522	40	20	M Pan 7	96.7	73.3
M Col 1752	90	75.5	M Pan 97	97	84.6
M Col 1805	100	71.2	M Par 100	95	90
M Col 2173	91.2	34.2	M Par 105	100	91.1
M Col 2215	50	22.7	M Par 41	90	85
M Col 2361	60.9	56.4	M Par 68	87.9	87.9
M Col 40	66.7	44.4	M Per 205	43.8	6.3
M Cr 35	100	63.12	M Per 255	97	41.5
M CR 59	100	73.3	M Per 295	93	20.7
M Cr 63	82.5	65.7	M Per 436	94.1	79.7
M Cub 16	89.6	89.6	M Per 597	90	73.3
M Cub 23	97.4	97.4	M Tai 1	75.6	62.2
M Cub 29	97.5	97.5	M Ven 117-b	73.3	26.2
M Cub 39	80.9	39.4	M Ven 90	76.7	50
M Cub 55	85	80			

Table 2 Reproducibility of the response of six cassava clones cryopreserved in liquid nitrogen across time.

Cassava clones	Survival (%)				Shoot recovery (%)			
	Plot 1	Plot 2	Plot 3	Control	Plot 1	Plot 2	Plot 3	Control
CM 4063-6	100	90.9	72.5	73.4	33	19.5	5	31.5
M Bra 69	100	96.6	91.6	86.3	50	60	73.3	33.7
M Bra 881	100	100	100	91.4	75	85	60	28.2
M Ecu 117	66.5	80	91.5	81.9	36.5	37.2	34	44.8
M Mal 2	86	94.4	80	71.1	67.5	78.3	60	61.7
M Par 71	100	100	100	96.7	85	100	95	96.7

Conclusions

- 66 cassava clones were cryopreserved and were recovered after frozen in liquid nitrogen.
- The adjusted protocol (described above) showed reproducible results across time.
- Using recovery media with IBA or IAA reduced callus induction.
- Standardizing the growth conditions of plants that served as source of shoots in material such as MNga 1, previously reported as recalcitrant (Annual Report 1999), improved their response after freezing. Other accessions such as MPar 101 and MPer 205 were still recalcitrant.

Future activities

- The Genetic Resource Unit (GRU) provided us with 100 cassava clones from the core collection to test them for cryopreservation with the adjusted protocol.
- We will work on recalcitrant materials with different desiccation procedures.
- We will test different cryopreservation times (2, 4, and 6 months) with selected materials.

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Annual Report 1999

2.3.4 Cryopreservation of friable embryogenic callus (FEC) of cassava

R. Escobar, P. Chavarriaga, L.D. Santos and W.M. Roca

Introduction

Taylor *et al* in 1996 established a technology to develop Friable Embriogenic Callus (FEC) for cassava. We also developed FEC for a Latin American cassava cultivar commercial use (this report). FEC can be maintained as a suspension culture or on solid medium. However, in vitro cultured plant cells are genetically unstable, and the labor and

cost of maintaining cell lines is high (Reinhoud *et al* 2000). Besides, the older the FEC the more difficult it is to recover normal plants.

Cryopreservation of FEC may provide a means of ensuring more genetic stability of cell lines. Therefore, our objective is to develop a method to cryopreserve FEC for cassava, to have a source of "fresh" FEC useful for genetic transformation.

Methodology

We used the encapsulation-dehydration procedure developed at CIAT (Annual Report 1999) to test it with FEC. Different steps, e.g., pre-growth, encapsulation and dehydration, were adjusted to obtain re-growth before the freezing phase. This allows knowing the effects of treatments previous to freezing, therefore selecting the best ones before the actual freezing step.

Result and discussion

We tested the effect of different sucrose concentrations (2, 6, 12 and 17%) on cell growth. It was observed that tissue growing on 2% developed faster, while with 17% sucrose it did not growth. Moderate growth was obtained at 6-12% sucrose.

Beads pre-treated with 6-12% sucrose for 3, 5 or 7 days were dehydrated on silica gel during 4, 6 or 8 hours. The following two treatments allowed to control the size of the beads and to recover tissues after the treatment:

- 6% sucrose, during 7 days, and 4 hours of dehydration
- 12% sucrose during 5 days, and 8 hours dehydration on silica gel

Different concentrations of sodium alginate (1.5, 2 and 3%) and calcium chloride (75 and 100 mM) were tested for bead formation. Beads prepared with 1.5% were unstable and did not resist manipulation. The best bead quality was obtained with 2% alginate and 75mM $CaCl_2$.

Conclusions

- We established a relation between FEC (1 gr.) and alginate (10 ml) for bead formation.
- FEC pre-grown on GD6-50Pi, before encapsulation, increased its weight to 21% (fresh weigh) in 14 days.
- FEC was recovered from beads pre-treated with sucrose (6-12%) during 5-7 days, and dehydrated on silica gel (4-8 hours).
- Differential growth response of encapsulated tissue was observde depending on gelling agent. Media containing agar was better than media with gel rite.

Future activities

- Establish procedures for freezing and initiate experiments.
- Recover growth of frozen tissue and reestablish the FEC culture.

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Annual Report 1999. Project SB-02. pp. 89-91

2.3.5 Development of a low cost, simplified, tissue culture system for cassava

R. Escobar, C. Hernandez, J. Restrepo. J. Tohme and W.M. Roca

Introduction

Based on CIAT's experiences in handling the cassava's world collection we modified and simplified the *in vitro* propagation method. Tissue culture related materials and equipment where replaced by those more frequently used in regional agriculture practices.

Methodology

A pilot experimental station was established in Santa Ana, Department of Cauca. A group of eleven women was selected to transfer a low-cost micropropagation methodology. C. Hernandez (a farmer selected by his own community) has been trainied at CIAT to transfer the technology to his community. In the same way, we implemented a low-cost facility at CIAT', where we are trying modifications to the propagation system, that allow increased propagation rates and reduce inputs.

Results and discussion

Propagation media, using local ingredients such as nutrients (salts) and growth regulators were designed (®Hormonagro 0.02 mg/l and ®Progibb 0.05-0.5 mg/l) (Annual Report 1999). Using these media we could obtain the same propagation rate (1:3) as with 4E medium (Roca 1984).

Santa Ana's tap water was analyzed (low ionic charge) and used as media component instead of commercial water bottles. Medium 9 (Table 1), supplemented with pineapple (0.5% w/v) that farmers cultivated it in the area, increased propagation rate, increasing also the general appearance of plants (healthier and stronger than without pineapple).

Table 1: Effect of supplementing media on propagation rate .

Media	Supplementation	Concentration (% W/V)	Propagation rate
9	Banana	1	1:2.6
		5	1:1.5
		10	1:2.7
	Coconut water	5	1:3.6
		10	1:3.2
	Tomato	0.5	1:3.3
		2	1:2.9
	Pineapple	**0.5**	**1:4.3**
		2	1:1.6
10	Tomato	0.5	1:3.4
		2	1:2.7
	Pineapple	0.5	1:3.5
		2	1:3.5

To prepare the media, we did not use commercial formulations like Murashigue and Skoog (1962). Instead we chose commercially available media components like sodium nitrate, magnesium sulfate, urea, sodium phosphate and ®Microcoljap-12. All of them were available in agrochemical stores at low price.

As with RITAs (see this report), the need of only one medium to induce roots, regardless of the type of explant, was also achieved here.

Conclusions

- FIDAR sign a Material Transfer Agreement (MTA) to obtain certified material of MCOL 1522, MBRA 383 and M523-7 from GRU to use with farmers
- The methodology is being validated with a women group in Santa Ana, Cauca
- All propagation media components were replaced by locally bought cheaper ingredients.

Future activities

- A scheme for production of certified planting material with MCol 1522 (a clone widely accepted by farmers) will be implemented
- The community is already planing on adapting crops like plantains to her facilities
- A cost analysis may need to be established
- Santa Ana's group is producing planting material for experimentation plots

Poster

Participatory research in the development of low-cost input, *in vitro* propagation method for cassava. R.H. Escobar, C. Hernandez, J. Restrepo, J. Tohme and W.M. Roca. *In:* Uniting science and participation in Research. III International seminar. Nairobi, Kenya 6-12 Nov. 2000

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2.3.6 Cassava micropropagation for rapid "seed" production using temporary immersion bioreactors.

R.H. Escobar, L. Muñoz and W.M. Roca.

Introduction

In the Northern Coast of Colombia cassava has been considered the most important alternative crop since it is the only crop that yields acceptably under marginal conditions with minimum inputs.

More than 250,000 ha are cultivated by small farmers, with cassava occupying a third of this area. According to CORPOICA, in the coming years, more than half of this area will be cultivated with cassava alone, which will make the quality and quantity of planting material a constrain. We are using a massive propagation system (RITA) to produce enough planting material of desired, indexed commercial clones.

Methodology

We adapted the RITA system for two clones: MCol 2215 (Venezolana) and MCol 1005 (Verdecita), which are widely accepted by farmers. We tested immersion periods of 1 minute every 4 or 6 hours, 5 minutes every 8 hours, and 10 minutes every 12 hours. We also tested different concentration of TDZ (0.024 and 0.001mg/l) as growth regulator. The objective of using RITAS is to find a propagation scheme to increase rates, reduce time, or both.

Results and discussion

The longest immersion period (10 min every 12h) reduced hiperhidricity, one of the most important problems associated with high growth regulator concentration and liquid propagation media. It also reduced propagation rate to 1:4 or 1:5, if compared to previous results obtained with RITA (1:6 to 1:8; Annual Report 1999). It is desirable to avoid hiperhydrated tissues since they usually have problems with propagation and post-flask management.
Tissue grown on 12h period had better appearance: solid green color, thick stems, although it had increased root formation. Excessive root formation is a problem since the roots penetrate deep into the foam and they get damaged when removed from the RITA. We are testing an alternative connection using two RITA recipients: one contains the medium and the other the tissues without foam (see explanation in Figure 1). Eliminating the foam will reduce damaging the roots and the excessive media retention, which increases hyperhydricity.

We found that high temperatures, possibly due to the light system set up in the growth room (tubes set at the side and not at the top of shelves), increased hyperhydration. We decided to set 3 light tubes at the top of each shelf, leaving 45 cm between the tubes and the bottom of the shelf (Berthouly, personal communication). These conditions aid to reduce temperature, light, humidity and hiperhydricity.

We also found that the evaluation time could be reduced to 45 instead of 60 days, saving roughly 25% of the time required. Also, adding Tween to the medium increased propagation rate with MCol 1505 but not with MCol 2215.

One more advantage of RITA for cassava propagation is the need of only one medium to induce roots, regardless of the type of explant. Previous protocols established for propagation needed two media: 4E (Roca 1984) for propagation using buds and shoots, and 17N (Roca 1984) for rooting shoots only; it does not work with buds (Escobar, 1991). With the RITA system, transfer of rooted plants to the greenhouse has taken as few as 20 days. We have already transferred rooted plants to the screen-house with high recovery rates.

CO_2 1min / 12 Horas Vs
$- CO_2$

Conclusions

- The use of TDZ as growth regulator improved propagation rates but increased hiperhydricity.
- Hyperhidricity was partially diminished by using different immersion periods. Longer elapse times between immersion periods reduced hiperhidricity, although it reduced propagation rate as well.
- The operational principle of RITA was changed from immersion to submersion. With submersion the tissues are bathed by the liquid without being completely immersed in it.
- Surfactant agents may be used in propagation media to increase propagation rates.
- Double RITA connections (Figure 1) may be used as a new propagation scheme. Explanting double RITA connections with longer, uncut stem sections, carrying multiple nodes, reduces explant manipulation.
- We are adapting some of the RITA conditions developed for cassava to other CIAT crops. RITA may help to improve embryogenesis in rice and Brachiaria (see this Annual Reports)
- Rooting could be done in RITAs without foam support.
- Our new conditions may reduce greenhouse management and costs per plant.

Future activities

- Keep fine-tuning conditions to improve propagation rates reducing hiperhydricity.
- Field test plants produced in RITAs.
- Initiate tests for other cassava clones. → Tabla Pasto
- Adapt low cost systems using local constructed bioreactor.
- Initiate test for vigor respond of RITA propagated plants

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NAI Prop × Llevar a Campo

8-6% 17N

160

CG1141-1 → 7 mzp. × 20 = 140
CM3306-4 → 13 mz × 20 = 260
(4) → CM3806-19 → 7 × 20 = 140
CM3555-6 → 20 × 20 = 400
M001/525 → 18 × 20 = 360
M001/215 → 8 × 20 = 160
SG13 765.2 → 3 × 20 = 60
SG13 761.4 → 4 × 20 = 80
TAI 8 → 22 × 20 = 440
MCOL 1505 → 12 × 20 = 240

Marzo 21/2001
5 tubos / 1 planta/ud

2.3.7 Temporary immersion system (RITA) for callus embryogenesis of rice

E. Tabares, G. Delgado, R, Escobar, Z. Lentini

Plant *in vitro* culture using temporary immersion offers all the advantages of a liquid medium system (automation, large scale production, easy changes of medium, filter sterilization, easy cleaning) without any of its drawbacks (reduced gas exchange, vitrification). The RITA device has the additional advantages of low cost and automation. And it is suitable for both industrial production and research use. This system has proved its efficiency for somatic embryogenesis of banana (Alvarat *et al*, 1993; Escalant *et al*, 1994), coffee (Berthouly *et al*, 1995; Etienne *et al*, 1997), citrus (Cabasson *et al*, 1997), oil palm and rubber plant (Etienne *et al*, 1997), and at CIAT for cassava (Escobar and Roca , 1999); for clonal propagation through micro-cuttings of coffee, and sugar cane (Lorenzo *et al*, 1998); for proliferation of meristems of banana, and pineapple; and for micro-tuberization of potato (Teisson & Alavarad, 1998). RITA system improves plant cell nutrition and gas exchange, and enhances the quantity and quality of micro-propagated tissue. One of the major bottlenecks for generating transgenic plants derived from zygotic embryos as in the case of rice, is the generation and maintenance of sufficient large number of embryogenic callus. Following is reported preliminary experiments directed to induce embryogenic callus of rice using the automated RITA system.

