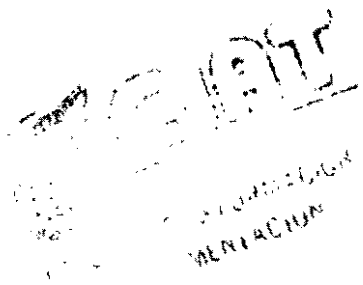


BIOTECHNOLOGY RESEARCH UNIT

ANNUAL REPORT 1995

Biotechnology Research Unit (BRU). 1995. Annual Report 1995. Centro Internacional de Agricultura Tropical (CIAT). Cali, Colombia. 87p.

BIOTECHNOLOGY RESEARCH UNIT
ANNUAL REPORT 1995

Executive Summary

In 1995 the Biotechnology Research Unit (BRU) further strived to integrate molecular and cellular genetic approaches and tools with CIAT research in genetic diversity and germplasm development, and strengthened CIAT collaboration with the NARS of developing countries and with advanced laboratories.

Most of the work highlighted here has been carried out in close cooperation with CIAT programs and units staff as well as with developing countries, particularly in Latin America and the Caribbean.

The work of the Unit in 1995 involved three project areas: 1. Molecular characterization of genetic diversity; 2. Molecular - biochemical studies of plant stress response mechanisms; 3. Gene transfer and conservation of genetic diversity. In addition, the Unit has also been involved in institutional development through cooperation with organizations in developing and developed countries.

1. PROJECT AREA: MOLECULAR CHARACTERIZATION OF GENETIC DIVERSITY.

1.1 **Molecular analysis of genetic diversity**. Using the newly developed DNA-based technology of AFLPs, the analysis of the wild *Phaseolus vulgaris* core collection was completed; the genetic structure of the main gene pools were characterized, and a third group in the Northern Andes with greater genetic diversity than the other gene pools was distinguished. AFLPs permitted much more detailed analysis than previously used markers with *Phaseolus* and with greater speed than RFLP and RAPDs. Similarly, AFLPs are used to analyse the diversity of the Rwanda common bean collection and to describe the phylogeny of *Phaseolus* species and eight *Brachiaria* species. Research carried out in collaboration with the Bean and Tropical Forages Programs.

1.2 **Genome mapping and gene tagging.** The first framework of the cassava molecular map has been constructed at CIAT. The map spans over 60% of the cassava genome, comprising 175 RFLP, RAPD, isozyme and microsatellite markers organized into 31 linkage groups. The latter markers have been identified thorugh collaboration with the Univ. of Georgia, Athens. With the RF support, work continues to saturate the map and its use in breeding for resitance to the African Cassava Mosaic Disease.

Tagging of resitance genes to rice blast fungus lineages was achieved with several crosses; and characterization of resistant var "Oryzica Llanos 5"has been initiated through molecular dissection of the resistant genes. Work in collaboration with the Rice Program, and funded by the RF.

Using RAPD and AFLP primer combinations, we have been able to confirm the ocurrence of a single dominant allele confering aposporic apomixis to *Brachiaria*. The AFLP methodology produced robust and highly reproducible bands from bulk DNA segregating analysis of parents and segregating populations for gene tagging.

2. PROJECT AREA: MOLECULAR-BIOCHEMICAL STUDIES OF PLANT STRESS RESPONSE MECHANISMS

2.1 **Tolerance mechanisms**. With the aim at characterizing plant response mechanisms to acid soils, the "acid soil stress syndrome"was simulated by ion strength nutrient solutions using three *Brachiaria* species. The influence of acid soil stress on the activity of ATPase was measued, and production of antitoxic compounds by roots was evaluated by seedling bioassayas and HPL analyses of phenolic compounds. PCR-based cDNA analysis will be used to identify the genes induced in root tips. Research carried out in collaboration with the Tropical Forages Program and the support of the Austrian government.

2.2 Work was carried out in collaboration with the Cassava Program to learn more about the biochemistry of cassava **root post harvest deterioration**. Our studies have involved the enzyme Phenylalanine ammonia lyase and the coumarin compound scopoletin. There is relationship between wound-response, post-harvest deterioration and scopoletin production.

2.3 In a collaborative work with the Cassava Program, the characterization of genetic diversity in the cassava germplasm with respect to **pro-vitamin A** was carried out. 900 land races have been screened and correlations of ß-carotene content with root color were determined; we have also initiated work to determine the genetics of ß-carotene accumulation in cassava roots.

3. PROJECT AREA: GENE TRANSFER AND CONSERVATION OF GENETIC DIVERSITY.

3.1 **Trangenic approaches**.

(i) Rice. With the aim of broadening the genetic base for managing rice hoja blanca virus (RHBV), in collaboration with the VRU and the Rice Program, we have initially generated *indica* rice plants expressing marker genes, and have studied their expression and inheritance in the transgenic plants. Based on these results, we have transformed rice plants with particle bombardment with the major RHBV structural protein (NS4) and with the nucleo capsid coat protein gene (NC). Molecular analysis of transgenic plants have shown the integration and expressions of the NS4 gene, and the integration of the NC gene. Work carried out in cooperation with the Virology Unit and the Rice Program, with support by the RF.

(ii) Cassava. This year we have obtained proof of integration and expression of transgenes in transformed cassava plants using an *Agrobacterium tumefaciens* - mediated methodology developed at CIAT. We have modified the CryIA(b) gene, acquired jointly with IRRI, into a construct using the pBGHC1 vector, for use in a transformation approach against the cassava stem borer. We have also initiated work to develop a gene construct for modification of the starch branching enzyme.

4. OTHER ACTIVITIES IN BIOTECHNOLOGY

This year the Unit's staff continued to dedicate significant efforts to meet CIAT's bridging role in biotechnology by various means of cooperation with developing and developed country institutions.

(i) One group **training course** and one workshop were offered for Latin American scientists. The former dealt with "biotechnology for the conservation fo agrobiodiversity" and the latter with the AFLP technology with the financial support from OEA-ICETEX-COLCIENCIAS and PROCISUR/AIB, respectively. Furthermore, a range of biotechnology applications have been hosted in the BRU as 1-6 month internships for 25 developing country scientists, mostly from Latin America and the Caribbean, and several research assistants from the Cassava and Bean Programs genetic sections.

(ii) In 1995, the BRU staff have participated, with **paper presentations**, in 9 international and regional scientific meetings.

On the other hand, as part of ongoing **collaborative work with advanced research** organizations, BRU staff visited 4 institutions and received the visit of collaborators-scientists from 6 organizations.

(iii) This year, BRU staff co-authored 6 **scientific publications** in international journals, one book article and one Laboratory Mannual in Spanish for training purposes.

CONTENTS

4062

1. PROJECT AREA: MOLECULAR CHARACTERIZATION OF GENETIC DIVERSITY

1.1 Project: DNA based methodologies for genetic fingerprinting and gene tagging.

The implementation of efficient and cheap DNA markers for large scale genome mapping has been a major research priority. So far, efforts have focused on improving standard RFLP techniques and more recently on PCR based markers such as RAPDs. Two new PCR based approaches, AFLP and microsatellites, offer great promise for the rapid construction of genome maps and genes tagging. Both techniques have received great attention during 94-95 and were integrated into several research projects dealing with germplasm characterization, construction of maps and gene tagging. The AFLP technique developed at Keygene in the Netherlands has been described in details in the annual report of 1994.

Microsatellites

Microsatellites or Simple Sequence Repeats (SSR) refers to very short DNA sequences of repeated di, tri tetra-nucleotides motifs with a variable in number and flanked by conserved sequences. Short 200 to 500 base pairs DNA sequence with a single occurrence, detectable by PCR, microsatellites are useful for gene tagging and serve as landmarks on for combining data from different mapping projects. The abundance of microsatellites is now well documented both in human (Litt and Luty, 1989, Hazan et al. 1992) and plants (Akkaya et al. 1992; Condit and Hubbel 1991; Morgante and Olivieri, 1993; Wu and Tanksley, 1993; Lagercrantz et al. 1993). Integration of microsatellites data with existing RFLP maps has started for several plant species (Bell and Ecker, 1994).

The advantages of microsatellites over markers such as RFLPs are that 1) they require small quantity of DNA and can be easily; 2) they can be rapidly typed with PCR; 3) As site specific primers, they provide a better resolution than RAPDs primers and 4) they are more polymorphic than both RFLPs and RAPDs for the analysis of intraspecific crosses. However numerous limitations to the application of microsatellites into large scale mapping projects remains. Isolating, sequencing and designing is still an expensive tasks. The availability of such markers is restricted to only few plant species such as rice, Arabidopsis, maize and soybeans. Three microsatellites projects are currently underway:

1) Detection, isolation and characterization of microsatellites in beans
2) Microsatellite diversity in the cassava core collection
3) Fingerprinting of the Latin America rice breeding core germplasm

1.1.1 Detection, isolation and characterization of microsatellites sequences in beans

Eliana Gaitán, Delkin Orlando González and Joe Tohme.
Biotechnology Research Unit.