Mature embryos were removed aseptically from the seeds of Ciac8 variety with the aid of a dissection microscope and plated onto 20 ml of solid callus culture medium in a petri plate. Fifty embryos per RITA (6 hours immersion) and 50 embryos (25 per plate, control) were evaluated. Two replications were tested per treatment.

Callus induction was noted 8 to 15 days earlier in the RITA system respect to the control (Table 1). A larger number of embryos showed callus formation, and between 2 to 4 fold increase in the number of embryogenic callus was seen in the automated system. Experiments are in progress to determine the reproducibility of these results with various *indica* genotypes, and the regeneration capacity of the callus induced from RITA system.

Table 1.- Induction of embryogenic callus of rice in RITA system

Treatment	Embryos cultured	Callus induction (days)	Embryos with callus	Embryogenic callus
RITA 1	49	20	49	1370
Control	49	35	35	380
RITA 2	50	35	46	700
Control	50	43	40	300

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2.3.8 Optimization of RITA system for an automated mass production of embryogenic callus of *Brachiaria*

C.P. Flores[1], R. Escobar, M.C. Duque, Z. Lentini
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Introduction

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 nutritional 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 back-cross 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 towards the establishment of a protocol for a reproducible and efficient method for *Agrobacterium* mediated transformation of *Brachiaria* species. One of the major bottlenecks encounter is the availability of sufficient starting material (embryogenic calli derived from mature zygotic embryos) for co-cultivation with Agrobacterium, and the low and variable rate of conversion from callus into plants. This year we report developments to optimize a protocol for the massive generation of embryogenic callus using the temporary automated RITA system, and to obtain a high rate of conversion from callus into.

To standardize a methodology for genetic transformation of *Brachiaria* it is important to have sufficient starting material in optimal stage of development. The experience with tissue culture of *Brachiaria* is limited to the works done at CIAT (Lennis, 1992 and Galindo, 1997). The availability of material for transformation has been one of the major limiting steps, therefore it is necessary to explore new alternatives to improve the quality and quantity of embryogenic callus required to conduct experiments for optimizing the transformation protocol.

The principal objective is to test the induction of somatic embryogenesis in *Brachiaria* using the RITA® system. The temporary immersion system , commercially known as RITA®, has been successfully used with some species of plants like banana (Alvarat *et al*, 1993; Escalant *et al*, 1994), coffee (Berthouly *et al*, 1995; Etienne *et al*, 1997), *Citrus* (Cabasson *et al*, 1997), rubber tree (Etienne *et al*, 1997), potato (Teisson & Alavarad,

1998) and sugarcane (Lorenzo *et al*, 1998). More recently at CIAT, the RITA® system has been used by Escobar and Roca (1999) for cassava micro-propagation. Some advantages of RITA® are mass production, decrease time production, synchronization of somatic embryogenesis and reduction in labor costs through a simplified handling of plants and medium.

Seven days old mature zygotic embryos of *B. decumbens* cv. basilisk from CIAT accession 606 were used. Fifty explants for treatment were used. A total of two replications were treatment were tested. One immersion period (1 min / 6 hours) was evaluated and compared with conventional induction system used for *Brachiaria*. In both cases *Brachiaria* induction M1 medium was used (Lenis, 1992; Galindo, 1997).

The evaluation was done for 35 days and expressed in terms of:

- Number of days to formation of first embryo
- Number of days to formation of embryos in 50% of explants
- Number of total embryos per treatment
- Shape of embryos (number of embryos of different developmental stages)

Preliminary results indicated that RITA® system reduced the time of embryo formation in one week (Figure 1), and increased by two fold the production of embryogenic respect to the conventional culture on solid medium (Figure 2).

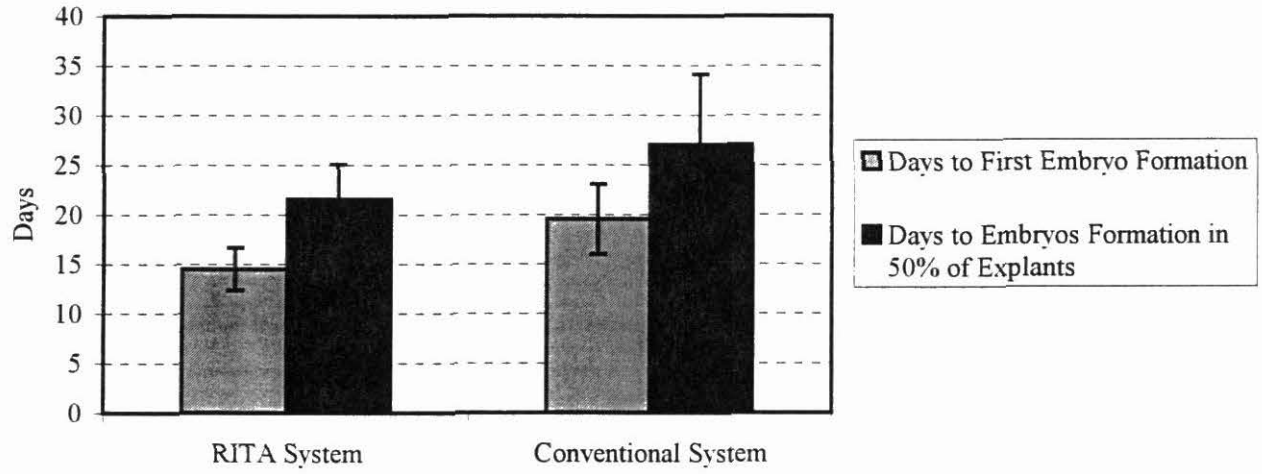

Figure 1. Embryogenesis of *B. decumbens* cv. basilisk in conventional and RITA® system.

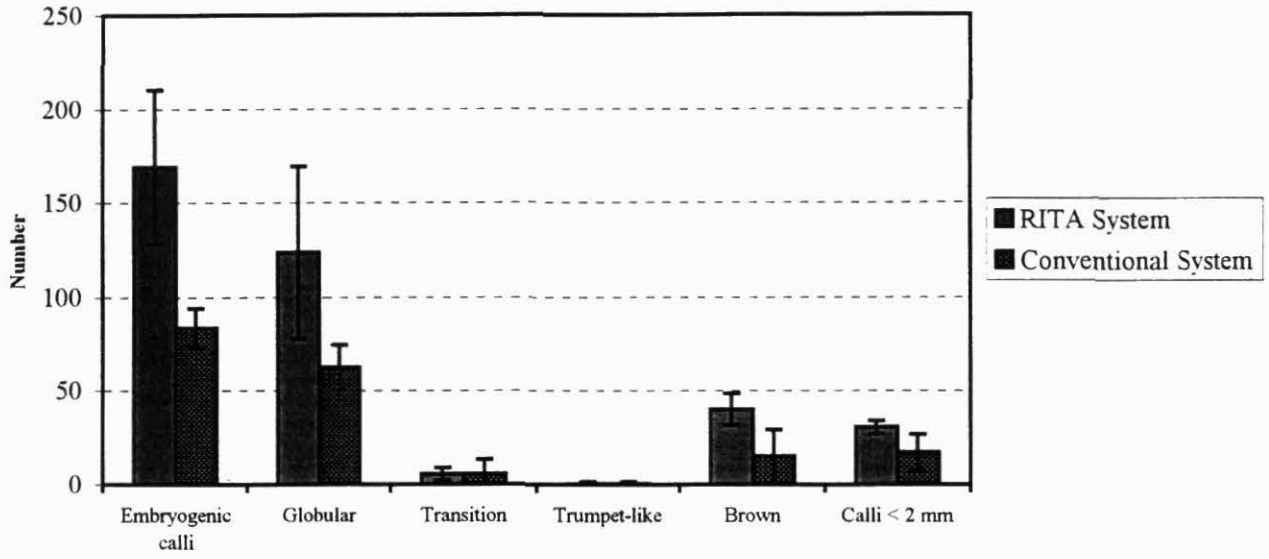

Figure 2. Number of embryos at number developmental stages using RITA® and conventional system for embryogenesis somatic in *Brachiaria.*

Future Prospects

- Increase the number of tests to the two systems.
- To use RITA® as a conventional methodology to induce somatic embryogenesis in *Brachiaria*.
- Use RITA® system to improve somatic embryo development in *Brachiaria*.

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2.3.9 Effects of medium composition on callus induction and plant regeneration in *Brachiaria decumbens*

C.P. Flores, M.C. Duque, Z. Lentini

B. decumbens is one of the most important pastures of tropical lowlands. Earlier work at CIAT included the establishment of some tissue culture methods for plant regeneration (Lenis, 1992) and genetic transformation by particle bombardment (Galindo, 1997). Last year we reported the progress made on the genetic transformation of *Brachiaria* via *Agrobacterium*. However, the efficiency of transformation is highly influenced by the physiological stage and the type of embryogenic callus. When using the conventional regeneration medium for *Brachiaria* (Lenis, 1992; Galindo, 1997), it is not possible to observe the different developmental stages of somatic embryos. In general, most callus show a pre-embryogenic development, and few follow a normal development and regenerate plants. Most of these pre-embryogenic callus turn brown after isolation, its growth is highly inhibited, and finally the tissue dies. Following is described a set of experiments directed to improve induction of somatic embryo formation, and the rate of conversion into plants.

Improving somatic embryogenesis in Brachiaria decumbens

Somatic embryogenesis of *Brachiaria* was induced using various culture media compositions. The basal medium consisting on MS salts and 3% sucrose, for somatic embryos induction was complemented with 2,4-D 2 mg/l (1). Somatic embryos a globular stage were transferred onto medium containing 1 mg/L and, embryos with advanced stage of development were placed on medium with BAP 0.4 mg/L, NAA 0.1 mg/L, and GA_3 2 mg/L for embryo elongation. Elongated embryos were then transferred to regeneration medium consisting on MS basal medium 0.1 mg/l NAA and 0.4 mg/l kinetin. Most embryos developed into plants when transferred to regeneration medium. The induction, maturation and germination of somatic embryos were reproducible using

this new protocol described above. For the first time, the three stages of development (globular, transition and trumpet-like) of *Brachiaria* somatic embryos were observed. The established protocol allowed plant regeneration at higher averages than those obtained with previous methodology.

Evaluation of Effects of Antioxidants Compounds on Brachiaria Callus Viability

Embryogenic calli of *B. decumbens* from CIAT accession 606 were placed on M1 medium (Lenis, 1992; Galindo, 1997), supplemented with antioxidant compounds as describe in Table 1.

Table 1. List of antioxidant compound added to M1 medium for callus induction

Treatment	Antioxidant Compound
T_1	Ascorbic Acid 100 mg/L, L-cysteine 100 mg/L, 2 mg/L Silver Nitrate
T_2	Citric Acid 150 mg/L
T_3	Citric Acid 100 mg/L, Ascorbic Acid 100 mg/L
T_4	Activated Charcoal 0.2%
T_5	none
T_6	PVP 1g/L

Callus viability was evaluated at 7, 14 and 21 days after. Rating were conducted using the following scale (0) 0% viability; (1) 25% viability; (2) 50% viability; (3) 75% viability, and (4) 100% viability. The initial size of callus was measured at time zero.
None of the antioxidant compounds did not decrease cell death rate in *Brachiaria* (Figure 3). Callus viability was highest at 7 days (75% viability) and 14 days (50% viability) when using activated charcoal (T_4), but not significant differences with the other treatments was noted at 21 days. There was a correlation between the size of the callus and its viability. Callus smaller than 2 mm, had a higher probability of browning and dying. Browning increased with time, and it was more evident after the first two weeks. Results suggest that activated charcoal maybe used to delay the browning process at early stages, but is likely that other medium components need to be added in order to stimulate the callus growth during this period. Next experiments will include the addition of various aminoacids and varying the NH4: NO3 ratio seeking a higher rate of embryogenic callus development.