Progress report

A random bean genomic library was constructed by digesting Ica Pijao with Sau 3AI. Restricted DNA fragments smaller than 500bp were ligated with Lambda Zap II DNA and packaged according to protocols described by Stratagene (BRU annual report 1994). Based on last year survey data to detect the presence and abundance of microsatellites in beans, a screening with the oligonucleotide (GGC)15 and GA(15) was conducted on the amplified library. End labeling of oligonucleotides was done by using gamma ^{32}ATP and T4 polynucleotide kinase reaction. Hybridization was carried at a temperature of 45°C. Duplicate membranes were used to eliminate false positives selection resulting from hybridization artifacts. Positive plaques were isolated using three rounds of screening and replating for further purification. Inserts of suitable plaques were excised in plasmid form and were sequenced with the T7 primer and the T3 primer using the Sequensae kit. Clones isolated with (GGC)15 did not gave any major GGC repeats. All of the insert isolated and screened with GA had a GA repeat sequence ranging from 18 to 31 perfect repeats. However, several of the inserts sequenced resulted in duplicates since an amplified library was used. Primers flanking each of the GA repeats were designed using the software Primer version .05 from the Whitehead Institute for Biomedical Research/MIT.

Future plans

The designed primers will be used to: 1) to estimate the number alleles obtained in beans; 2) test the usefulness of the microsatellites in beans for germplasm classification with and between gene pools of *P. vulgaris* ; and 3) map them on the RFLP bean map. Sequencing of additional GA clones is underway. Screening of Lambda Zap II library with (CA)15 repeat has been initiated to increase the number of microsatellites obtained. An enriched library strategy has also been initiated to increase the efficiency of microsatellites selection.

Reference

Akkaya, M. S., A. A. Bhagwat and P. B. Cregan. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. Genetics 132:1131-1139.

Bell, C. J. and J. R. Ecker. *1994.* Assignment of 30 microsatellite loci to the the linkage map of *Arabidopsis.* Genomics 19:137-144.

Condit, R. and S. P. Hubbel. 1991. Abundance and DNA sequence of two-base repeat regions in tropical tree genomes. Genome 34:66-71.

Hazan, J., C. Dubay, M. P. Pankowiak, N. Becuwe and J. Weissenbach. 1992. A genetic linkage map of human chromosome 20 composed entirely of microsatellite markers. Genomics 12:183-189.

Lagercrantz, U., H. Ellegren and L. Anderson. 1993. The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. Nucleic Acids Research 21:1111-1115.

Morgante, M. and A. M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. The Plant Journal 3:175-182.

Wu, K. S. and S. D. Tanksley. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241:225-235.

1.1.2. Microsatellite diversity in the cassava core collection.

Paul Chavarriaga-Aguirre[1], Meredith Bonierbale[2], Gerardo Gallego[3] and Joe Tohme[3]
[1]Visiting scientist, University of Georgia, [2]Cassava Program and [3]Biotechnology Research Unit

Progress report

18 Cassava microsatellites, with different repeats sequences, were isolated by Paul Chavarriaga-Aguirre at the University of Georgia as part of a PhD thesis under the supervision of Dr. Gary Kochert. The objective of the work conducted at CIAT by Paul Chavarriaga-Aguirre was to evaluate the polymorphism of 18 microsatellites in 200 individuals of the Cassava Core Collection. In an attempt to reduce the cost of the screening, it was decided to make the scoring using 10 % (28.5:1.5 Acryl/Bisacryl) Polyacrylamide Gel Electrophoresis (PAGE) and staining with Ethidium Bromide (EB). However, small assays were also done using ^{32}P-labeled PCR (hot-PCR) to compare levels of resolution. The work was carried out at CIAT mainly because of the availability of the core collection, PAGE-equipment (six Protean II chambers), and several PCR machines.

An initial screening using hot-PCR and five individuals from the core showed that 10 out of 14 microsatellites were polymorphic, having between two and five alleles each. Only one microsatellite (GA-13) was found to be monomorphic, and two more gave a high background of nonspecific products. These results indicated that the majority of the microsatellites evaluated were polymorphic in different degrees.

Another diversity study using wild cassava species, hot-PCR, 39 individuals from the core and the microsatellite GA-12 (C. Roa, CIAT, unpublished data) showed 15 alleles, seven of them present in the core and eight in the wild relatives. When the microsatellite GA-12 was scored in 178 individuals of the core using PAGE, only 3-4 (?) alleles could be detected. This indicated that almost 50% of the alleles were not well resolved on polyacrylamide gels stained with EB.

A subsample of 40 individuals from the core, different from the one mentioned above, was screened with four microsatellites (GA-12, GA-126, GA-127 and GA-140) using hot-PCR. The purpose of this experiment was to have an idea of how many different alleles to expect, and to make comparisons with the results obtained with cold-PCR. The microsatellites GA-12 and GA-126 revealed at least eight and

revealed at least eight and four alleles respectively. Although there was a higher background of nonspecific bands, the microsatellite GA-140 showed not less than eight alleles in the subsample scored. GA-127 did not amplify with any sample. It was probably due to a human mistake since this microsatellite produces unique bands under the conditions tested here.

Cold-PCR.

From six polymorphic microsatellites (GA-12, GA-21, GA-131, GA-134, GA-140 and GAGG-5) evaluated in the entire population using cold-PCR and PAGE , only two could be scored with acceptable accuracy. However, in all cases, there were individuals for which it was not possible to determine if their alleles were different from the rest of the population, or if they had true null alleles. In one case, with the microsatellite GAGG-5, it was found that the land races from Panama had null alleles. However, these results have to be confirmed using hot-PCR since more individuals also showed very weak bands or complete absence of them when they were scored with PAGE and EB.

Four microsatellites, polymorphic in the male parent of the mapping population, were mapped after scoring them on PAGE or Metaphor Agarose gels. In these cases it was easier to score the progeny since we knew the parental polymorphism. Microsatellites that are polymorphic in the female parent will be mapped as soon as the data base are available.

Specificity of Microsatellite Primers.

Surprisingly, the specificity of the primers used to amplify the microsatellites in cassava depended upon the primers themselves and the PCR machines among other factors. The Perkin-Elmer thermocycler was the most consistent for cold-PCR amplification and scoring with PAGE. All the amplifications for hot-PCR were made in a MJ/Hot Bonnet thermocycler. The use of hot-PCR and sequencing gels greatly reduced the number of non specific products for most microsatellites. Similar results were obtained in a parallel study with 140 rice varieties (G. Gallego and J. Tohme, pers. comm.). Besides, genetic diversity studies with microsatellites in rice, barley and tomato confirm that hot-PCR and sequencing gels give the best resolution.

1.1.3 Fingerprinting of the genetic base of rice in Latin America and the Caribbean

Gerardo Gallego[1], Pedro Rocha[1], E. Guimaraes[2], C. Martinez[2] and J. Tohme[1]
[1]Biotechnology Research Unit and [2]Rice Program

Progress Report

The fingerprinting of 150 rice genotypes from the genetic core of rice programs in Latin America was analyzed in 93-94 using RAPDs primers (BRU annual report 93-94). Most of these genotypes are commercially planted in Latin American countries under either irrigated or rainfed conditions. Pedigree analysis using the coefficient of parentage between and within countries suggested a

narrow genetic base in certain countries (Cuevas et al. 1992). Based on RAPDs analysis, Indica, Japonica and a Surinam-Guyana groupings were identifying. The lack of discrimination with each main grouping , specially in the Indica cluster suggested the need for additional screening to refine the germplasm classification.

Due to availability of rice mircosatellites (Wu and Tanksley, 1993; Susan McCouch, personal communication) a screening of the same genotypes was initiated to improve the resolution of the germplasm classification and to compare the level of detection between RAPDs and microsatellites. The 25 primers used for the analysis of microsatellite polymorphism were purchased from Research Genetics, Inc. The microsatellites were originally isolated and characterized by Oliver Panaud in Susan McCouch's lab, Cornell University. The microsatellites of various repeat motifs have already been mapped on the Cornell RFLP map. At present only the chromosomes number assignment is available for each microsatellite.

The resolution of microsatellites detection was compared using 10 % (28.5:1.5 Acryl/Bisacryl) polyacrylamide gel electrophoresis (PAGE) stained with Ethidium Bromide and ^{32}P-labeled using sequencing gels (Hot PCR). Several methodological steps like annealing temperature, Mg concentration and % of acrylamide were optimized. Hot PCR gave by far the best resolution. The number of alleles detected using polyacrylamide gel was lower and several bands could not be scored properly. So far, 140 genotypes have been scored using 7 microsatellites with hot PCR. The number of alleles ranges from 4-15 (Table 1).

Table 1. Results of rice microsatellites amplification of 140 genotypes

Microsatellites	Chromosome location	Repet sequence	Annealing Temperature	Number of alleles
RM1	CHR1	GA12	56°C	~ 8
RM4	CHR 11/CHR12	GA25	56°C	15
RM5	CHR1	GA273	56°C	5
RM7		GA304	56°C	7
RM18	CHR7	GA97	56°C	4
RM167	CHR11	(GA)	56°C	15

Future plans

The analysis of the rest of the microsatellites is underway. The microsatellites data will be analyzed to determine the variability within and between the Indica, Japonica and Javanica groups and to compare the degree of resolution of RAPDs and microsatellites. In order to reduce cost and biosafety hazards, a sliver staining protocol of the gels will be tested and if possibly implemented.

Reference

Cuevas-Perez, F.E.; Guimaraes, E.; Berrio, L. and Gonzalez, D. 1992. Genetic base of irrigated rice in Latin America and the Caribbean, 1971-1989. *Crop Sc.* 152:

Wu, K. S. and S. D. Tanksley. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241:225-235.

1.1.4 Toward the implementation of an efficient and quick DNA extraction method

Mauricio Corredor, Jerson Lopez and Joe Tohme
Biotechnology Research Unit

Background

DNA extraction of large numbers of samples is one of the main bottleneck facing the implementation of molecular markers in a breeding program. Using simple extraction protocols, hundreds of samples have to be processed very quickly, at a low cost and must yield high amount of quality DNA. Most of the protocols used at CIAT (Gonzalez et al., 1995), while providing adequate yield still lack the efficiency that will be needed in a breeding program. An assessment of several protocols was undertaken this year to identify a feasible and efficient DNA extraction method.

Progress report

Several protocols used at CIAT and in various labs were tested and modification were attempted to: 1) reduce the storage space in -80 ultra freezer; 2) speed up the time of grinding tissue; 3) reduce or eliminate the use of organic and toxic reagents. Most protocols require the use of liquid nitrogen to assist in grinding plant material for DNA extraction (Dellaporta et al., 1993). Although this step consistently allow the extraction of good quantities of high quality, the use of liquid nitrogen presented some problems. We tested several drying methods. Drying temperature at 55 °C for 24 hr seems to work for most of CIAT crops. The second step we looked at was the grinding of tissue. Instead of using a paint shaker (Tai and Tanksley,1987) we tested successfully the use of a home made seed grinder. The machine has been used at CIAT to grind bean seeds and leaf tissue for nutrient analysis. The grinder can handle a large amount of samples and the pulverized tissue is highly suitable for DNA extraction. The liquid nitrogen and drying-

mechanical grinding methods were compared for different species (5 g fresh tissue of cassava; rice; bean; maize; and *Brachiaria*. Preliminary data indicates that dehydration and mechanical grinding of tissue greatly increases the efficiency of the extraction similar to data obtained by Tai and Tanksley, 1987. Several CTAB (cetyl trimetyl ammonium bromide) based protocols (Stewart N. and Via L. 1993; Tai and Tanksley, 1987) are currently being evaluated (Table 1) with some degree of success with the various CIAT crops.

Future plans

Efforts will concentrate on the implementation of a quick CTAB DNA extraction protocol using dried tissue and grinded mechanically and without the heavy use of chlorofom.

Table 1. Comparison of the different methods for DNA extraction of dry plant tissue

Methods	Purity	Average X 1 G Tissue	Time of Extraction	Amount High	Amount Medium	Amount Small	Efficiency
SDS extraction	Poor		8 Hours or more		Rice	Others	Low
CTAB extractions (High concentration)	Regular	3.3 mg	6 Hours	Cassava Maize	Rice B. Ruzi		Good
CTAB extraction (Low concentration)	Good	2 mg	6 Hours	Cassava B. Briza	Bean Rice		Excellent
CTAB miniprep extraction	Regular	1 mg	4 Hours or Less	Maize B. Ruzi	Others	Rice	Good
ORSTOM method	Good	1.5 mg	6 Hours	Cassava B. Briza	Bean Rice		Excellent
CTAB concentrate with DEAE-CELLULOSE	Good	1 mg	8 Hours or More	Cassava Maize	Rice B. Ruzi		Good

References

Dellaporta, S. L., J. Wood and J. B. Hicks. 1983. A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1:19-21.

Gonzalez, D.O., Palacios N. and Tohme J. 1995. Protocolos para Marcadores Moleculares. Unidad de Investigacion en Biotecnología. Centro Internacional de Agricultura Tropical (CIAT). Cali, Colombia, 78p.

Stewart N. and Via L. 1993. A rapid CTAB isolation technique useful for RAPD Fingerprinting and other PCR applications. BioTechniques 14(5):748-749

Tai , T. and Tanksley S.D. 1987. A rapid and Inexpensive method for Isolation of total DNA from dehydrated plant tissue. Plant Molecular Biology Reporter. 8(4):297-303.

1.2 Project: Molecular characteization of *Phaseolus* diversity

1.2.1 AFLP analysis of the genetic structure between and within gene pools of the wild *Phaseolus vulgaris* core collection

D. Orlando Gonzalez[1], Steve Beebe[2], Myriam C. Duque[3] and Joe Tohme[1]
[1]Biotechnology Research Unit, [2]Bean Program and [3]Biometry Unit

Background

The wild ancestor of the cultivated common bean, *Phaseolus vulgaris* L., is a herbaceous climbing annual, found in the sub humid premontane forests of tropical and sub tropical America from Chihuahua, Mexico, to Salta, Argentina (Toro et al., 1990). Comparisons of wild and cultivated beans have shed light on bean domestication. Gentry (1969) suggested that common bean was domesticated in Mexico and then transported to South America and other regions, but recent evidence indicates that the crop was domesticated several times and in distant regions. Gepts et al. (1986) showed that landraces of Mexico displayed distinct phaseolin morphotypes than South American cultivars. However, the phaseolin types of the landraces are the same as those of their respective local wild populations. Based on similar evidence, Colombia was also suggested to be a center of domestication (Gepts and Bliss, 1986).

Wild bean populations are believed to represent more genetic variability than cultivated beans. For example, some morphotypes of phaseolin are found in wild beans but not in cultivars. Another seed protein, designated arcelin, was identified in Mexican wild beans but is absent from cultivated. The occurrence in wild populations of seed proteins that are absent from cultivated beans suggests a founder effect in the domestication process (Ladizinsky, 1985). This could have excluded from cultivars valuable genetic variability for many traits (Debouck and Tohme, 1989).

Besides seed proteins, other methodologies have been used to study the structure of wild bean populations. Isozyme analysis permitted the study of a broader number of loci than phaseolin, and suggested the existence of two principal gene pools in both wild bean (Koenig and Gepts, 1989) and cultivated bean (Singh et al., 1991). Isozymes analysis has also revealed a smaller group of wild germplasm in the northern Andes, which appears to combine isozymes of the two major gene pools (Debouck et al., 1993). DNA analysis with RFLPs confirmed the existence of two gene pools in wild bean (Becerra Velasquez and Gepts, 1994).

Objectives and expected outputs

Several aspects of the relationship between wild and cultivated bean need to be explored more fully. Which populations of wild bean were indeed domesticated and where? What is the degree of the founder effect? What populations are truly distinct from one another and merit further study of their potential to improve cultivated bean, for example, for yield potential? Other questions relate to germplasm conservation. Where can we find diversity, and what are the best strategies for *ex situ* and

in situ conservation? A first step toward answering all these questions is to gain a better understanding of the genetic structure of wild populations. In the present study, we analyzed DNA fingerprints from a core collection of wild *P. vulgaris* established at CIAT, utilizing Amplified Fragment Polymorphism (AFLP) technology (Vos et al., 1995) to determine the genetic structure of the populations.

Methodology

The genotypes selected for this study were drawn from a core collection of wild *P. vulgaris*, which was established according to similar criteria as the cultivated core collection (Tohme et al., 1994). Based on the geographic and agroecological origin of wild bean accessions and on variability in their seed proteins, a tentative core collection of 114 lines derived from individual plants was selected to represent the diversity derived from 96 collection sites.

Double digestion and adapters ligation

AFLP fingerprinting was performed as described by Vos et al. (1995) with some minor modifications. Five hundred ng of DNA from each sample were double digested with 5 units each of EcoRI (Pharmacia) and MseI (New England Biolabs), using the One Phor All reaction buffer provided by Pharmacia and supplemented with 5mM of DTT in a final volume of 50µl. The reaction was initially incubated at 37°C for 1 h. Then, 10 µl of a mixture containing 5 pmoles of EcoRI adapter, 50 pmoles of MseI adapter, 1.2mM ATP (Pharmacia), 1X One Phor All reaction buffer, DTT, and 1 unit of T4 DNA Ligase (Pharmacia) were added and incubated for 3 more h at 37°C. The adapter ligation is performed in such a way that restriction sites are not restored.

Selective restriction fragment amplification

Sixteen primer combinations for EcoRI and MseI adapters were carefully designed, by adding 1 and 3 randomly selected nucleotides to the 3' end, and were tested to select two combinations of primers. The primer extensions for the first combination were AAC for the EcoRI site and GTA for the MseI site. The second combination consisted of AGT for the EcoRI site and GAC for the MseI site.

Two consecutive amplifications with one and three extra nucleotides per primer, respectively, were carried out. All the amplifications were carried out in a PTC-100 programmable thermal controller (MJ Research); 5µl of double digested and adapter ligated DNA were amplified in a final volume of 50µl, containing 75 ng of each primer with an extra nucleotide, 200µM of dNTPs, 1x buffer for PCR (10 mM Tris-HCl pH:8.0, 1.5 mM MgCl2, 50 mM KCl), and one unit of Taq Polymerase. The cycle profile was: 94°C, 30 sec; 55°C, 30 sec and 72°C, 60 sec, during 35 cycles. A slight smear was observed when 20µl of the PCR product were loaded on a 1.5% agarose gel. For the second amplification, 5 ng of primer for EcoRI with three extra nucleotides were end-labeled by using 0.2 µl of gamma ^{32}P-ATP (5000 Ci/mmol), 1X One Phor all buffer (Pharmacia), and 0.1 unit of T4 Polinucleotide Kinase. This was then incubated at 37°C for 30 min and the enzyme inactivated by heating at 70°C for 10 min. Thirty ng of the non labeled primer for MseI were added and mixed with

200µM of dNTPs, 1X buffer for PCR (10 mM Tris-HCl pH: 8.0, 1.5mM MgCl2, 50 mM KCl), 0.4 units of Taq Polymerase, and 10µl of a 1:20 dilution of the initial PCR product in a final volume of 20µl. This second amplification was carried out by programming a Touchdown Cycle Profile (Don et al., 1991), as follows: 94°C, 30 sec; 65°C (-0.7°C /Cycle), 30 sec. and 72°C, 60 sec during 12 cycles, until reaching the optimal annealing temperature of 55°C. At this temperature 24 more cycles were achieved to complete the second amplification.