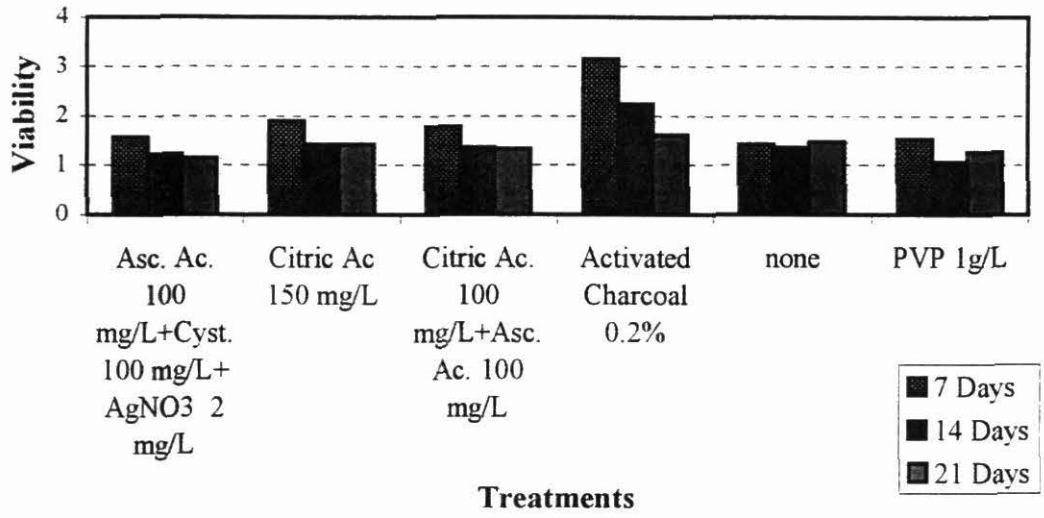

Figure 3: Response of Brachiaria embryogenic callus to different treatments with antioxidant compounds (see table 1 for treatments)

Induction of callus embryogenesis using two types of carbon source (sucrose and maltose)

The effects of two different concentrations of maltose (3% and 4%) and one of sucrose (3%) were tested on the induction of somatic embryos. Three seed batches, from CIAT accession 606, were also compared. The following seed lots were used :lot 95-065 (harvested on 13-10-95) , lot 99-034 (harvested on 18-06-99), and lot 99-005 (harvested on 23-03-99). Nine treatments (three per each seed lot) were evaluated, using 15 mature germinated embryos per treatment. The evaluation was expressed as total number of embryogenic callus developed.

No differences in response between seed batches were noted in all three treatment (Figure 4). Maltose 4% maltose seemed to increase embryogenic callus production (Figure 4, batches 2 and 3). But the difference was not statistically different. More replications using maltose 4% will be tested to evaluate the reproducibility of this increased response. Plant regeneration from callus induced with these various concentrations of carbon source is in progress, to determine its effect on regeneration capacity.

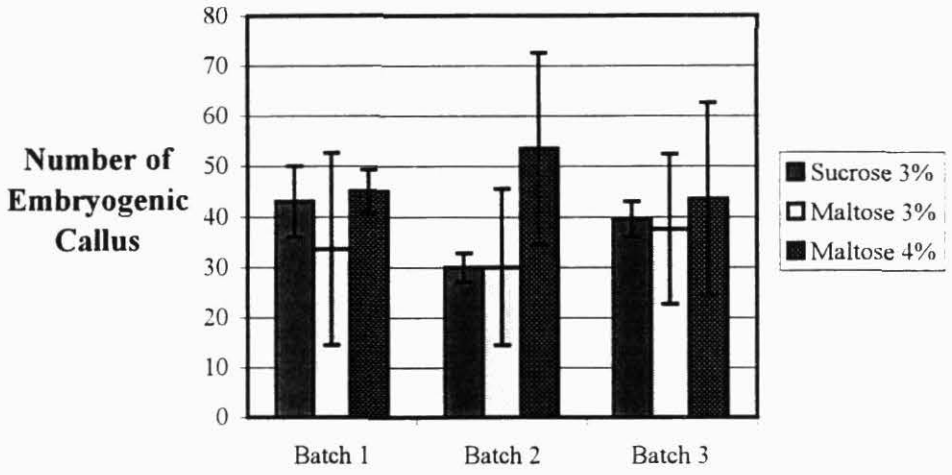

Figure 4.- Induction of embryogenic callus using maltose and sucrose and different seed batch.

Future Prospects

- Test the effects of N and NH_4 / NO_3 on the browning process
- Test regeneration (conversion to plantlets) of embryos induced on maltose
- Test the transformation potential of embryos in different stages using *Agrobacterium* as vector

References

Galindo, L. 1997. Transformación genética de la gramínea forragera *Brachiaria spp*. Mediante la técnica de bombardeo de partículas. Tesis Ing. Agr. Universidad Nacional de Colombia. Sede Palmira. 130 p.

Lenis, S. 1992. Regeneración de plantas de la gramínea forragera tropical *Brachiaria spp* a partir de tejidos cultivados *in vitro*. Tesis Biología Universidad Santiago de Cali. 80 p

2.3.10 *In vitro* propagation and plant regeneration of lulo (*Solanum Quitoense* Lam.)

[1]V. Segovia, [1]I. Sánchez and W. Roca.
[1]Corpoica

Biotechnological advances in the improvement of tropical fruits is progressing albeit slowly, the need for the development of improved varieties of these species through genetic transformation has been identified as an important tool for achieving this goal. This essentially requires the development of an efficient system of plant regeneration, in addition to the establishment of an efficient methodology for the genetic transformation. Equally important is the necessity to standardize *in vitro* propagation techniques as a method for producing disease free planting material of novel hybrid varieties.

Research efforts initiated in July 1999, has resulted in the development of an efficient regeneration protocol and an optimized *in vitro* propagation procedure for selected varieties.

Methodology

In vitro shoot cultures of five different accessions of lulo were kindly provided by the Centro Frutícola Andino-CEFA (La Union, Valle, Colombia). For *in vitro* propagation different media and techniques were used. These were developed based on the methodologies used for cassava at the BRU (table 1).

For *in vitro* regeneration, the explants were pretreated with a sonicator (Branson™ Sonifier 450 watt). For this, whole leaves were submersed in liquid RAS medium containing 2mg/l of BAP or Zeatine, and treated for 1 minute at output level 4. Once sonicated, the explants were divided in leaves and petiols, and placed several culture media in petri dishes (Table 1).

Results

Optimization of the methodology of *in vitro* micropropagation

The first regeneration report on organogenesis in lulo involved the use of tissues isolated from plants obtained from *in vitro*-germinated seeds (Hendrix. *et al* 1987). However, the use of this kind of tissues is not advisable due to the high variability generated through sexual reproduction of this allogamous species.

For producing genetically, and physiologically homogeneous tissue for plant regeneration and transformation experiments, a methodology for *in vitro* propagation was optimized.

Initially, a methodology similar to the one used routinely at the BRU, to micropropagate cassava, was applied with some success. This consisted in dividing *in vitro* cultured plants in micro-cuttings of one inter-nodal segment with one node, and inserting them in agar solidified 4E medium (Mafla, personal communication). However, only two of five different varieties could be propagated on this medium. The other did not produce adventitious roots and remain small and weak.

After testing several modifications of the culture medium, and different culture conditions, we found one that was optimal for the development of lulo plantlets in vitro for all 5 different clones tested. These conditions included culturing micro-cuttings in: (i) 4E medium with half of the concentration of the MS-Salts; (ii) at a temperature of 24 oC and (iii) in tubes covered with vented plastic caps (with polyurethane foam) in order to allow gas exchange.

Table 2. Culture media used for the induction of organogenesis in lulo.

Media components*	**RAS**	**T1**	**T2**	**T3**			**T4**
		Litz	*modified Litz*	*Latin Square*			*Ultzen*
MS** salts (g/L)	2.25	4.5	4.5	4.5			4.5
Vitamins MS (mg/L)	-	104	-	-			-
Vitamins B5 *** (mg/L)	112	-	112	112			112
Sugar (g/L)	20	30	10	20			10
Glucose (g/L)	-	-	10	-			10
Gel Rite (g/L)	-	2	2				3
Agar (g/L)	-		-				1
Casein enzymatic hydrolysate (mg/L)	200						
L-Asparagin (mg/L)	750						
pH	5.7-5.8	5.7	5.7	5.7			5.8 - 5.8
BAP or Zeatine (mg/L)	2.0						
BAP (mg/L)		-	2	0.3	1	3	-
ANA (mg/L)		-	-	0	0.1	0.3	-
KIN (mg/L)		5	-		-		-

* Premixed media and vitamins purchased from Duchefa
** Murashigue and Skoog basal salts (1962)
*** Gamborg et al. 1968

After 4 weeks of culture of the micro-cuttings under these conditions, the youngest developed leaves were taken for the plant regeneration and transformation studies.
A rate of hundred percent survival of the *in vitro* propagated plantlets was obtained if they were transferred after six weeks of culture to the greenhouse.

The optimized micropropagation methodology can be used for multiplying lulo varieties for producing disease-free planting material. Some existing varieties (like "La Selva"; Bernal *et al.* 1998), and new ones that are being developed (M. Lobo, Corpoica, pers.

Communication), are not pure lines but early generations of interspecific hybrids, and would lose their agronomic characteristics if propagated sexually.

Development of an *in vitro* regeneration protocol through organogenesis

Methodologies for inducing of organogenesis in lulo leaf explants, described by Hendrix *et al.* (1987), were tested with several modifications. There was no success in regenerating plants. The reason for this could be that we used different genotypes in the experiments. Only after applying an ultrasonic pretreatment to the explants (petiole and leaf blade of the first or second youngest leaves of *in vitro* propagated plants) in a liquid BAP- or Zeatine-containing medium, before they were placed on the solidified medium, an organogenic response was induced. Supplementing the medium with glucose (Ultzen *et al.* 1995; H. Ramirez, pers. communication) seemed to be also important for the induction of organogenesis. Under the best-tested conditions, 25% of the explants produced adventitious buds. These buds developed plants easily after subculture on hormone-free medium.

Conclusions

The methodology for micropropagation of lulo was optimized to produce vigorously growing, well-rooted plantlets of all five genotypes tested. This optimized methodology can be used for both, massive *in vitro* propagation and the production of genetically and physiologically homogeneous tissue for plant regeneration and genetic transformation. Also, a protocol for in vitro plant regeneration through organogenesis was developed. This is the first protocol developed using tissues from selected, Colombian varieties of lulo. The use of this protocol, or an optimized version of it, may allow the production of transgenic plants of this species.

Future Plans

- To carry out experiments for increasing the efficiency of regeneration.
- Publish results from studies on micropropagation and regeneration.
- Develop a methodology for genetic transformation of selected varieties.

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Ultzen, T.; Gielen, J.; Venema, F.; Westerbroek, A.; Haan, P.; So, M.; Schram, A.; Grinsven, M. and Goldbach, R. 1995. Resistance to tomato spotted wilt virus in transgenic tomato hybrids. Euphytica. 85: 159-168.

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OUTPUT 3 COLLABORATION WITH PUBLIC AND PRIVATE PARTNERS ENHANCED

Main Achievements

- CIAT obtained approval of a project from BMZ, Germany, to evaluate gene flow from beans and rice into weedy and wild species relatives. Partners include scientist from the European Biosafety Commission and from Costa Rica and Peru, where local experiments will also be conducted. With this project will initiate studies on environmental biosafety jointly with the generation of transgenic plants to set guidelines for their safe use in agriculture.

- During the period of Oct 1999-Sept 2000 a total of 59 researchers received training with Sb-2 staff.

- The First Workshop on the Molecular Genetic Diversity of Cassava Network was organized at CIAT by M. Fregene

- A training course on bioinformatic was organized for the SB-02 assistants and post doc. The course was given by a Cornell graduate and ex CIAT assistants from the BRU. As a results, a series of bioinfrmatic activities were conducted to provide CIAT with local data search capacities fro GenBank sequences.

- FloraMap was officially released in 2000. Some 190 registered users from several countries have obtained a copy.

- Two assistants from the genome lab received full fellowships to attend two advanced training courses on sequencing and bioinformatics at the Cold Spring Harbor Lab US.

- A patent on propagation of *Annona* was filled in collaboration with BIOTEC.

- In the period of Oct 1999-Sept 2000, SB-02 projects members published 19 Scientific papers in referred journal and books, at least 10 abstracts and posters in conference proceedings and 3 theses. At he same time staff members gave keynotes presentations at international and regional meetings.

- In the period of Oct 1999-Sept 2000, projects members increased the contacts with private sector at the regional and international level to establish collaborative projects on technology transfer and to obtain freedom to operate for key technologies. Same kind of activities were established with regional NGOs in order to transfer CIAT technologies.

- In the same period, 8 proposals were approved and 5 more submitted

- A total of 19 organizations contributed to funding projects in SB-2.

ACTIVITY 3.1 New collaborative arrangements and organization of workshops and training courses

3.1.1 First Workshop on the Molecular Genetic Diversity of Cassava Network (MOLCAS), held At CIAT August 22-24, 2000.

M. Fregene

Introduction

The molecular genetic diversity network of cassava (MOLCAS) comprises of scientists drawn from institutes in Malawi, Uganda, Tanzania, Nigeria, Brazil, Sweden, Colombia, France, and the USA, and it is funded by the International Chemical Sciences Program (IPICs), University of Uppsala. The goal of the network is to enhance the enduring and emerging roles of cassava as a food security crop and industrial crop by the assessment and exploitation of genetic variation. The network assists members, with tools, information, and in some instances funds, with the collection molecular and agronomic characterization of cassava land races in Africa and Latin America.

Studies concluded to date by members of the network include:

- A SSR study of cassava land races in Northern Malawi (Bvumbwe Agricultural Research Station Malawi, SLU, Uppsala, Sweden)
- A SSR study of cassava land races in Southern Tanzania (CIAT, SLU, ARI-Mwanza, ARI-Mikocheni Tanzania)
- A molecular marker SSR study of cassava land races from the Amazonian basin and North Eastern coast of Brazil (ICA-Campinas, Brazil)

Ongoing studies include:

- SSR study of a cassava collection from the Amazonian region of Colombia (CIAT, Uni Valle Colombia)
- SSR study of land races in Nigeria (IITA, NRCRI Nigeria, CIAT)
- Test for heterotic (hybrid vigor) patterns in clusters of previous collections (CIAT).

Summary of Workshop Report

After 2 years of the existence of the network, it was decided to hold a workshop to review progresses, and discuss future perspectives. The first MOLCAS workshop was therefore planned for August 22-24, 2000 at CIAT headquaters, Cali, Colombia. Invitations were sent out in May to members and interested CIAT participants. Two days were spent reviewing the results of cassava collections and their analysis with molecular markers in Brazil, Malawi, Tanzania, CIAT, and IITA collections, the third day was spent discussing future projects and deciding on priorities.

A total of 8 presentations were made during the workshop. Three talks dwelled on new methodologies, namely: recent advances in plant genomics and the implications for study and use of genetic diversity; GIS tools for guiding collection of genetic diversity; and the need to involve farmers in the study of genetic resources. There were 3 genetic diversity assessment reports, conducted in Brazil, Malawi and Tanzania respectively; the other 2 presentations were from CIAT on the study of genetic diversity in wild relatives and the CIAT cassava collection. The presentation on advances in plant genomics, given by Dr Matthew Blair, CIAT bean geneticist, on behalf of Prof Steve Kresovich, was a good introduction to advances and powerful new tools of plant genomics that are redefining traditional thinking on genetic diversity. In particular, the new methods of uncovering bi-allelic, highly abundant single nucleotide polymorphisms (SNPs) promises to provide unprecendented resolution for genetic studies.

The studies of genetic diversity assessment of cassava land races in two African and one Latin American country provided new evidence on the elucidation of the genetic structure of cassava in its primary and a secondary center of diversity. The studies, conducted with SSR, AFLP, and RAPD markers revealed genetic differentiation in the land races along lines that are not fully understood at the moment, although some evidence points to bitter and sweetness of roots, and multiple introductions. Questions raised about the underlying reasons for observed organization of genetic variation and the preservation of the genetic structure in the primary and secondary centers of will be the objective of further studies. Finally, we were reminded about the wisdom of properly marrying farmers' knowledge of cassava with molecular tools to make the best interpretation of results.

Future Activities

IPICs, the principal donor of MOLCAS has invited the network to apply for funding during the period 2001-2003, the last session of the workshop was therefore spent brainstorming projects the network considers priority for the future. Anke Van de Hurk, IPGRI scientist based at CIAT, served as a very able facilitator in this process. In the period 2001-2003, the network has prioritized the following studies:

- SSR marker study of the role seedlings play in preserving genetic diversity during the disease epidemics (Uganda)
- A study of broad adaptation of cassava genotypes in Southern Africa (Malawi, Tanzania, Mozambique, Uganda)
- Farmer keys for identification of their varieties (Malawi).
- A study of broad adaptation of cassava genotypes in Brazil (Brazil)
- Continuation of the country study of genetic diversity structure (Democratic Republic of Congo, Ghana and Mozambique)
- Development of a set of highly polymorphic markers for studying genetic diversity in cassava

3.1.2 Collaborations with public and private sectors

- Three members of SB-2 (W Roca, A Bellotti and P Chavarriaga) visited Novartis Agribusiness Biotechnology Research, Inc., (NC-USA) to advance collaboration on technology transfer for cassava transformation (genes for insect resistance and non-antibiotic selection), as well as for bio-assaying gene products against cassava's main insect pests.

- Four members of SB-2 (S. Beebe,M. Blair, C. Martinez and J. Tohme) visited Novartis facilities in Minnesota to discuss marker assisted selection and the Univ of Minnesota to discuss bioinformatics and legume genomics. Previously, J. Tohme had visited Novartis in Toulouse, France to discuss MAS and databases.

- Two members of SB-2 (M. Blair and J. Tohme) visited Monsanto and the Danforth center to discuss possible collaboration on cassava and molecular biology.

- Novartis-Colombia held conversations with SB-2 members on greenhouse testing of transgenic maize lines against maize's major insect pests (i.e., *Spodoptera* spp).

- Aventis-Colombia also held conversations with SB-2 members for greenhouse- and field-testing of transgenic rice lines with resistance to herbicides. They also were interested in developing bioassays for in-vitro testing of fungicides against *Mycosphaerella fijiensis* (Sigatoka negra).

- CIAT jointly with Corporación BIOTEC filed a patent, in Colombia, for in vitro propagation of *Anonna muricata* (Soursop) via cyclic micro-grafting (SIC # 00076031, 06-10 of 200).

- Two staff (W. Roca and J. Tohme) attended the sustainable agriculture in the new millennium the impact of biotechnology on developing countries organized by NGOS (OXFAM and Friend of the Earth). J. Tohme gave a plenary presentation on the role of biotech for sustainable agriculture.

- Z.Lentini visited DuPont to explore possibilities of strategic alliance collaboration was conducted on April 2000. Main contact Dr. Barbara Valent. . ZL also visited Pioneer to explore possibilities of strategic alliance. Main contact Dr. Paul Olson and Larry Beach. A follow up will continue to define possible collaboration in the area of quality traits.

- SB-02 staff took an active part in the IP audit from coordinating activities to participation in the review of CIAT IP.

- Two members of SB-2 (R. Escobar adn J. Tohme visited Maizena- Corn Product International, Seccional Barranquilla. to discuss the transfer of CIAT cassava

propagation technologies to the Coast of Colombia. A agreement was reached for training.

- The collaboration with the Humboldt coninued in the forms of joint projects and training.

- Two assistants from the genome lab received a full fellowship to attend two advanced training courses on sequencing and bioinformatics at the Cold Spring harbor Lab US. The courses a very competitive and only 12 -15 participants are accepted.
- Three staff (C. Mba, E. Gaitan and A. Almeida) received training in the US on the use of the microarray system of Hitachi.

- SB-02 staff collaborated with CorpoICA, Cenicana, Corporación BIOTEC and Cenicafe researchers and provided molecular biology and transformation expertise to these centers.

3.1.3 Scientific Meetings

- CGIAR workshop on nutritional quality, IRRI, October, 1999. Talk entitled "Research on Trace Minerals in Common Bean", (Steve Beebe, Alma Viviana Gonzalez, Judith Rengifo)

- Beebe, S., A. Velasco, y F. Pedraza. 1999. "Marcaje de genes para rendimiento en condiciones de alto y bajo fósforo el las accesiones de frijol G21212 y BAT 881". Poster presented at VI Meeting of RENAFE, November, 1999, Salvador, Brazil.

- Cortéz D, Almeida A, Escobar R and Chavarriaga P. Invited speakers at "IV Congreso Colombiano de Genética, Popayán" 21-25 February 2000. The following talks were given: "Conociendo el Germoplasma Vegetal Mediante Marcadores Moleculares", "Mapeo Genómico", "Plantas Transgénicas: La Polémica", "Preservando y Propagando la Diversidad del Germoplasma"

- Chavarriaga P, "Herramientas de la Biotecnología para Reforzar la Agricultura en Colombia: El Caso de la Yuca", invited speaker at "Seminario Tópicos Actuales de Investigación en Genética Conmemorativo 15 Años Grupo GETEG", sponsored by GETEG-UNIVALLE-ACCB, June 6-7, 2000, Cali, Colombia.

- Escobar R, "El uso de la biotecnologia en la conservacion y multiplicacion de la yuca", invited speaker at "Seminario Tópicos Actuales de Investigación en Genética Conmemorativo 15 Años Grupo GETEG", sponsored by GETEG-UNIVALLE-ACCB, June 6-7, 2000, Cali, Colombia.

- Chavarriaga P. Invited speaker at "XXXIV Congreso Nacional de Ciencias Biológicas, Octubre 27 al 30 de 1999, Santiago de Cali, Colombia". Panel I: Genetically Modified Organisms.

- Chavarriaga P. Invited speaker at "X Congreso Brasileiro de Mandioca. Mandioca: sua Importancia frente a globalizacao da economia" October 11-15 of 1999; Manaus, Brazil.

- Chavarriaga P, Tohme J and Roca W. Invited speakers at "IV Congreso Peruano de Genética" August 9-12 of 2000; Lima, Peru.

- Blair MW "Selección Asistida por Marcadores en Frijol", invited speaker, Univ Nacional de Cajamarca, Cajamarca, Peru

- Blair MW "La Importancia de los Marcadores Moleculares y Estudios del Genoma en el Fitomejoramiento", invited speaker, IASA and Sta Catalina Experiment station Quito Ecuador

- Mejía A and Royero N, invited speakers at seminar "Avances Tecnológicos en el Cultivo del Guanabano", organized by SENA-Valle, September 21-22, 2000, Buga, Colombia.

3.1.4 Workshops, training and theses

- A pre-graduate student from the University of Tolima was supervised and completed his laboratory work toward obtaining his undergraduate agronomist degree. His thesis involved the identification of QTL for agronomic traits that had been introgressed from wild bean to cultivated bean.

- Manrique NC. "Respuesta Varietal de 95 genotipos de la colección núcleo de yuca (*Manihot esculenta* Crantz) a la crioconservación usando la técnica de encapsulación-deshidratación" Undergraduate Thesis, Universidad Nacional, Palmira, Colombia, 2000.

- Luis Orlando Duque. Agronomy Engineering Undergraduate thesis. Thesis completion 2000. Universidad Nacional. Sede Palmira. Outstanding Recognition. Advisor: Zaida Lentini.

- A Cuban trainee, Odile Rodriguez, spent 6 weeks in CIAT to learn the use of a SCAR for Marker Assisted Selection of a gene for Bean Golden Mosaic Resistance.

- "Curso Internacional para Mejoradores y Patologos de la Región Andina - tecnicas modernas en el mejoramiento y estudios de patologia de frijol." October 25 - November 5, 1999, CIAT, Palmira, Colombia (22 participants)

- Corporación BIOTEC organized the workshop "Exploración de Oportunidades de Inversión y Negocios de Base Biotecnológica, en Cadenas Productivas Bio-Industriales", CIAT, June 30, 2000, Palmira, Colombia

- First Workshop of the Cassava Molecular Diversity Network (MOLCAS). August 21-25 of 2000; CIAT, Cali, Colombia. Invited speakers from SB-2.

- Z.Lentini, W. Roca. Coordination of Workshop on Agriculture Biosafety for the Colombian National Biosafety Committee. April 13-15, 2000. CIAT, Cali, Colombia. A total of 25 participants representing the main committee members and replacement attended the meeting. SB-2 members particpated as speakers.

- C. Martínez (IP4, SB2), Z.Lentini (SB2, IP4), J. Tohme (SB2). Advanced course on integrated application of plant breeding and molecular techniques for rice breeding. September 2000. CIAT, Cali, Colombia. SB-2 members participated as speakers.

- Z.Lentini. Coordination of International workshop. Development of Insect and Fungal Resistant Rice: Introgression of genetic resistance to pests and diseases which are dependant on chemical control". April 23-28. Porto Alegre, Brazil.

Graduate students (current):

- Oscar Gomez - PhD program, Ecology and Crop Production Science Dept. SLU - Upsala - Sweden and Univ Nacional Agraria – Nicaragua.
- Ma. Ximena Rodriguez; Biochemical markers for PPD in cassava – PhD Program, Biology and Biochemistry- University of Bath, UK.
- Juan F. Fernandez; Molecular markers and population genetics of *Quercus* –PhD Program, Tropical Ecology, University of Missouri in Saint Louis –UMSL, USA.
- Alejandra Jaramillo; ITS sequencing for *Piper* systematics –PhD program, Systematic Biology, Duke University, USA.
- Hernando Ramirez; Tomato transformation for insect resistance –PhD program, Agronomic Sciences, Universidad Nacional de Colombia.
- Claudia Patricia Florez; *Agrobacterium*-mediated transformation of *Brachiaria* – PhD program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.
- Gerardo Gallego; Gene cloning of rice disease resistance genes - PhD program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.
- Eliana Gaitán; Molecular markers and diversity of palm trees - PhD program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.
- Roosevelt Escobar; Genotypic stability of cryopreserved cassava plants- MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.
- Nelson Royero; Molecular markers and diversity of *Anonna* spp - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.
- Fabio Escobar; Molecular markers to certify seeds of rice - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.
- Edgar Barrera; Molecular markers for ACMD resistance- MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Juan J. Ruiz; Field evaluation of in vitro propagated *Annona* - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- **Undergraduate students (current):**

  - Gloria Iriarte, Universidad de Tolima.
  - Hector Fabio Buendia, Universidad de Tolima.
  - Carolina Ramirez Rodriguez, Universidad del Tolima.
  - Carolina Astudillo, Universidad del Valle.
  - Wilfredo Pantoja, Universidad del Valle.
  - Olga Ximena Giraldo, Universidad del Valle.
  - Gladys Perdomo, Universidad del Valle.
  - E. Gonzales, Universidad del Valle.
  - Luis Guillermo Santos, Universidad Nacional -Palmira.
  - Andrés Bolaños, Universidad Nacional –Palmira.
  - Ana Karine Martinez, Universidad Nacional –Bogotá.
  - Eduardo Tovar Luque, Universidad Nacional -Bogotá.

- A total of 59 trainees, 25 of them international trainees, came through the SB-2 for training in different areas and/or for courses and workshops. Their backgrounds were diverse, having from BS to pos-doctoral degrees.

ACTIVITY 3.2 Assembling databases, genetic stocks, maps probes and related information

3.2.1 Flora map: a computer tool for predicting the distribution of plants and other organisms in the wild

Peter Jones

Introduction

FloraMap maps the predicted distribution, or areas of possible adaptation, of natural organisms when little is known of the detailed physiology of the organism. The climate at the collection point of a set of individuals is assumed representative of the environmental range of the organisms. For plants, these are usually germplasm accessions or herbarium specimens.