Electrophoresis in 6% polyacrylamide denaturing gels

Selectively amplified fragments were mixed with an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylen cyanol FF), denatured at 95°C for 3 min, and incubated in an ice bath for 5 min. Four µl of each sample were loaded onto a 6% polyacrylamide denaturing gel (acrylamide: N,N'-methylene bis acrylamide weight ratio of 19:1, in 0.5X TBE and 7.6M urea) cast in a Sequi-Gen 0.04 x 38 x 50 cm sequencing cell (Bio-Rad). Electrophoresis was carried out in 1X TBE at 40V/cm for 2 h at 50°C. Gels were wrapped in Saran Wrap, dried for 1 h at 80°C, and exposed to a Kodak X-OMAT LS overnight at room temperature. A common sample of DNA was included three times in each gel to establish a standard for comparison of bands among gels. Bands were scored as present or absent.

Data analysis

The data matrix obtained for presence or absence of bands was analyzed by two methods. First, genetic distance was calculated according to the Nei-Li (1979) definition of similarity. The matrix of distances was analyzed by the UPMGA method of the software NTSYS. Dendrograms were created with the TREE program of NTSYS. Groups so formed were examined for internal consistency with respect to country and region of origin and to data for seed proteins and morphological characters.

In addition, Multiple Correspondence Analysis was applied, using the CORRESP procedure of SAS version 6.09 to visualize the dispersion of individuals in relation to the first three principal axes of variation (SAS Institue, 1989). For this purpose the active variables were those corresponding to the presence or absence of bands. Country of origin was used as a supplementary variable, but conserving the geographic value of each individual so as to appreciate the position of individual genotypes. Otherwise, each country would be represented by a single point.

Results

AFLP methodology

The AFLP methodology gave highly reproducible bands, based on replicate lanes of DNA from a control check, which were run on each gel. A total of 110 bands for combination 1 and 93 bands for combination 2 were scored, of which over 90% were polymorphic among the genotypes tested. This is a high rate of polymorphism and somewhat greater than that obtained with RFLPs (Becerra Velasquez and Gepts, 1994; Chase et al., 1991).

The maximum diversity among groups formed by the NTSYS analysis was 0.31, as measured as Nei's distance (Fig 1). This is considerably greater than the diversity observed among wild and cultivated beans with isozymes (Singh et al., 1991) and is comparable to that found with RFLP analysis (Becerra Velasquez and Gepts, 1994). Chase et al. (1991) reported slightly higher values for genetic distance with both isozymes and RFLPs in a group of 14 breeding lines and landraces, but many of the polymorphisms was observed in a breeding line derived from an interspecific cross. In general, different methods and genotypes give rather similar maximum values for genetic distance, in the range of 0.3 to 0.4.

Classification of wild beans

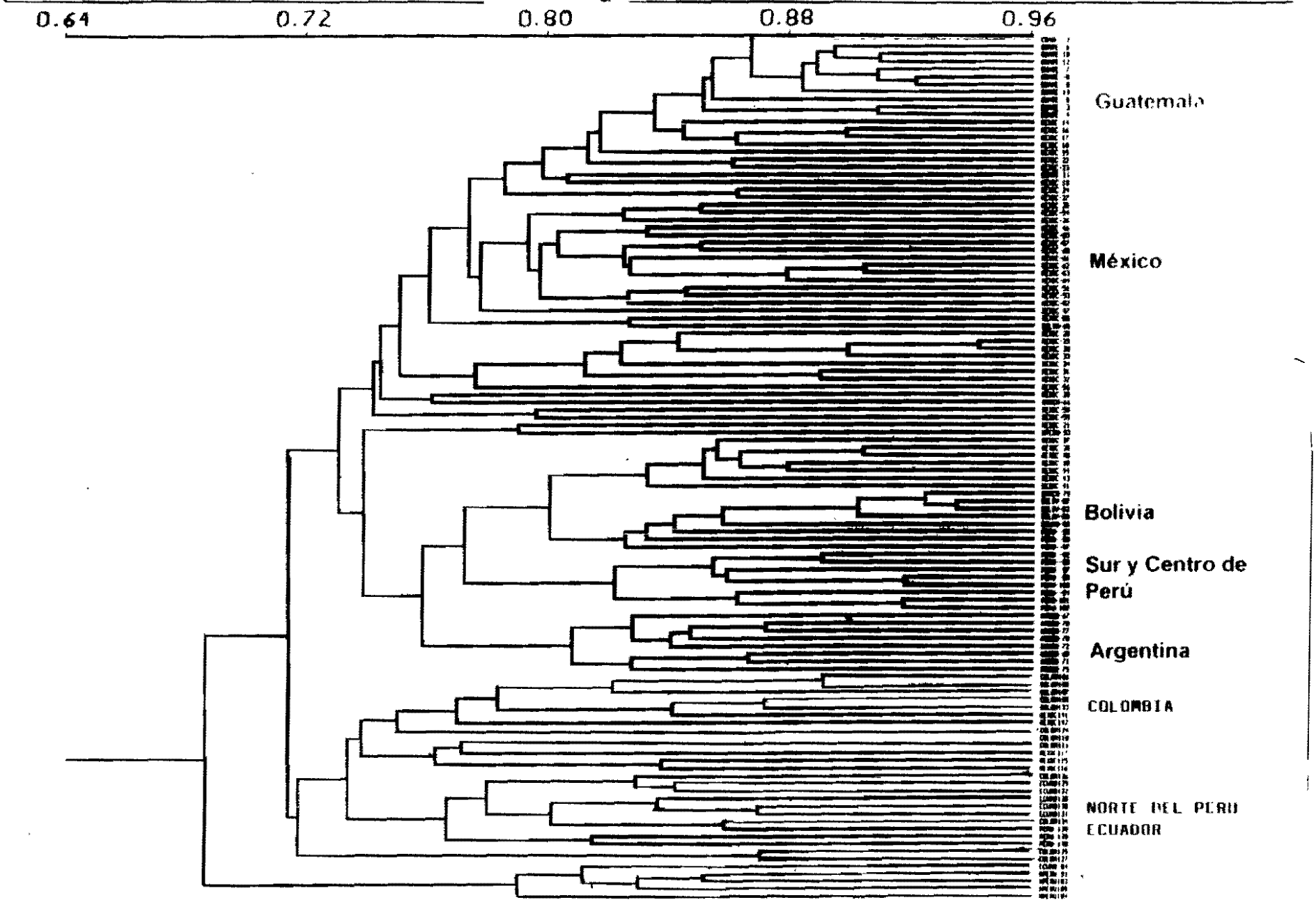
Major groups or gene pools were recognized in Mesoamerica, Colombia, the Northern Andes of Peru and the Southern Andes, although the separation among gene pools was not wide (Fig 1). A unique segment of germplasm in Northern Peru showed the greater genetic diversity from the other gene pools. Although most Mexican wild beans formed a rather homogenous group, a small number of accessions clustered with the Andean gene pool, and accessions from Guatemala tended to cluster apart from Mexican accessions. In the Southern Andean gene pool more discrete groups were formed which were associated with certain regions such as Argentina, possibly reflecting greater geographic and genetic isolation. Colombian accessions appeared to be highly introgressed with germplasm from other areas. AFLP analysis produced a large amount of data in a short period, thus permitting greater insights into the genetic structure of wild beans than had been possible with other methods of analysis.

Compared to other methods of germplasm classification utilized previously, AFLP analysis allowed much more detailed analysis of the genetic structure of *P. vulgaris* germplasm. Since only a limited number of loci can be studied with isozymes and their degree of polymorphism is relatively low, their power of discrimination is limited. Phaseolin presents very broad polymorphism and has been very useful for broad definitions of gene pools of cultivated bean. However, for finer definition phaseolin is of limited value. In the present study, there was not always a good correlation between phaseolin type and grouping by AFLP analysis. For Mexican germplasm particularly the distribution of phaseolin types among the groups formed seemed nearly random.

DNA analysis allows one to sample a greater number of sites in the genome than do phaseolin or isozymes. RFLPs have been used successfully to study wild beans (Becerra Velasquez and Gepts, 1994), but AFLPs were faster than either RFLPs or RAPDs. To obtain a data set of 203 bands for 114 genotypes took approximately 20 person-days of work, including DNA extraction and data reading. With RAPDs this could have taken about 45 days, assuming six bands per primer pair. The quality and reproducibility of the information were greater than for RAPDs. However, with AFLPs as with RAPDs, bands of the same weight may be considered monomorphic, when in fact they represent different sites on the genome.

In many ways the results with AFLPs agreed with those obtained by other methods. AFLPs thus produce reliable results for studies of genetic diversity. In this study, AFLPs gave greater detail and permitted additional insights into the genetic structure of wild bean germplasm. This was due at least in part to the speed with which data can be generated. It would have been difficult to study such a large group of accessions with so many bands in such a short time with any other method. AFLPs should be especially useful for analyzing the genetic diversity of the so-called "orphan crops", about which little or no knowledge is available.