The climate at these collection points is extracted from a climate database, and used as a calibration set to calculate 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 model. This is used to map the probability surface from a set of interpolated climate grids.

Software release January 2000

Alexander Gladkov, our Windows Application Developer left CIAT in October 1999, but the job was so well in hand that writing up and publishing the manual and CD-ROM went ahead without major hitches. The software was officially released on 18th January 2000 and an email notice was sent out to potentially interested scientists. A World Wide Web page was available at the time of release and took 1785 sessions with download of 1,552Mb of data in the first week. This has now fallen of to a steady rate of about 120 sessions per week.

FloraMap Users

The Users Group listserver at http://www.FloraMap-CIAT.org now has 102 registered users. It is being used to service the software with answers to questions from users and to fix a few problems that have occurred with the installations. Of the 92 registered members whose country of origin can be identified from their email address, the numbers in the following list shows that the users of FloraMap are widely dispersed.

Country	No. of users	Country	No. of users
USA	22	Austria	1
Mexico	8	Bolivia	1
Brazil	7	Botswana	1
U.K.	7	Canada	1
Australia	6	Denmark	1
India	5	France	1
Colombia	4	Honduras	1
Germany	4	Israel	1
Belgium	3	Perú	1
Italy	3	Switzerland	1
Argentina	2	Thailand	1
Burma	2	Turkey	1
Holland	2	Taiwan	1
South Africa	2	Venezuela	1

It should be noted however that these are only those users who have registered with the listserver. Many more copies of FloraMap have, in fact, been distributed.

Many of the users are employing FloraMap to map plant species distributions from germplasm collections, herbaria or from sample surveys. One user in Bolivia is mapping the distribution of many hundreds of endemic species. He requested a custom made climate grid for Bolivia to speed up the work and we were happy to oblige. Other users are finding new ways to use FloraMap.

One set of users in Mexico is using it to predict the spread of Cactoblastus which is threatening native cacti in northern Mexico and over the border into the USA. I hope shortly to make a 1km climate grid available to them for Mexico. This is being produced by Oswaldo Tellez-Valdez in the laboratory of Dr. Mike Hutchinson in Canberra, Australia. He is using data from the CIAT climate database and data from the ERIC database that have been pre-processed in CIAT.

More adventurous users are attempting to use FloraMap for determining, invading weed populations, small mammal distributions and even coastal and estuarine marine life. It remains to be seen how successful some of these efforts will turn out.

Future plans

- Extend FloraMap coverage world wide using IWMI 10 min World Water & Climate Atlas files.

- Incorporate high precision climate grids, especially for mountainous areas. Moving to a 1km grid will increase the data density almost 400 times.

- Rewrite FloraMap data storage structures and data retrieval routines to cope with increased data density.

- Further refinements to the probability analysis, including, amongst other things, an automatic transform for rainfall which will take the onus off the user to normalize this variate.

References

Jones, P.G. & Gladkov, A. (1999) FloraMap: A computer tool for predicting the distribution of plants and other organisms in the wild; version 1, 1999. Edited by Annie L. Jones - CIAT CD-ROM Series. Cali, Colombia: Centro Internacional de Agricultura Tropical.

Jones, P.G.; Segura, S.; Guarino, L. and Peters, M. (2000) FloraMap. A computer tool for predicting the distribution of plants and other organisms in the wild. Poster presented at the International Conference on Science and Technology for Managing Plant Genetic Diversity in the 21st Century. June 2000. Kuala Lumpur, Malaysia

Collaborators

International Plant Genetic Resources Institute.	IPGRI
Centre for Resource & Environ. Studies. Australian National University	CRES-ANU
University of Oregon	
International Water Management Institute.	IWMI
International Livestock Research Institute	ILRI

Instituto de Hidrologia Meteorologia y Estudios Ambientales, Colombia. IDEAM

International Fertilizer Development Centre IFDC

3.2.2 Regional yield trials extrapolation: A method for extrapolating probable varietal yield from large regional trials networks

P. Jones and S. Beebe

Introduction

Work has started on the problem of extrapolating and mapping yields from regional trials in a manner similar to the way FloraMap operates. The analogue with FloraMap is however a tenuous one and it soon became evident that completely different algorithms would be necessary. The main problem is that FloraMap uses a binary relationship to construct the probability models. When we introduce the factor of yield, or rank of adaptation, or of any other ordinate measure this model breaks down

I believed that this problem might be conquerable if we could cluster the trial sites into relatively dense groups within which the varieties respond in consistent ways. However this is not at all easy. I first tried to develop a Very Sparse Matrix Clustering (VSMC) algorithm in the early '80s. This was based on viewing the clusters and their members in a multidimensional space, of which only a subspace of restricted dimension was handled for each member to member and member to cluster distance measure. I proposed using the probability of similarity as a dimension free distance measure.

Unfortunately I had to give up because I could not produce a dimension free probability measure. In August 1998 I finally succeeded in doing this for the implementation of FloraMap. Late last year I realized that this was exactly what I had lacked in 1984. I have therefore written cluster algorithm which can create a dimension independent distance measure in the variable dimensioned subspaces. It works surprisingly well.

I believe that the crux to the working of this algorithm is what is known as the 'Small World' or the 'Kevin Bacon' effect. The essence of the Kevin Bacon game is to link any Hollywood actor to Kevin Bacon by the pairings of actors working together in films in the least number of steps.

> **Alfred Hitchcock** was in **Show Business at War** (1943) with **Orson Welles**,
> **Orson Welles** was in **A Safe Place** (1971) with **Jack Nicholson**,
> **Jack Nicholson** was in **A Few Good Men** (1992) with **Kevin Bacon**.

The link from Hitchcock to Bacon is made in three steps even though there are many thousands of actors who have worked in Hollywood. This is becoming widely researched in the investigation of network connectivity. The important result is that regular networks can show a very poor connectivity on large scales, but by adding a few long range connections the performance of a regular network can be made to approximate very

closely to a truly random network. Random, common varietal entries have this effect in the trials network,

There is still a pressing need for much testing of the algorithm. but from extensive testing on specially created test data sets there are indications that it can extract (with 100% accuracy) clusters embedded in a variety by trial yield matrix with over 85% of the data missing and coefficients of variation in the region of 30%. This data structure is remarkable similar to the IBYAN (International Bean Yield and Adaptation Nursery) data for black beans that I am using as a test set

I have now developed algorithms, based on those in FloraMap, for assigning a climate probability distribution to the resulting clusters. The next step was to relate these pixel by pixel probabilities to the probabilities of obtaining a given yield as expressed in the IBYAN's yields and variances.

A first test of the algorithm produced the following two varietal yield probability maps. The two varieties are both small black beans and in fact one was bred from the other, so they are very closely related. They should both have very similar adaptation ranges. In fact this could be one of the hardest tests of the system. If it can differentiate between these two very closely related materials, it should be able to handle most comparisons.

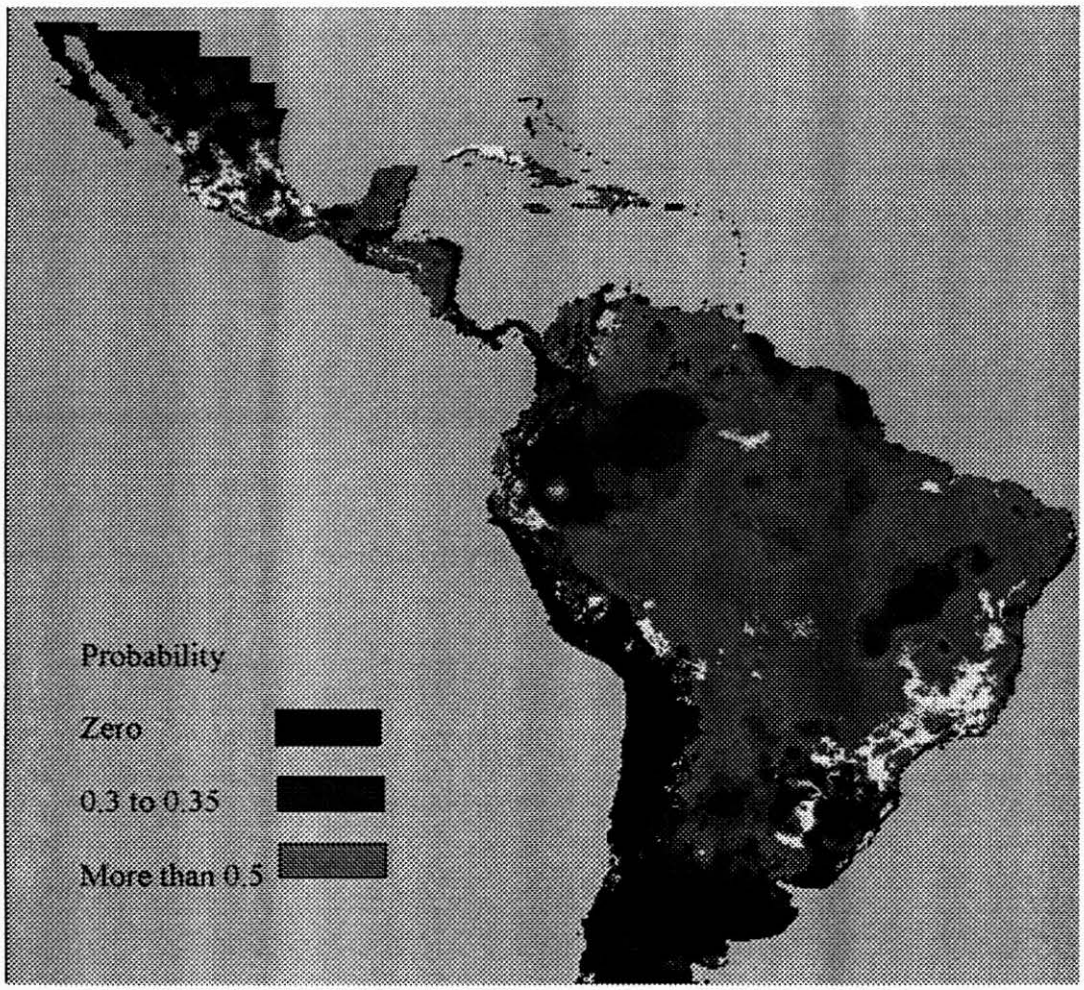

Figure 1. Bean variety Ica Pijao. Probability of yielding more than 2 tons per hectare

There are subtle differences in the adaptation range of the two varieties. Ica Pijao is generally thought to have a better adaptation to cooler climates, hence the better adaptation in Mexico, the Central American highlands and southern Brazil. Whereas Porilllo Sintetico would marginally out perform it in the Brazilian Cerrados. These maps were plotted from the test algorithms without any adjustment of the adaptation ranges that were calculated directly from the IBYAN yield and variance data.

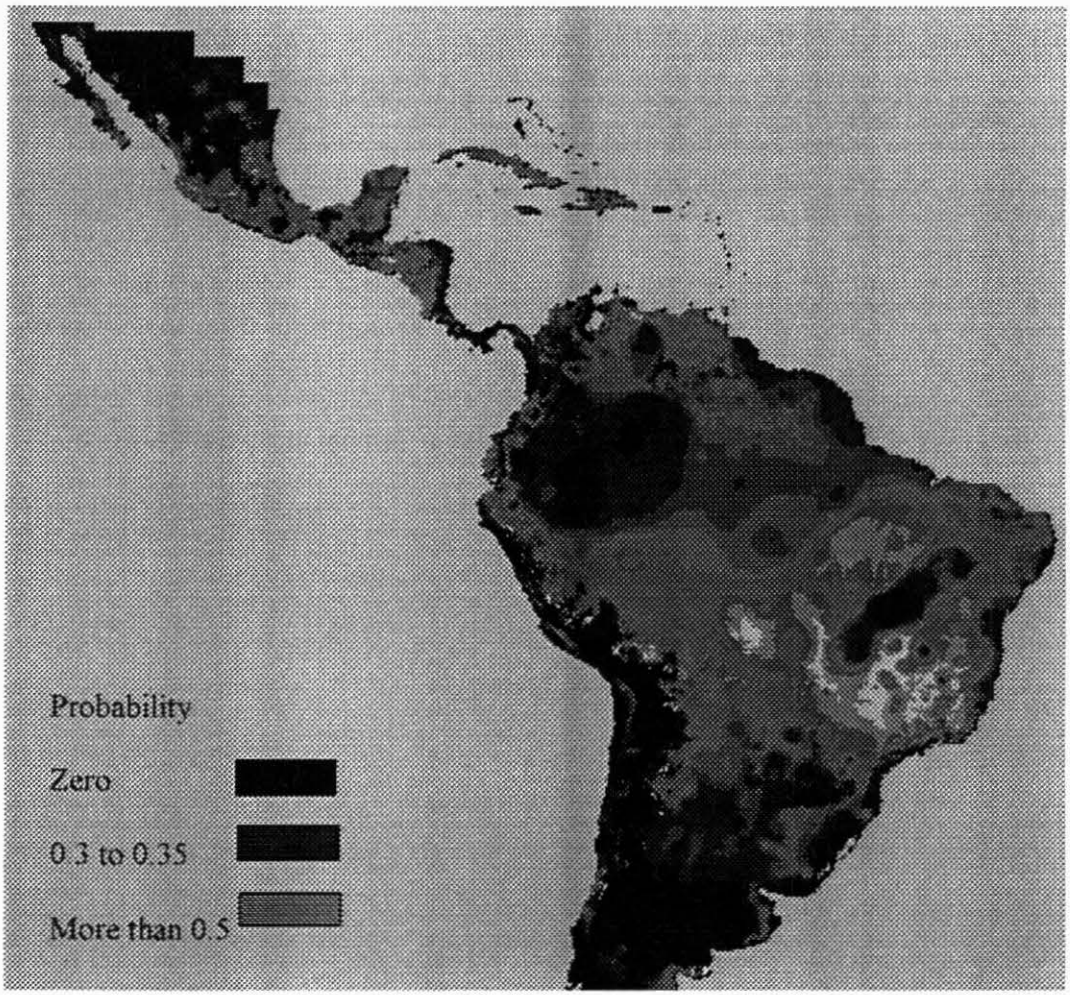

Fig 2. Bean Variety Porillo Sintetico. Probability of yielding more than 2 tons per hectare

Future work

- There is a great deal to do in testing the algorithms, the tests to date on the clustering algorithm have been on random networks. The fact that they work the same on the IBYAN network leads me to think that the 'Small Worlds' effect is effectively allowing the IBYAN network to behave as random. This needs to be rigorously tested.

- The probability calculations have to be put on a sound mathematical footing. Including validation from the available yield data sets.

- Ways have to found of making do with few points in a probability cluster. This work is also needed for FloraMap when using small accession data sets.

- Although we may be able to use some of the software controls from FloraMap, there will be considerable work in designing and coding new software for the application.

3.2.3 Local BLAST implementation and bioinformatics applications

D.F. Cortes, F. Rojas, J. Tohme

Introduction

We are establishing a Computational Molecular Biology Laboratory (CMBL) in order to provide CIAT with the necessary tools and training to approach biological questions from a systems-wide perspective

Three different operating systems as platform for data analysis software wee established: Unix, Macintosh and Microsoft. The following program have been either acquired from offiicial dealers or from the public domain: . Sequencher, Clustal, PHYLIP, Paup, PHRAP, PHRED, Primer design, MapMaker and Consed.

A Sun Server was acquired in order to increase the speed in the BLAST search and formatting database with the sequences obtained in the BRU work,. A copy of the NCBI database as well as the BLAST programs were installed in the server (http://gene2/BLAST/inicio.htm). In addition to a local web site had been created to make available databases and BLAST programs through Internet browsers into CIAT, as well as more accessible and friendly the work in the BLAST searches.

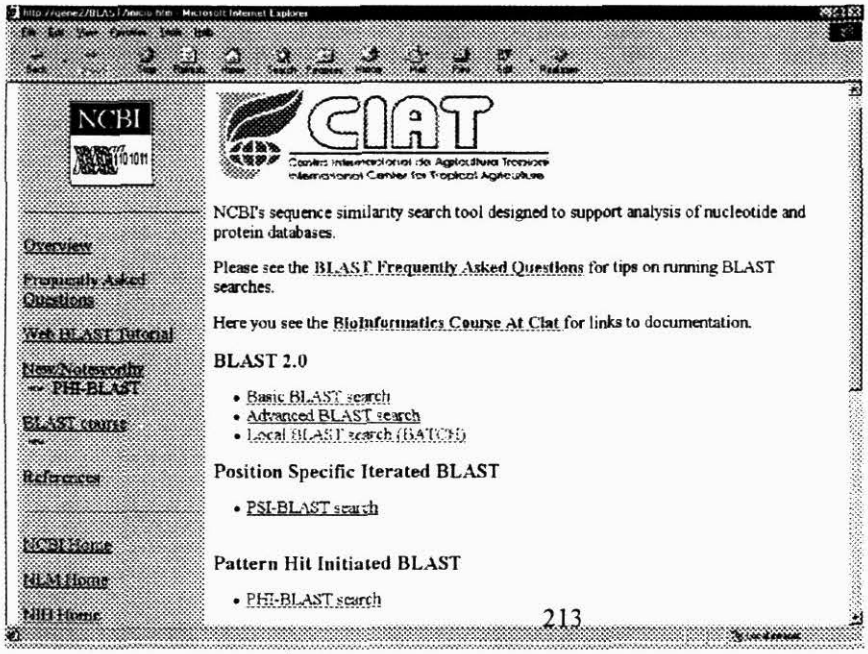

Figure 1. Local BLAST home page appearance

3.2.4 Molecular genetics database constructed for a RAPD survey of Andean *common* bean germplasm

M.W. Blair, M. Muñoz, S. Beebe

We are developing a database to store the information from the RAPD analysis of genetic diversity of the core collection of Andean common beans. Our first step was to find a software system for storing, handling and presenting images within a relational database. Fortunately, a new version of the Oracle software, v. 7.3.2, has been released which has these capabilities, as well as a more user-friendly interface and the capacity to be loaded onto the web. As a relational database, Oracle has the advantage of being an efficient program for organizing and managing data that has multiple layers of relational structure and which is based on a series of data tables. This software provides a set of tools for building new interfaces for a database and asking new questions of that database. The three principal components are tools to generate worksheets, reports and graphic images. The program DEVELOPER/2000, part of the Oracle suite, has a web-compatible format that uses windows and buttons to allow for interactive searches and queries. Oracle software is the standard program for databasing the information from the breeding programs at CIAT. We hope that this preliminary database will be the basis for collecting genotypic information on common bean and a dynamic analysis tool allowing researchers to ask such questions as: how many polymorphisms can I expect when comparing two varieties that might be potential parents? Which polymorphisms distinguish one variety from another?

A total of 330 photographs of RAPD banding patterns were scanned and analyzed using the software package Quantity One from Biorad. These represented 10 primers run on 680 genotypes. A total of 151 bands were scored, averaging 15 polymorphic bands per RAPD primer. Molecular weights were estimated by comparing the most intense bands to the lambda-*Pst*I molecular weight size standard. The annotated gel images were loaded into the database using the Oracle graphics development tool and the estimated band sizes were loaded using the Oracle worksheet development tool. The finished database has three main windows for "gel", "accession" and "bands" as shown in Figure 1. Each of these has a datasheet format with columns and entries. The "gel" window indicates the center of origin for the genotypes, the molecular technique used, the primer used and shows all of the genotypes run together in a given gel. Clicking on a genotypes entry brings up all the sized bands for that individual. Embedded windows are used to call additional items such as the gel images as shown in Figure 2. The "accession" window can be used to compare the markers present in two genotypes (Figure 3). Activities are realized either through the menu bar or action buttons. A console line indicates the status and location of the user. The first version of this database was written in Spanish.

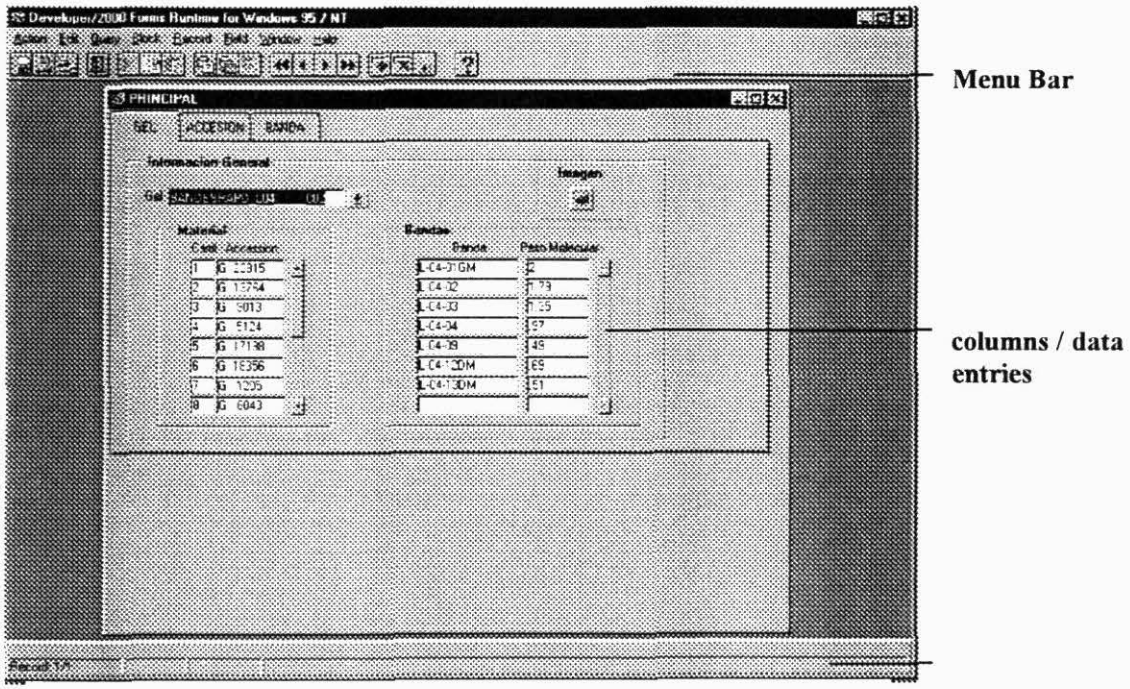

Figure 1. Components of the main window

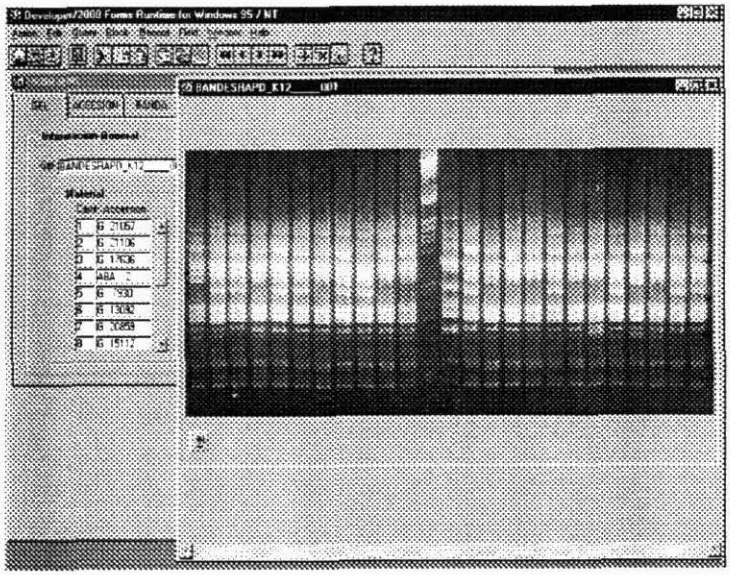

Figure 2. Gel-image window linked to accession and band data.

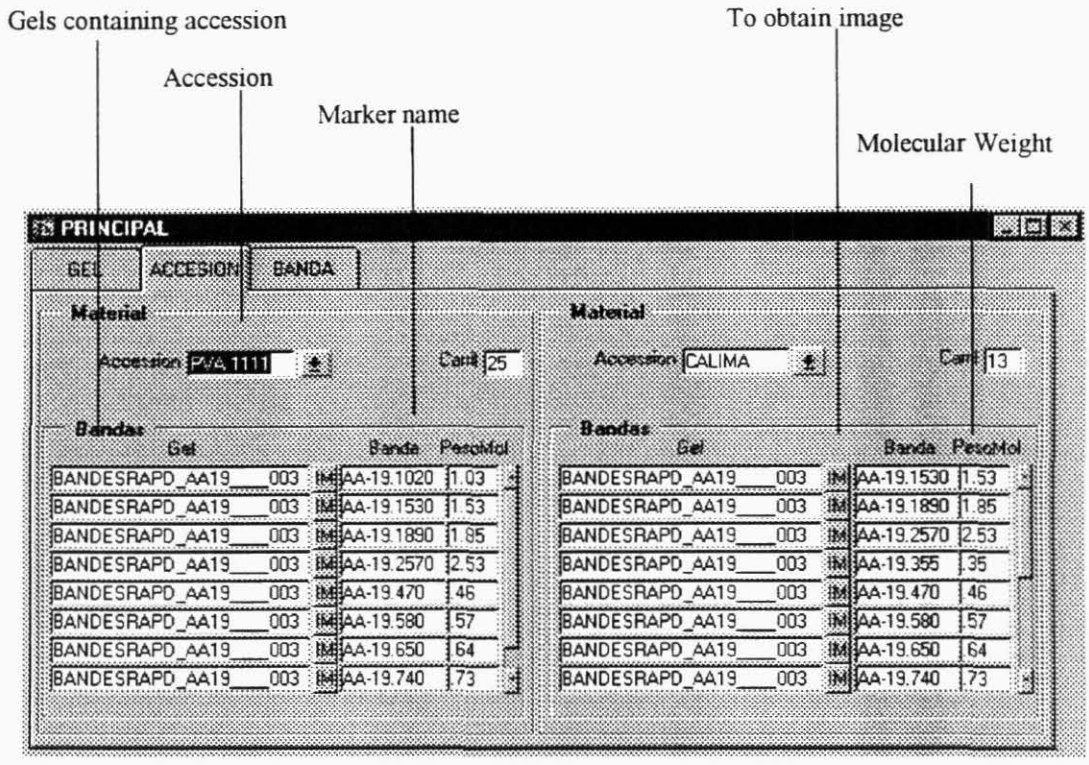

Figure 3. Comparison of markers present in two accessions

Future activities

Future plans are to load additional molecular marker data into the present database. We hope to place the database on the internet using Microsoft Interdev or with the Oracle software tool, Web-DB. With either system, the database can be accessed from any type of computer, via common web-browsers such as Netscape or Internet Explorer. To date, the database contains RAPD fingerprints but could also accommodate data from other multiple-copy marker systems such as AFLP fingerprints. The information stored in this Oracle database could be amenable to incorporation into databases that use other software applications. The basic components of this database will be easy to transfer to ICIS (International Crop Information System http://www.cgiar.org/icis), which is the database system for managing and integrating genetic resource, crop improvement and crop management information of the CG-system. IPHIS (International Phaseolus Information System - http://www.ciat.cgiar.org/icis/) is the database that has been developed at CIAT to hold bean-breeding data. Molecular data is foreseen to be an important part of these databases in the future. It would also be interesting to link the data on genetic diversity with the location of individual markers on the bean genetic map. Many of the RAPD primers used in the diversity study have also been genetic mapped at CIAT and therefore the chromosomal location of the markers can often be inferred. A comparative mapping

tool would allow researchers to compare the position of the markers in the CIAT population to mapping results from other laboratories (notably with the University of Florida and the University of California - Davis). It would also be important for this database to be interactively linked with the BeanGenes database (http://beangenes.cws.ndsu.nodak.edu/), which was established by the USDA - Plant Genome program to specialize in the genetic information relating to common bean. To realize the maximum potential of a molecular marker database, it should also be linked with other existing databases that contain germplasm data on *Phaseolus* including SINGER (http://singer.cgiar.org), the principal database on genetic resources held in the CGIAR system and GRIN (http://www.ars-grin.gov/npgs/). The potential for linkages between all these databases is shown schematically in Figure 4.