AFLP fingerprinting: Cluster analysis using UPMGA of 114 accessions of wild *P. vulgaris*



References

Becerra Velasquez, L. and P. Gepts. 1994. RFLP diversity of common bean (*Phaseolus vulgaris*) in its centers of origin. Genome 37:256-263.

Chase, C. D., V. M. Ortega and C. E. Vallejos. 1991. DNA restriction fragment length polymorphisms correlate with isozyme diversity in *Phaseolus vulgaris* L. Theor. Appl. Genet. 81:806-811.

Debouck, D. G., and J. Tohme. 1989. Implications for bean breeders of studies on the origins of common beans, *Phaseolus vulgaris* L. *In* Current topics in breeding of common bean (S. Beebe, ed.), Vol. 47, pp. 3-42. Bean Program, Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Debouck, D. G., O. Toro, O. M. Paredes, W. C. Johnson and P. Gepts. 1993. Genetic diversity and ecological distribution of *Phaseolus vulgaris* (Fabaceae) in northwestern South America. Econ. Bot. 47:408-423.

Gentry, H. S. 1969. Origin of the common bean, *Phaseolus vulgaris*. Econ. Bot. 23:55-69.

Gepts, P., and F. A. Bliss. 1986. Phaseolin variability among wild and cultivated common beans (*Phaseolus vulgaris*) from Colombia. Econ. Bot. 40:469-478.

Koenig, R. and P. Gepts. 1989. Allozyme diversity in wild *Phaseolus vulgaris*: further evidence for two major centers of genetic diversity. Theor. Appl. Genet. 78:809-817.

Ladizinsky, G. 1985. Founder effect in crop-plant evolution. Econ. Bot. 39:191-199.

Nei, M. and W. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5269-5273.

Singh, S. P., R. Nodari, and P. Gepts. 1991. Genetic diversity in cultivated common bean: I. Allozymes. Crop Sci. 31:19-23.

Tohme, J., P. Jones, S. Beebe, and M. Iwanaga. 1994. The combined use of agroecological and characterization data to establish the CIAT *Phaseolus vulgaris* core collection. in T. Hodgkin, A.H.D. Brown, Th. J. L. van Hintium and E. A. V. Morales (Eds.), Core collections of plant Genetic Resources, J. Wiley & Sons, Chichester, U.K., pp. 95-107.

Toro, O., J. Tohme, and D. G. Debouck (1990). Wild bean (*Phaseolus vulgaris* L.): Description and distribution, International Board for Genetic Resources (IBPGR) and Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van der Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acid Research (Submitted).

1.2.2 Seeds of hope: assessment of *Phaseolus vulgaris* genetic diversity in Rwanda

Eliana Gaitain[1], [2]Steve Beebe and Joe Tohme[1]
[1]Biotechnology Research Unit, [2]Bean Program

Background

A study of genetic diversity was initiated on Rwandan bean landraces as part of the Seeds of Hope project, to determine if and to what degree genetic diversity had been altered during the past 11 years, possibly as a result of the civil war in that country. The study will analyze diversity at the morphological/phenotypic and at the genetic level.

Progress report

Morphological assessment

With the collaboration of several NGO's established in Rwanda, grain samples were collected on-farm in 8 prefectures. As an initial step to determine possible loss of diversity, grain types in the 1995 collection were compared with those reported in 1984 by Lamb and Hardman (*Survey of bean varieties grown in Rwanda*, and *Catalog of bean varieties grown in Rwanda*). These reports utilize two sorts of "identifiers" for the grain types collected: 1) a description based on seed form, color, color pattern, seed coat brilliance and size; 2) color photos which are likewise linked to the forementioned descriptors. Since the interpretation of grain color, form, etc based on word descriptions is always somewhat subjective, the grains from the SOH collections were compared with the photos in most cases. Then, the word description of that grain type, as defined by Lamb and Hardman, was used to compare to data on frequency of those same types on a prefecture level.

Lamb and Hardman reported those grain types which appeared in 25% or more of the samples collected in a prefecture. No data is available on types with a lower frequency. We were therefore comparing the common types in 1984 with those in 1995, looking for those which had either dropped from prominence or perhaps had increased in frequency. Of these latter types, in most cases we cannot say that these are "new" since these may have existed previously but at a frequency less than 25% .

In general, these results suggest that there has been an overall loss of on-farm variability compared to what existed in 1984. In part this was expected based on pre-war experience in Butare, where farmers were observed to be abandoning Andean genotypes due to susceptibility to root rots. This observation was consistent with present results, since loss of Andean types was especially acute in Butare, as well as in Gikongoro. In this latter prefecture soils are reported to be especially poor, and this may have contributed to the abandonment of Andean types, especially if some degree of root rots is/was present which exacerbated the effect of the poor soil fertility. It may be difficult to distinguish well between pre-war loss due to conscious or unconscious elimination by farmers, and that which resulted from the civil war. Some imaginative work may be necessary with farmers to determine what

what variability they would like to recover, of that which is now apparently lost or at low frequency.

Molecular assessment

The ex situ collection is being fingerprinted using the AFLP technique which is widely used at CIAT for the genetic diversity of wild and cultivated bean core collection. So Far, DNA was extracted from 500 accessions from the collection held at CIAT. Some 350 accessions have been screened with one AFLP primer combination that gave an average of 75 well scorable bands. Variance will be estimated from marker bands to compare variability in ex situ collections made formerly, with variability collected from farmers' fields during the 95 collection trip. AFLP fingerprinting will permit recognizing unique genotypes in ex situ collections which are absent or in low frequency in farmers fields and which require re-establishment.

Future Plans

We plan to continue the AFLP analysis with two primers combinations for the CIAT collection plus accessions recently collected and received from the quarantine facilities. The data on the Rwandan germplasm will also be compared with a selected sub-set of Latin American germplasm from the core collection representing a wide range of diversity. The comparison will allow us: 1) to have a set of reference point for classifying the Rwandan accessions into a Mesoamerican and Andean gene pools; 2) to determine the extend of the loss of diversity before and after the various collections; 3) to assess the extend of diversity that exist in Rwanda as compared to the primary centers, thus allowing a better targeting of new introduction into Rwanda if needed.

1.3 Project: Molecular characterization of diversity in cassava and associated organisms

1.3.1 A genetic linkage map of cassava (*Manihot esculenta* Crantz) based on molecular markers

Martin Fregene[1], Fernando Angel[1], Fernando Rodriguez[1], Meredith Bonierbale[2], and Joe Tohme[1].
[1]Biotechnology Research Unit and [2]Cassava Program

Background

Our objectives were to construct a genetic linkage map of the cassava genome with DNA markers, relatively more abundant, non-epistatic and non-pleiotropic, and elucidate genome organization at the molecular level using an intraspecific *Manihot esculenta* F1 cross. The development of a molecular map of cassava is a starting point to finding molecular markers linked to traits of interest in cassava; which would lead to marker assisted selection in the enhancement of selection efficiency of complex traits in the highly heterozygous genetic background encountered in cassava improvement schemes.

Progress Report

The cassava mapping population comprised of 90 F1 plants from an intra-specific cross between Ng 2, an elite cassava clone developed at the International Institute of Tropical Agriculture (IITA), Nigeria, as female parent and CM 2177-2, a successful cassava cultivar from breeding efforts in Colombia, as male parent. About 900 low copy sequences from two large Pst I libraries, 100 genomic clones from the four relatively smaller libraries and 75 cDNA clones have been so far screened between the parents of the mapping population with the five restriction enzymes Eco RI, Eco RV, Hae III, Hind III and Pst I. A total of 360, or 36%, genomic clone detected a unique fragment (single dose marker) with at least one restriction enzyme in the male parent, while 330 genomic clones, or 33%, revealed single dose markers in the female parent with at least one restriction enzyme. 16 cDNA clones, or 20%, detected single dose markers in the male parent and 20 cDNA clones, or 26%, identified a unique allele in the female parent with at least one restriction enzyme (the cDNA libraries were developed at Dr Gary Kochert's group, University of Georgia, by Paul Chavariagga). Of 740 RAPD primers screened between the parents of the mapping population 250 or 33% single dose markers were obtained from the female parent and 200 single dose markers, or 27% was for the male parent. Eighteen GA repeats, ranging in length from 12 to 134, were screened for unique fragments between the parents; 5 microsatellite, or 28%, were heterozygous in both the male parent and the female parent. On the other hand 9 isoenzyme loci detected 4 or roughly 50%, single dose markers in the female but only one, or 13% in the male parent.