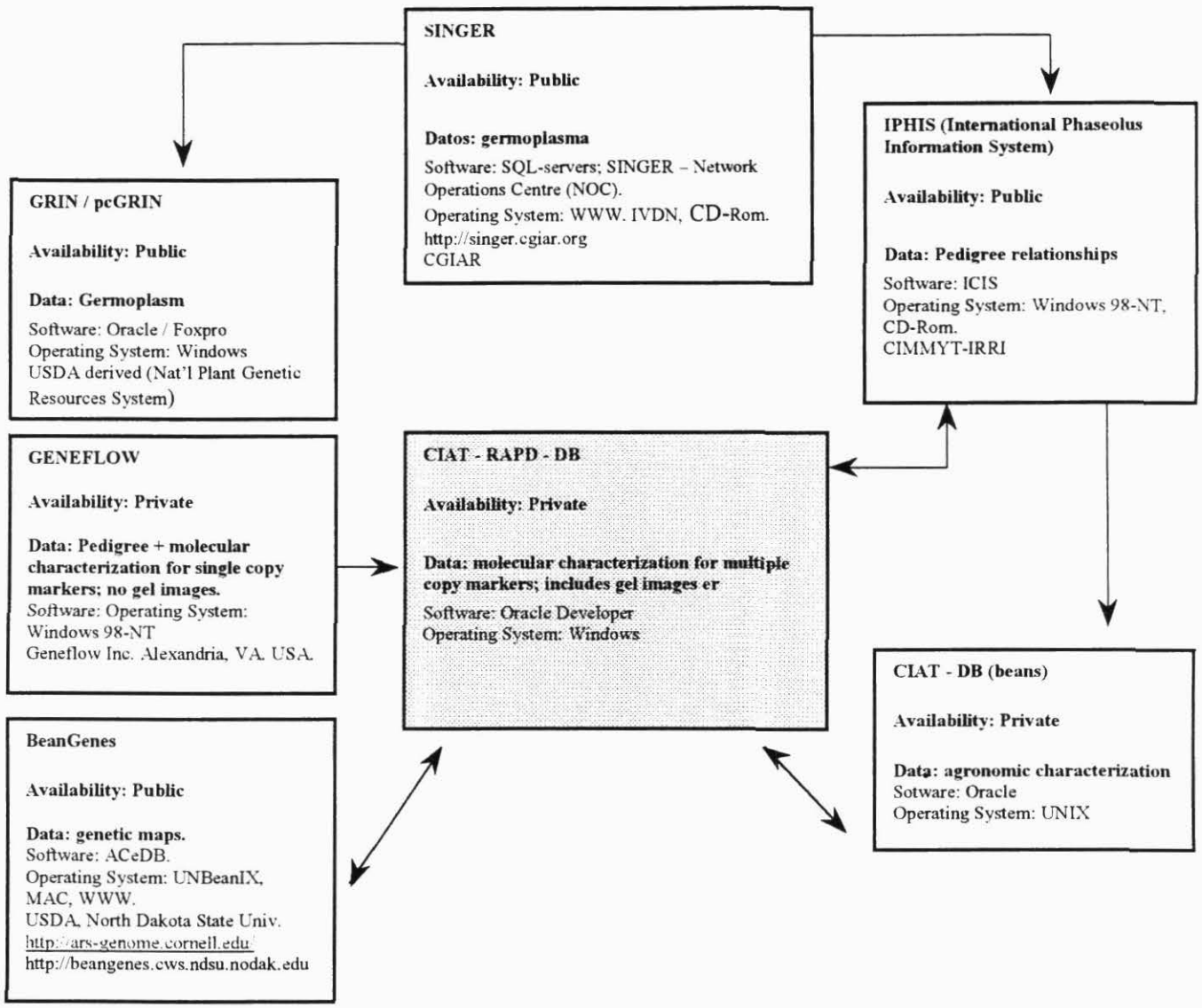

Figure 4. Databases for common bean germplasm and genetic information. Websites are given for those databases on the internet.

3.2.5 Genetic constructs

- 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. These plasmids were placed into E.coli and *Agrobacteriun tumefaciens* Agl1 strains.

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

- 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. E.coli and Agrobacteriun tumesfaciens Agl1 strains.

- Ten different constructs carrying the RHBV NS4 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. E.coli and Agrobacteriun tumesfaciens Agl1 strains.

- 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. E.coli and Agrobacteriun tumesfaciens Agl1 strains

3.2.6 Transgenic and haploid lines

- Transgenic Cica 8, Palmar, Cimarrón, and Fundarroz PN1 carrying PAP and NS4 genes.

- Total of 7,200 doubled haploids lines were generated from rice anther cultured for the various breeding efforts stationed at CIAT. Three hundred R2 lines and nine hundred R3 lines were distributed this year to national program in Latin America.

ACTIVITY 3.3 Publications and project proposals

3.3.1 Projects approved

- Capacidad de regeneración y potencial de transformación de variedades de yuca de importancia comercial en Colombia, approved March 2000 by the Colombian Ministry of Agriculture and Rural Development.

- Establecimiento de un sistema de propagación *in vitro* de material genético promisorio de yuca usando bioreactores (RITA), approved March 2000 by the Colombian Ministry of Agriculture and Rural Development.

- The Cassava Biotechnology Network in Latin America Strategies for Integrating Small-Scale End-Users in Research Agenda-Setting, Testing and Evaluation was submitted to and approved by DGIS and IDRC

- Evaluación de la compatibilidad entre clones de guanábano (*Annona muricata* L.) y patrones de diferentes especies de anonáceas, microinjertados in vitro", approved by "Fundación para la Promoción de la Investigación y la Tecnología -Banco de la República

- Caracterización molecular y agromorfológica de la variabilidad genética nativa de guanábana (*Annona muricata* L.) y especies de anonáceas relacionadas" approved by Colciencias.

- Integration of biofertilisation in bean cultivation by optimising the use of the *Rhizobium*-bean symbiosis": a project to work with Mexico and with the Catholic University of Leuven on the topic of Symbiotic Nitrogen Fixation (SNF) was tentatively approved by the Belgian government. This will permit follow-up on the gene tagging work initiated several years ago to improve SNF under conditions of low P availability.

- An Integrated Approach for Genetic Improvement of Aluminum Resistance of Crops on Low-fertility Acid Soils", to explore aluminum tolerance in bean and Brachiaria was approved by BMZ-Germany. The bean component includes on-site selection of aluminum resistant genotypes in Africa, and will serve to maintain contact between headquarters and African breeders.

- Broadening the Genetic Base of Common Beans (*Phaseolus vulgaris* L.) through Biotechnology": A project was approved by AGCD-Belgium to extend activities carried out previously with Ghent University on interspecific hybridization and transformation.
- Wallace Genetics Foundation "The development of molecular markers for breeding of common beans". Approved, April, 2000.

- Project proposal: Gene Flow Analysis for Assessing the Safety of Bio-Engineered Crops in the Tropics. BMZ. Approved September 2000. Euro 766,938 for three years.

3.3.2 Projects submitted, in preparation, concept notes

- Sustainable management of P and N to improve production and quality of peanut (*Arachis hypogea* L.) in Latin America", a project submitted to the European Union with partners in Belgium, France, Scotland, Spain, Cuba, Argentina, Mexico and Brazil, that will permit exploring further applications in the use of FloraMap in predicting crop productivity across environments.

- Broadening the Genetic Base of Common Beans (*Phaseolus vulgaris* L.) through Biotechnology", the same project that was approved by AGCD-Belgium with Ghent University, was submitted for extension.

- Interspecific solutions to intractable problems of common bean: Understanding the genome of the secondary gene pool to facilitate interspecific transfer of genes and broadening the genetic base of common bean". This is a request for extension of a project that was ongoing for the previous four years, in partnership with Gembloux University, to exploit the diversity of *P. coccineus* and *P. polyanthus*.

- Breeding beans for resistance to drought, mites and root rots for Iran, a proposal for extension of collaboration with the Ministry of Agriculture of Iran that would incluse gene tagging for drought resistance.

- Establishment and operation of a pilot in vitro base gene bank under cryopreservation in liquid nitrogen using cassava (*Manihot esculenta*) as a model crop", a project submitted to the GTZ in partnership with CORPOICA and two German Institutions (DSMZ and FAL), presented by COLCIENCIAS in the World Fair Expo-Hannover 2000.

- Collaborative project CIAT-CIBCM for deployment of transgenic rice plants resistant to rice hoja blanca disease: from field testing to cultivar release under tropical environments, currently under review by Dr. Gary Toenniessen. The Rockefeller Foundation

- Real digestibility and stimulating effect of phaseolin, the bean storage protein, on endogenous secretion in rats", a proposal developed with the Universidad Nacional de Palmira, to study the effects of different morphotypes of phaseolin protein on endogenous protein loss from the gut, to be submitted to the Volkswagon Foundation.

- Exploiting diversity among genes pools of common bean for underprivileged farmers in the highlands of Mesoamerica and Ecuador", a pre-proposal developed with NARS of Guatemala, Mexico and Ecuador, and with Michigan State University and submitted to the McKnight Foundation, to extend the benefits of breeding to farmers that have been bypassed by past efforts in varietal development.

- Breeding staple crops for improved micronutrient value", a concept note submitted to the Gates Foundation, to improve the nutritional status of bean consumers in Africa and Middle America.

- Mineral-rich grains for better nutrition: Beans with higher zinc and iron for African farmers and consumers": a concept note developed for submission to USAID-Uganda.

- Avances en la elucidación del mecanismo de acción del fungicida Pyrimethanil en el control de Sigatoka Negra (*Mycosphaerella fijiensis*) en Musaceas", a concept note submitted for financing to AVENTIS-Colombia, in partnership with AVENTIS.

3.3.3 Publications

3.3.3.1 Refereed publications and books

- Beebe, S., P. W. Skroch, J. Tohme, M.C. Duque, F. Pedraza, and J. Nienhuis. 2000. Structure of genetic diversity among common bean landraces of Mesoamerican origin based on Correspondence Analysis of RAPD. Crop Sci. 40(1): 264-273.Beebe, S., J. Rengifo, E. Gaitan, M.C. Duque, and J. Tohme. Diversity and Origin of Andean Landraces of Common Bean. Crop Sci. Accepted.

- Tohme, J., S. Beebe, and C. Iglesias. 1999. Molecular characterization of the CIAT bean and cassava core collections. *In*: R.C. Johnson and T. Hodgkin. Core collections for today and tomorrow. International Plant Genetic Resources Institute. Rome, Italy.

- Welch, R.M., House, W.A., Beebe, S., and Cheng, Z. 1999. Genetic selection for enhanced bioavailable levels of iron in bean (*Phaseolus vulgaris* L.) seeds. J Agr Fd Chem 48:3576-3580.

- Ana C. Roa, Paul Chavarriaga-Aguirre, Myriam C. Duque, María M. Maya, Merideth W. Bonierbale, Carlos Iglesias, and Joe Tohme (2000) Cross-species amplification of cassava (*Manihot esculenta*) (Euphorbiaceae) microsatellites: allelic polymorphism and degree of relationship. American Journal of Botany (In press) 87(11):000-000

- Sarria R., E. Torres, F. Angel, P. Chavarriaga and WM. Roca (2000) Transgenic plants of cassava (*Manihot esculenta*) with resistance to Basta obtained through *Agrobacterium*-mediated transformation. Plant Cell Reports 19:339-344.

- Wenzl, P., Mancilla, L., Mayer, J., Rao, I.M., Heberle-Bors, E. (2000). Selective enrichment of low-abundance cDNAs by "Asymmetric Self-Subtraction": A proof of principle through mathematical simulation. Analytical Biochemistry, In press (accepted may 2000).

- Lentini Z., Lozano I, Tabares E., Fory L., Domínguez J., Cuervo M., Calvert L. 2000. Expression and inheritance of hypersensitive resistance to rice hoja blanca virus mediated by the viral nucleocapsid protein gene in transgenic rice. Theoretical and Applied Genetics (submitted June 2000, accepted with revision September 2000)

- Lentini, Z. 2000. Biotecnología en el fitomejoramiento del maíz. 207-243. In: H. Fontana and C. González (Eds.). Maíz en Venezuela. Fundación Polar. Caracas, Venezuela. 529 p.