132 RFLP, 124 RAPD, 3 microsatellite and 1 isoenzyme single dose markers, segregating from the gametes of the male parent in our F1 mapping population were tested for linkage using the Mapmaker computer package with a linkage threshold of LOD 5.0 and recombination fraction of 0.25. 118 RFLP loci, corresponding to 115 RFLP probes, 58 RAPD loci, 1 microsatellite and 1 isoenyme loci define 31 linkage groups with an average marker density of one marker every 10.8cM. 17 RFLP markers, 64 RAPD loci and 2 microsatellite markers remained unlinked. The 31 linkage groups are broken down into 14 relatively large groups with between 6 and 12 markers; 7 medium sized groups of 4 or 5 markers and 10 small groups of 2 - 3 markers (Table 2 and Fig 1). The largest linkage group, group I is 122.8cM long and contains twelve markers at an average interval of 10.8cM. The most densely populated linkage group, group V has one marker every 5.7cM, indicating differences in saturation of groups with markers. One hundred and thirty four markers or 75%, attached to linkage groups by a bold horizontal bar, make up the LOD 2.5 frame work map of cassava; the rest of the 44 or 25% markers, displayed in square brackets, show an approximate position on the frame work map as their precise positions could not be ordered with a LOD>2.5. An estimate of how much of the cassava genome is presently covered by our frame work map was calculated using the method described by Hulbert et al (1988). Our estimate of the cassava genome, based on segregation data reported here yielded a genome length of 2439.3 cM. It therefore implies that our present framework map of the cassava genome roughly covers 60% of the genome.

Future plans

We are presently scoring over 150 RFLPs, 4 microsatellite, 4 isoenzyme markers and several AFLP primer combinations heterozygous with respect to the female parent in the mapping population, with an eventual aim of adding them to the present frame work map developed from the male parent using the computer package Join Map 2.0.

The cassava map can serve as an important tool for introgressing traits based on linkage of molecular markers with useful genes. Presently development of genetic stocks to find associations between useful traits such as resistance to ACMV, CBB, White fly, cyanogenesis, culinary quality etc and markers on the map are being pursued.

TABLE 2 Description of linkage groups of the frame work map of cassava with maximum likelihood LOD> 2.5 for all intervals

Linkage group	Number of loci	Length CM	Average CM between loci	No of marker linked in repulsion	Duplicate loci
I	12	122.8	10.2	2	PN 564a, PN 564b
II	11	99.7	9.1	4	CDY123b
III	11	80	7.3	4	pm64b
IV	10	82.9	8.3	2	
V	10	57.3	5.7	5	
VI	9	69.0	7.6	4	
VII	9	57.8	6.4	3	
VIII	8	66.7	8.3	2	
IX	7	77	11.0	3	CDY 123a
X	7	76.6	10.9	3	
XI	10	62.4	8.9	3	
XII	7	50.1	7.2	1	
XIII	7	38.7	5.5	1	
XIV	6	59.3	8.5	2	
XV	5	53.6	10.7	-	
XVI	5	48.4	9.7	2	
XVII	5	30.5	7.6	1	
XVIII	4	62.6	15.7	1	
XIX	4	41.0	10.2	1	
XX	4	36	9	2	
XXI	4	21.5	5.4	1	
XXII	3	23.8	7.9	1	
XXIII	3	20.3	6.8	1	
XXIV	3	12.7	4.2	1	
XXV	3	11.9	4.0	1	
XXVI	2	31	15.5	1	pm64a
XXVII	2	20.0	10.0	1	
XXVIII	2	23.5	11.8	-	
XXIX	2	15.3	7.6	1	
XXX	2	9.3	4.7	-	
XXXI	2	3.1	1.6	-	
TOTAL	178	1465cM	10.8	51	

1.3.2 Molecular genetic characterization of cassava green mite and its natural enemies.

Adriana Bohórquez[1], Lincoln Smith[1] and Joe Tohme[2]
[1]Cassava Program and [2]Biotechnology Research Unit

Molecular genetic techniques promise to be very powerful tools for helping to resolve taxonomic questions. The resolution of species and strain identities, for both pests and natural enemies, is a critical hurdle in the process of finding successful biological control agents.

Characterization of cassava green mite.

Results of genetic sequencing of the ITS1 and ITS2 regions of ribosomal DNA of 5 populations of *Mononychellus tanajoa* and one of *M. caribbeanae* were reported last year. The results indicated that populations from Colombia, Uganda, Venezuela and Brazil were not significantly different (≤ 6 bases out of 580). This supports the theory that there is only one species of *M. tanajoa* s.l., which has been a subject of debate. However this sample set was very small and we running AFLP which is a more sensitive genetic techniques. Collaborators at IITA have collected 30 samples from national program collaborators throughout Africa, and a similar number are being collected by collaborators in Brazil at CNPMA and PROFISMA.

Characterization of *Neozygites*.

Neozygites sp. is an Entomophthoralean fungal pathogen of the cassava green mite which has been observed in Colombia, Brazil and West Africa by scientists associated with CIAT, EMBRAPA and IITA. In northeast Brazil rapid, virulent epizootics have been observed in cassava green mites. But such epizootics do not appear to occur in Africa. IITA would like to introduce strains from South America; however, we do not currently have any method to identify strains. Even taxonomy of species is not fully developed. Therefore we are developing molecular genetic methods to permit the identification of strains and species.

Since the availability of a efficient DNA extraction protocol is a first step toward the molecular characterization, an *in vitro* culture methods developed by Richard Humber (Boyce Thompson Institute, Ithaca, New York) and Italo Delalibera (PROFISMA, Cruz das Almas, Brazil) was implemented for the isolation of axenic cultures of *Neozygites*. Grace's medium was inoculated with hemolymph from surface-sterilized infected mites and incubated for 10 days. It is not clear whether the fungal hyphal bodies actually replicate, but they increase in size and there was no sign of contamination. This has allowed to obtain enough pure DNA to extract for molecular genetic analysis. Preliminary work shows that the ribosomal DNA amplified from *in vitro* cultures using ITS4-IST5 and NS7-NS8 primers can be detected on agarose gels. These bands were compared to those from infected and uninfected *T. urticae* mites and from *Fusarium* and *Phytophthora* fungal

hyphae. These NS7-NS8 region is highly conserved but bands from the ITS4-IST5 region showed similarity between Neozygites and Fusarium, but differences from *T. urticae* and *Phytophthora*. The medium M-199 was found to be less suitable than Grace's for culturing the hyphal bodies, with none surviving beyond a few days.

Future plans

We plan to analyze populations of the Cassava Green mite, predators mites (Phytoseiidae) and *Neozygites* to learn more about the diversity and population genetics of each of the species. The AFLP technique has already been implemented with representative samples of phytophagus and predators mites (table 1). Due to the small genome of the species studied, a amplification of +2/+3 combinations was tested along with the well established +3/+3 combination with two AFLP primers. Preliminary results indicate that the AFLP technique with a of +2/+3 combinations produced on the average 30-40 reproducible bands and will provide a quick and efficient methods for DNA fingerprinting of mites. We plan to screen a large number of samples of mites and predators mites from various countries using several AFLP primer combinations. The AFLP techniques will also be used for *Neozygites* as the DNA extraction become well established.

Table 1. Number of scorable bands obtained by +2/+3 combinations AFLP analysis of phytophagus and predators mites.

Species	# Samples (Populations)	# Scorable bands	
		PM1 C	PM1D
Phytophagus mites (Tetranychidae)			
Tetranychus urticae	1	30	26
Mononychellus caribbeanae	1	40	29
Mononychellus tanajoa	31	34	32
Predators mites (Phytoseiidae)			
Neoseiulus idaeus	6	37	51
Typhlodromalus teniuscutus	5	36	26
Typhlodromalus manihotae	5	40	47

1.4 Project: Mapping of resistant genes to Colombian rice blast MGR lineages.

Gerardo Gallego[1], Jershon Lopez[1], Mauricio La Rota[1], Fernando Correa[2] and Joe Tohme[1]
Biotechnology Research Unit[1] and Rice Program[2]

Background

The characterization of the genetic diversity and virulence spectrum of *P. grisea* populations and the identification of markers linked to resistance genes provides a major opportunities to develop resistant and durable rice cultivars. Results to date indicate that more stable and durable resistance to rice blast under chronic epidemic conditions and high pathotype can be achieved through massive genetic crosses and evaluation and selection of large numbers of segregating populations at "hot spot" sites (Correa-Victoria & Zeigler, 1993a). The integration of the analysis of the genetic and virulence diversity of the blast population in Colombia using DNA-fingerprinting and the use of molecular markers (Correa-Victoria & Zeigler, 1993b; Levy et al. 1993; Correa-Victoria et al. 1993) have allowed the development of a resistance breeding strategy (Zeigler et al. 1993). Such strategy should help rice breeders to select adequate resistance sources, make proper cross-combinations and reduce evaluation and selection efforts. The objectives of this blast research carried in close cooperation with the rice pathology section continue to be:
Characterize the genetic diversity of *P. grisea* populations in Colombia. and
determine the relationship between virulence spectrum and genetic families in the rice blast fungus.

Tag resistance genes to the different lineages of rice blast in Colombia.

Develop PCR based markers for tagged gene (s) of resistance to rice blast
Colombian lineages and to integrate molecular makers in a breeding selection program for durable rice blast resistance.

Progress report

Doubled haploid lines were generated from the cross between Fanny, a susceptible cultivar to most MGR lineages in Colombia and Colombia 1 a resistant cultivar to several MGR lineages. The individual doubled halpoid were screened with blast isolates belonging to MGR lineage SRL-1, SRL-3 SRL-5, SRL-6, ALL-7, ALL,10 and ALL 12. RFLPs markers from the rice molecular map developed at Cornell (McCouch et al. 1988) and in Japan were used. Out of 274 probes screened, 79 resulted polymorphic between the two parents and were subsequently used to screen the mapping population. RFLPs markers were identified to be linked to a cluster of resistance genes to isolates from lineages SRL-6 on chromosome12 and to one gene resistant to ALL-7 isolates on chromosome 11. Fine mapping of the gene (s) located on chromosome 12 is underway. The data of linkage on chromosome 11 confirmed the data obtained last year form the cross Irat 13 x Fanny. The gene mapped at the same location of Pi-1 (Yu et al 1991). Based on virulence and pathogencity data ALL-7 gene seems to be allelic to Pi-1.