- R.H. Escobar, D. Debouck and W.M. Roca (2000) Development of cassava cryopreservation. *In*: Cryopreservation of tropical plant germplasm: Current research progress and application. F.Engelmann and H. Takagi (ed). pp 222-226. IPGRI, Rome, Italy.

- W.M. Roca, D. Debouck, R.H. Escobar, G. Mafla and M. Fregene (2000) Cryopreservation and cassava germplasm conservation at CIAT. *In*: Cryopreservation of tropical plant germplasm: Current research progress and application. F.Engelmann and H. Takagi (ed).pp. 273-279. IPGRI, Rome, Italy.

- R.H. Escobar, G. Mafla and W.M. Roca (2000) Cassava Cryopreservation I. *In*: Cryopreservation of tropical plant germplasm: Current research progress and application. F.Engelmann and H. Takagi (ed).pp. 404-407. IPGRI, Rome, Italy.

- R.H. Escobar, J.D. Palacio, M.P Rangel and W.M. Roca (2000) Cassava Cryopreservation II. *In*: Cryopreservation of tropical plant germplasm: Current research progress and application. F.Engelmann and H. Takagi (ed).pp. 408-410. IPGRI, Rome, Italy.

- Verdier, V. and Mosquera G. 1999. Specific detection of *Xanthomonas axonopodis* pv. *manihotis* with a DNA hybridization probe. Journal of Phytopathology, 147: 417-423

- Ojeda, S. and V. Verdier. 2000. Detection of *Xanthomonas axonopodis* pv. *manihotis* in cassava true seeds by Nested-Polymerase Chain Reaction assay (N-PCR). Can. J. Phytopathology 22: 1-7

- Jorge, V.; Fregene, M. A.; Duque, M. C.; Bonierbale, M.W.; Tohme J. and Verdier, V. 2000. Genetic mapping of resistance to bacterial blight disease in cassava (*Manihot esculenta* Crantz). Theor. Applied Genetics, 101: 865-872

- Restrepo, S.; Velez, C. M. and Verdier, V. 2000. Measurement of the Genetic Diversity in *Xanthomonas axonopodis* pv. *manihotis* within different fields in Colombia by rep-PCR, RFLP and AFLP analyses. Phytopathology 90:638-690

- Jorge, V.; Fregene, M.; Vélez, C.M,· Duque, M.C.; Tohme, J.,·Verdier, V. 2000. Genetics of field resistance to *Xanthomonas axonopodis* pv. *manihotis* in cassava. TAG, in press

- Restrepo, S.; Duque, M C and V Verdier. 2000. Characterization of pathotypes among isolates of *Xanthomonas axonopodis* pv. *manihotis* in Colombia. Plant Pathology, in press.

- Verdier, V. ; Ojeda, S. and Mosquera G. 2000. Methods for detecting the cassava bacterial blight pathogen: a practical approach for managing the disease. Euphytica, in press

3.3.3.2 Proceedings, Abstracts, Working Documents, Newspapers

- Rengifo, J., A. V. Gonzalez, M. Muñoz, M. Blair, S. Beebe. 2000. "Análisis de dos poblaciones de líneas recombinantes para el estudio de la herencia del contenido de minerales (hierro y zinc) en semilla de frijol (*Phaseolus vulgaris* L.)". Paper presented at XLVI Annual Meeting of the Programa Cooperativo Centroamericano para el Mejoramiento de Cultivos Alimenticios (PCCMCA), May, 2000, San Juan, Puerto Rico.

- Terán, H., C. Quintero, F.J. Morales and S. Beebe. 2000. "Selección de líneas rojas de frijol mediante el mejoramiento asistido por marcadores moleculares en CIAT, Cali, Colombia: caso mosaico dorado". Paper presented at XLVI Annual Meeting of the Programa Cooperativo Centroamericano para el Mejoramiento de Cultivos Alimenticios (PCCMCA), May, 2000, San Juan, Puerto Rico.

- Osorno, J.M, S. Beebe, M. Blair, A. Hoyos, H. Terán. 2000. "Aumento en la productividad del frijol comun, mediante la introgresion de materiales silvestres". Paper presented at XLVI Annual Meeting of the Programa Cooperativo Centroamericano para el Mejoramiento de Cultivos Alimenticios (PCCMCA), May, 2000, San Juan, Puerto Rico.

- Chavarriaga, P; R Escobar, J Tohme and WM Roca (2000) "Plant Biotechnology in Colombia", In: "Colombia-Germany, A Scientific Alliance Endowed with Tradition and Future: Biotechnology State of the Art (chapter one)", Working Document. COLCIENCIAS (ed.), Bogotá, Colombia, October 2000, pp 11-34.

- Chavarriaga P, Escobar R, Tohme J and WM Roca. "El Potencial de la Biotecnología para Contribuir al Manejo de Plagas en la Yuca" Invited speaker an paper presented at "XXVII Congreso Sociedad Colombiana de Entomología" July 26-28 of 2000, Medellín, Colombia.

- Blair MW, Pedraza F, Beebe S, Weeden N (2000) Nuevos marcadores moleculares para la seleccion asistida de caracteres multiples del frijol (Phaseolus vulgaris L.). XLVI Annual Meeting PCCMCA. San Juan, Puerto Rico. May 1-5, 2000. Proceedings.

- Blair MW, Gonzalez AV, Iriarte G, Buendia HF, Beebe S (2000) Analisis molecular de poblaciones derivadas de retrocruzas avanzadas entre acessiones silvestres y variedades cultivadas de frijol común (Phaseolus vulgaris L.) XLVI Annual Meeting PCCMCA. San Juan, Puerto Rico. May 1-5, 2000. Proceedings.

- Gomez OJ, Blair MW, Gullberg U, Frankow-Lindberg B, Gaitan E, Tohme J (2000) Diversidad genética de variedades locales de frijol en Nicaragua. XLVI Annual Meeting PCCMCA. San Juan, Puerto Rico. May 1-5, 2000. Proceedings.

- Rengifo J, Gonzalez AV, Muñoz M, Blair M, Beebe S (2000) Análisis de dos poblaciones de líneas recombinantes para el estudio de la herencia del contenido de minerales (hierro y zinc) en semilla de frijol (Phaseolus vulgaris L.). XLVI Annual Meeting PCCMCA. San Juan, Puerto Rico. May 1-5, 2000. Proceedings.

- T. Pratt, Z. Lentini (SB2). Information Systems for Biotechnology (ISB) News Report. May 2000 by Timothy Pratt. Colombia Biosafety Council takes on training. Report on biosafety workshop offered to the Colombian Biosafety Committee.

- Lentini, Z. 2000. Biotechnology Applied to Rice Germplasm Development. XI Fenarroz (Feria Nacional de Arroz y Muestra Comercial e Industrial). Invited Speaker. April 25, 2000.

- Lentini, Z. 2000. Transgenic Crops: Environmental and Food Biosafety. XI Fenarroz (Feria Nacional de Arroz y Muestra Comercial e Industrial). Invited Speaker. April 25, 2000.

- Lentini, Z. 2000. Biotechnology for Broadening the Genetic Base of Rice in Latin America. Workshop. Development of Insect and Fungal Resistant Rice: Introgression of genetic resistance to pests and diseases dependant on chemical control". April 23-28. Porto Alegre, Brazil. April 26, 2000.

- Lentini, Z. 2000. Forum on "Transgenic plants an alternative for plant pathogen management: Risks and Benefits". XXI Ascolfi Congress. August 20- September 1, 2000. CIAT Cali, Colombia. September 1,2000.

- Lentini, Z. 2000. Industrial uses of transgenic plants: Current and Perspectives. Forum on Food Biosafety and Biotechnology Development: Risk and Opportunities. ILSI-Nor Andino. Universidad Javeriana. Bogotá, Colombia. September 21,2000.

- Lentini, Z. 2000. From germplasm banks to farmer fields: Role of CIAT biotechnology in research and training in Latin America. Invited Speaker at Pioneer Hi-Bred Seeds. Johnston, Iowa. USA. October 16, 2000

- Gonzalez, C.; Restrepo, S.; Verdier, V. 2000. Caractérisation de souches pathogènes et non pathogènes de *Xanthomonas axonopodis* pv. *manihotis* par AFLP. 4èmes rencontres de Phtyobactériologie, Aussois, 17-20 Janvier 2000.

- Jorge, V.; Fregene, M.; Vélez, C., Duque, M.; Tohme, J.; Verdier V. 2000. Cartographie de la résistance à la bactériose vasculaire du manioc au champ. 4èmes rencontres de Phtyobactériologie, Aussois, 17-20 Janvier 2000.

- Restrepo S., M. Velez C., Duque M.C. et Verdier V. 2000. Etude de la structure des populations de *Xanthomonas axonopodis* pv. *manihotis* en Colombie (1995-1999). 4èmes rencontres de Phtyobactériologie, Aussois, 17-20 Janvier 2000.

- Restrepo, S.; Velez, C.M.; and Verdier, V. 2000. Population dynamics of *Xanthomonas axonopodis* pv. manihotis populations in Colombia from 1996 to 1999. Phytopathology 80(6) : S64-65. Abstr.

ACTIVITY 3.4 Donors contributing to Project SB-02 in the period Oct 1999-Sept 2000

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.

2. CEGA-DGIS, Colombia

- Development of a bio-reactor (RITA) system for rapid in vitro clonal multiplication of cassava. Collaboration with CORPOICA.
- Transgenic resistance to cassava stem borer. Collaboration with CORPOICA.

3. 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

4. COLCIENCIAS

- Analysis of diversity and resistance to CBB pathogen (*Xam*)
- Viral disease diagnosis in sugar cane

5. A. von Humboldt Institute- ColCiencias

- Molecular and morphological assessment of biodiversity of endangered Colombian palm trees

6. Centro Tecnológico Polar, Caracas, Venezuela

- Rice transgenic resistance to *Rhizoctonia*.

7. Corporación BIOTEC

- Soursop micrografting

8. Colombian Ministry of Agriculture and Rural Development (MADR)

- Regeneration and transformation potential of commercial varieties of cassava
- Rapid propagation of commercial varieties of cassava

9. **Wallace genetic Foundation, Inc.**
- Molecular markers and breeding for common beans

10. **Agropolis Advance Research Platform**
- EST's for cassava starch and CBB resistance

11. **CENIPALMA**
- Ethiology and epidemiology of oil palm angular spot

12. **AGCD/BABC**
- Genetic improvement of common bean using exotic germplasm

13. **Instituto Amazónico de Investigaciones Científicas -SINCHI**
- Molecular characterization of peppers (Capsicum)

14. **Natural Resources International –NRI**
- Control of PPD in cassava

15. **International Livestock Research Institute –ILRI**
- Molecular genetic markers for feeding value of cereal crop residues

16. **CYTED. España.**
- For Coordination of International workshop. Development of Insect and Fungal Resistant Rice: Introgression of genetic resistance to pests and diseases which are dependant on chemical control". April 23-28. Porto Alegre, Brazil

17. **Institute de la Recherche pour le Development - IRD - France**
- Cassava Bacterial Blight Project

18. **Ministere des Affairs Etranjeres - MAE - France**
- Cassava Bacterial Blight Project

19. **European Community (INCO-DC)**
- Cassava Bacterial Blight Project

ACTIVITY 3.5 PROJECT SB-02 STAFF (2000)

Name	Discipline	Area	Dedication %
S. Beebe	Breeding	Breeding	30
T. Bellotti	Entomology	IPM	20
M. Blair	Genetics	Mol. Markers	60
P. Chavarriaga	Genetics/Mol. Biology	Transformation	100
H. Ceballos	Breeding	Breeding	60
D. Debouck	Botany	Genetic Resource	20
M. Fregene	Molec. Genetics	Molecular markers	60
Z. Lentini	Cell Biology/genetics	Tissue culture/genetic transformation	80
C. Mba	Breeding	Molec. Marker	100
A. Mejia	Biology	Tissue culture.transformation	20
W. Roca	Physiology	Tissue culture/transformation	80
I. Sanchez	Genetics	Diversity-CORPOICA	100
J. Tohme	Genetics	Molec. Markers	100
V. Verdier	Molec. Pathology	Microbial diversity	100

Genome Modification

L.F. Galindo	Research Assistant
L.I. Mancilla	Research Assistant
J.J Ladino	Research Assistant
R. Escobar	Research Assistant
E. Tabares	Research Assistant
I. Acosta	Research Assistant
P. Herrera	Technician
O.X. Giraldo	Technician
C. Dorado	Technician
M. Valenciano	Technician

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
A. Bohorquez	Research Assistant
J. Vargas	Research Assistant
C. Quintero	Research Assistant
M. Giraldo	Research Assistant
F. Pedraza	Research Assistant

D.F. Cortés	Research Assistant
N. Reyes	Technician

Plant-Stress interactions

A.L. Chaves	Associated Research
A. P. Zuluaga	Visiting Researcher
M. Santaella	Research Assistant
C. Gonzalez	Visiting Researcher
S. Ojeda	Visiting Researcher
R. P. Pineda	Visiting Researcher
V. Jorge	Visiting Researcher

Administrative

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

A.V. Humboldt

J.D. Palacio	Visiting Researcher

Corporación BIOTEC

A. Mejía	Visiting Researcher
N. Royero	Visiting Researcher

CORPOICA

I. Sanchez	Visiting Researcher

CENICAÑA

P. Rangel	Visiting Researcher