The AFLP techniques for rice blast gene tagging was implemented using 25 MseI-EcoRI primer combinations and the bulk segregant method. Highly reproducible AFLP bands were obtained. Several putative bands are currently being looked at on the whole mapping population. The level of polymorphism was however extremely low as compared to the level obtained in beans, Cassava and *Brachiaria*. To increase the level of polymorphism, amplification of +1/+3 and +2/+3 combinations were tested along with the well established +3/+3 combination with several AFLP MseI-EcoRI primer combination. Still the level was well below expected and might not, at least on our mapping populations, justify the cost of running AFLP with present combinations for gene tagging. One possible explanation of such low level of polymorphism might be due to the small genome size of rice and the frequency and size of MseI-EcoRI restriction sites. Rather than testing additional MseI-EcoRI primers combination we are currently testing other restriction enzymes combinations such as PstI and TaqI along with MseI.

Future plans

Screening of the doubled haploid lines from the cross Fanny x Carreon is underway. The identification of polymorphic probes between Fanny and O. Llanos 5 has been initiated and will be used to screened the recombinant inbred lines. The RIL from Fanny and O. Llanos 5 will be available by early December 95 for screening with RFLP, RAPDs and isolates from the different Colombian lineages .

References

Correa-Victoria, F.J., and Zeigler, R.S. 1993. Pathogenic variability in *Pyricularia grisea* at a Rice Blast "Hot Spot" Breeding Site in Eastern Colombia. Plant Disease. 77:1029-1035.

Correa-Victoria, F.J., Levy, M., and Zeigler, R.S. 1993. Virulence characteristics of genetic families of *Pyricularia grisea* in Colombia. Proc. Intl. Symp. on Rice Blast Disease. R.S. Zeigler, S. Leong and P. Ten, eds., Commonwealth Agriculture Bureaux, International, UK, and IRRI, Los Baños, The Philippines.

Levy, M., Correa-Victoria, F.J., Zeigler, R.S., Shizong, X., and Hamer, J. 1993. Genetic Diversity of the Rice Blast fungus in a Disease Nursery in Colombia. Phytopathology. 83:1427-1433.

McCouch, S.R., G. Kochert, Z.H. Yu, Z.Y. Wang, G.S. Khush, W.R. Coffman and S.D. Tanksley. 1988. Molecular mapping of rice chromosomes. Theor. Appl. Genet. 76: 815-829.

Michelmore, R.W., I. Paran and R.V. Kesseli. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc. Natl. Acad. Sci. USA. 88: 9828-9832.

McCouch, S.R., R. Nelson, J. Tohme and R.S. Zeigler. 1994. Molecular mapping of blast resistance genes in rice. Proc. Intl. Symp. on Rice Blast Disease. R.S. Zeigler, S. Leong and P. Ten, eds.,

Commonwealth Agriculture Bureaux, Int., UK, and IRRI, Los Baños, The Philippines page 167-186.

Yu, Z. H., D. J. Mackill, J. M. Bonman and S. D. Tanksley. 1991. Tagging genes for blast resistance in rice via linkage to RFLP markers. Theor Appl Genet 81:471-476.

Zeigler, R.S., J. Tohme, R. Nelson, M. Levy and F. Correa-Victoria. 1993. From Gene to Population: The road to Durable Blast Resistance? Proc. Intl. Symp. on Rice Blast Disease. R.S. Zeigler, S. Leong and P. Ten, eds., Commonwealth Agriculture Bureaux, International, UK, and IRRI, Los Baños, The Philippines page 267-292.

1.5 Project: Identifying PCR-based markers linked to the apomixis gene in *Brachiaria*

N. Palacios[1] J. W. Miles[2], G. Gallego[1] and J. Tohmé[1]
[1]Biotechnology Research Unit, [2]Tropical Forages Program.

Background

Apomixis, which is asexual reproduction through seed, allows faithful propagation of even highly heterozygous genotypes of seed-propagated crops. For that reason there is growing interest in the improvement of natural apomicts and in the introduction of apomixis to sexually reproducing crops (Asker and Jerling 1992). Apomictic reproduction is uncommon among crops. Although it occurs in a number of fruit species (e.g., *Citrus* spp.), they are generally propagated vegetatively. Two main types of gametophytic apomixis occur: diplospory and apospory. In the former embryo sacs develop from an unreduced megaspore mother cell; while in the latter the megaspore mother cell (or its meiotic products) degenerate, and an embryo sac develops from somatic cells arising from the nucellus.

Over the past 25 years, several African species of *Brachiaria* have been used commercially as forages in the tropics, particularly on the acid, infertile soils of the neotropical savannas. Commercial *Brachiaria* species, with the exception of *B. ruziziensis*, a diploid sexual, are generally polyploid apomicts. Their apomixis is of the aposporous type (Valle 1986) which involves the development of an unreduced embryo sac from a somatic cell of the nucellus.

Objectives and expected outputs

The objectives of our *Brachiaria* genome research are: 1) to tag and fine map the apomixis gene in different backgrounds using two PCR-based technologies, RAPD (Williams et al., 1990) and AFLP (Vos et al., 1995; Lin and Kuo, 1995), 2) to construct a *Brachiaria* genetic map based on heterologous probes from the rice, maize, and wheat RFLP maps; 3) to integrate PCR-based markers in a breeding program using sexual and apomictic populations; 4) to establish a transformation protocol, a needed step toward possible cloning the apomixis gene using map-based cloning or transposoon tagging.

Methodology

Plant materials

An induced tetraploid, sexual *B. ruziziensis* clone derived from the Belgian material (Swenne et al. 1981) was used as the female parent in crosses with a natural tetraploid apomictic of accession *B. brizantha* (CIAT 26646). These genotypes were crossed in the field by allowing open pollination of a potted plant of the female *B. ruziziensis* within a small, isolated plot of the apomictic male *B. brizantha*. Selfs of the female clone were identified on the basis of α, ß-esterase isozyme and morphological traits. A population of 115 true hybrid individuals was produced for the analysis.

Reproductive mode phenotyping

Reproductive mode was phenotyped through microscopic examination of the structure of cleared embryo sacs. Cleared ovaries were examined through interference contrast microscopy. Individual embryo sacs were classified as sexual, apomictic, or aberrant. Sterile ovaries, containing no embryo sac, were also found. Hybrids were classified as sexual if no apomictic sacs were observed and as apomictic if any apomictic sacs were observed.

Results

The results allowed us to a refine our monogenic model for control of apomixis in *Brachiaria*. RAPD primers and AFLP primer combinations were screened by bulk segregant analysis (Michelmore et al., 1991) on parental and on bulk hybrid populations to detect promising bands consistently associated with the apomixis phenotype. To date, 600 RAPDs and 64 AFLP primers have been screened on bulk DNA. Each individual hybrid genotype was screened with 4 RAPD primers and 7 AFLP primer combinations that gave informative bands. Combined data on the mode of reproduction and a total of 200 distinct fragments were analyzed with MAPMAKER to determine possible linkages. A LOD score of ≥ 3.5, recombination value of 30 % and the Kosambi function were used. We detected RAPD and AFLP bands that segregate 1:1 and are to be linked with the apomixis locus at a distance of ca. 12 cM. Our analysis strongly supports the existence of a single dominant allele which confers the potential for apospory in *Brachiaria*. The AFLP methodology produced robust and highly reproducible bands. A total of 80-90 bands were obtained per primer combination providing a very useful and especially fast means for gene tagging.

Future Plans

Fine mapping of the apomixis gene is underway. An additional set of 30 AFLP primer combinations is currently being used to screen the whole mapping population. We are also trying to confirm our linkage results in a second mapping population, using an unrelated, *B. decumbens* apomictic parent. Genome mapping of *Brachiaria* has been initiated, using polymorphic RAPDs and AFLPs along with mapped clones from the rice (McCouch et al. 1988), and maize (Coe et al. 1990) maps. Preliminary screening indicates good homology between *Brachiaria*, rice and maize. A set of 70 rice and maize

clones has been identified to be polymorphic between the parental genotypes of two *Brachiaria* mapping population. Comparative mapping will provide a genetic framework to assess the relationship between the apomixis locus in *Brachiaria, Tripsacum and Pennisetum*

References

Asker SE, Jerling L (1992) Apomixis in plants. CRC Press, Boca Raton, Florida

Coe EH,. Hoisington DA, Neuffer MG (1990) Linkage map of corn (*Zea mays* L.),. In: O'Brien, SJ (ed), Genetic Maps, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp 6.39-6.67

Lin, J-J and Kuo J. 1995. AFLP: a novel PCR-based assay for plant and bacterial DNA fingerprinting. Focus 17: 66-70.

McCouch SR, Kochert G, Yu AH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815-829

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc Natl Acad Sci USA 88:9828-9832

Swenne A, Louant B, Dujardin M, (1981) Induction a la colchicine de formes autotetraploides chez *Brachiaria ruziziensis* Germain et Evrard (Graminee). Agro Trop 36(2): 134-141

Valle CB do (1986) Cytology, mode of reproduction, and forage quality of selected species of *Brachiaria* Griseb. Ph.D. Thesis. University of Illinois: Urbana, IL, USA. pp 89

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van der Lee, M. Homes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acid Research (Submitted).

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are use useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

2. PROJECT AREA: MOLECULAR-BIOCHEMICAL STUDIES OF PLANT STRESS / RESPONSE MECHANIMS.

2.1 Project: Molecular basis of resistance to the bean weevil *Acanthoscelides obtectus*

P. Rocha[1], L. Destefano[1], C.E. Posso[2], C.Cardona[2], J. Mayer[2]
[1]Biotechnology Research Unit and [2] Bean Program

Background

The bean weevil [*Acanthoselides obtectus* (Say) (*Coleoptera: Bruchidae*)] is a major pest of stored beans in the Americas and in Africa. It has not been possible so far to transfer resistance genes, naturally found in some wild Mexican Phaseolus species, into commercial varieties. Inheritance seems to obey to a complex pattern. However, F7, F8 and F9 containing individuals segregating for resistance coming from crosses between resistant and suceptible plants are available. These crosses could be very useful in order to attempt to isolate and to clone the gene(s) involved in resistance to Achantoselides obtectus.

Progress Report

The methodology to extract and to purify total RNA and mRNA (poly A) during pod filling has been established. Several protocols were assayed, being the method described by Hall et al (1978) the most suitable in order to obtain total RNA from different genotypes. mRNA (poly A) was efficiently isolated using Hybond mAP (Amersham).

mRNA isolated from resistant and suceptible genotypes (G12954 and ICA-Pijao repectively) were used to establish the differential display technique, largely used in mammalian cells (Liang and Pardee, 1992).

Furthermore, a cDNA library from G12954 genotype was constructed in I-MOSSIox phage, from the same mRNA used for differential display. This cDNA library is available and could be useful to carry out further studies on messengers synthesized during pod filling present in the resistant genotype.

Future plans

F7, F8, F9 individuals coming from a cross between G12952 and ICA-Pijao are available. mRNAs from suceptible and from resistant individuals will be bulked in two different groups to try to minimize differences present in both groups. Selected mRNA will be used in differential display assays. mRNA present in resistant but absent in suceptible individuals will be isolated from the gel, sinthesized in double strand cDNA and cloned in a suitable vector.

References

Hall TC, MA Y, Buchbinder BU, Pyne, JW, Sun, SM, Bliss FA .1978. Messenger RNA for G1 protein of french bean seeds: cell-free translation and product characterization. Proc Natl Acad Sci. U.S.A. 75:3196-3200.

Liang P, Pardee AB. 1992. Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction. Science 257 :967-971

2.2 Project: Biochemical studies of cassava root post-harvest physiological deterioration

G. M. O'Brien[1], Jorge E. Mayer[2], A.L. Chaves[2], O. Orozco[1]
[1] Utilization, Cassava Program and [2] Biotechnology Research Unit

Abstract

Cassava is highly perishable after harvest; visible symptoms of post-harvest physiological deterioration (PPD) often become apparent within 24-48 hours of harvest. We are analysing the role of the phenylpropanoid pathway in this deterioration. Studies have involved components of the phenylpropanoid metabolic pathway (PPP).The enzyme PAL (phenylalanine ammonia lyase) showed promise as PAL levels correlated well with visible PPD. The coumarin compound scopoletin has show notable potential: experiments involving 6 cassava clones showed strong correlation between mean scopoletin content and mean visible PPD. A wounding experiment involving a waxed cassava root indicated a clear relationship between wound-response, PPD and scopoletin production. Research into a possible causal link between scopoletin and cassava root PPD is continuing.

Background

Cassava root post-harvest physiological deterioration (PPD) is a purely physiological process, and should not be confused with microbiological deterioration, which generally follows after PPD. The phenylpropanoid pathway (PPP), a metabolic pathway, is strongly implicated in cassava root (PPD) (Beeching et al., 1994). two enzymes (phenylalanine ammonia lyase-PAL and chalcone synthase - CHS) and one metabolite (scopoletin),(Rickard, 1985; Wheatley,1982), involved in the PPP, were selected for research regarding:

(i)Their potential for genetic manipulation in order to suppress PHD
(ii) suitability for use as a biochemical indicator of PHD, in conventional plant breeding and genetic manipulation.

Expected Outputs

(i) Anticipated outputs include not only increased knowledge regarding the metabolites, but also the application of new assays and/or novel applications for existing protocols.

(ii) Characterization of the enzymes and/or metabolites according to correlations between their concentrations and visually observed PHD symptoms in cassava parenchymal tissue.

Progress Report

PAL levels in cassava roots during 6 days after harvest. Seven freshly-harvested cassava roots of the clone CM 3306-4 were cut into 2 halves, each of which wrapped in clingfilm, but leaving the central wound uncovered (to promote PHD and wound healing response, WHR). The root pieces were stored under ambient conditions. At 24 hour intervals, two pieces were selected. From each piece, three 1-cm layers were transversely cut, starting at the wound surface. Figure 1 show the results where during the first 72 hours after harvest, the PPD values on the first and second layers together showed slightly higher correlation with PAL activity than those of the second and third layers together (R^2 = 0.930 as compared with 0.895). Notably higher correlation with DMC was observed during this period, although with a fairly narrow DMC range (45-55%) and a high value intercept on the DMC axis, any interpretation would have to be limited.

After the 72-hour period, the correlation between PAL and PPD decreased sharply (R^2 = 0.485 during the overall 6-day period), while that between DMC and PPD remained strong (R^2 = 0.875). The "R^2" values relating to results from the first and second layers together appear to imply strong causal influences of both PAL activity and moisture loss with regard to PPD.

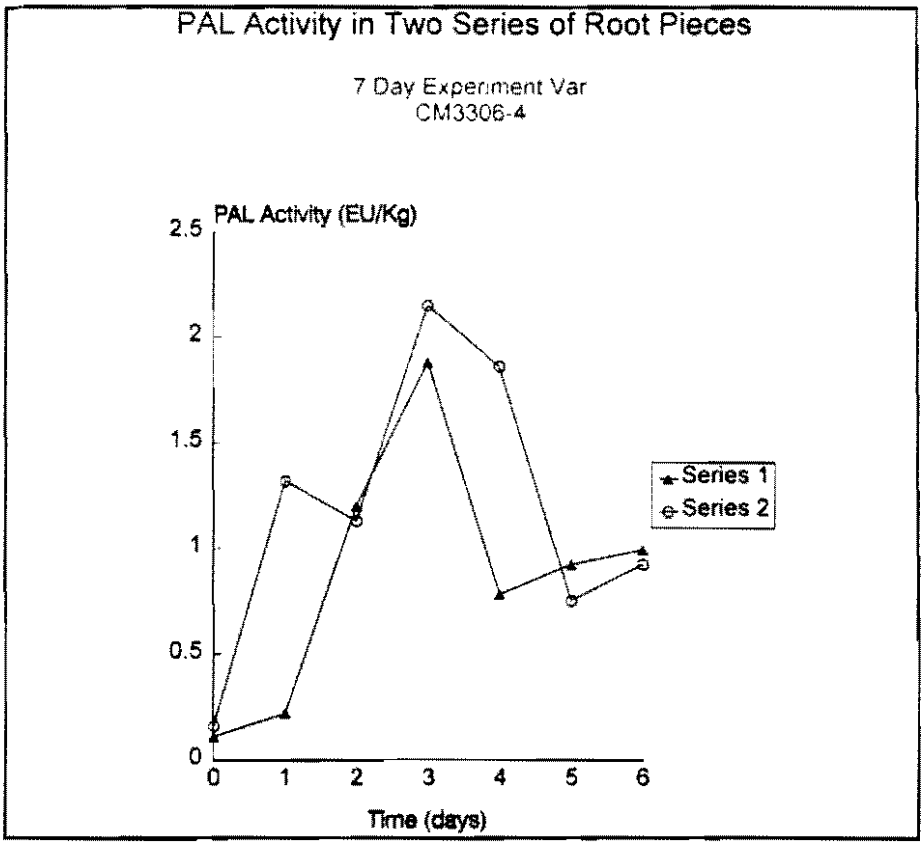


Fig. 1

Screening of cassava clones for scopoletin, DMC and visible PPD. Six cassava clones were chosen according to previous DMC and PPD assays carried out at CIAT (C. Iglesias, pers. comm., Aug. 1993). Five plants of each clone were harvested at 12-13 months maturity. At 0, 24 and 48 hours two roots of each clone were randomly sampled. Triplicated subsamples of parenchyma were taken from each root for scopoletin extraction (80% ethanol, 70 °C, 1h). Extracts were concentrated under vacuum until dryness and redissolved in 80% ethanol for HPLC assay (Risner, 1993). Analysis of scopoletin contents at harvest, 24 and 48 hours after harvest revealed that mean scopoletin content generally reached a peak value at 24 hours after harvest, with a reduced value at 48 hours. Figures 2 and 3 show that:

(i) there was strong correlation, at 24 hours post-harvest between mean scopoletin content and mean PPD; the clones studied showed a range of mean PPD from 0-80% with a corresponding range of mean scopoletin contents from 0.5 - 22 mg/kg.

(ii) there was wide variation in PPD response between roots of the same variety with some roots showing much higher PPD than others, and in almost every case this difference was reflected in the scopoletin contents of the roots.

(iii) there was notable but weaker correlation between DMC and visible PPD (R = 0.72, p = 0.1037).

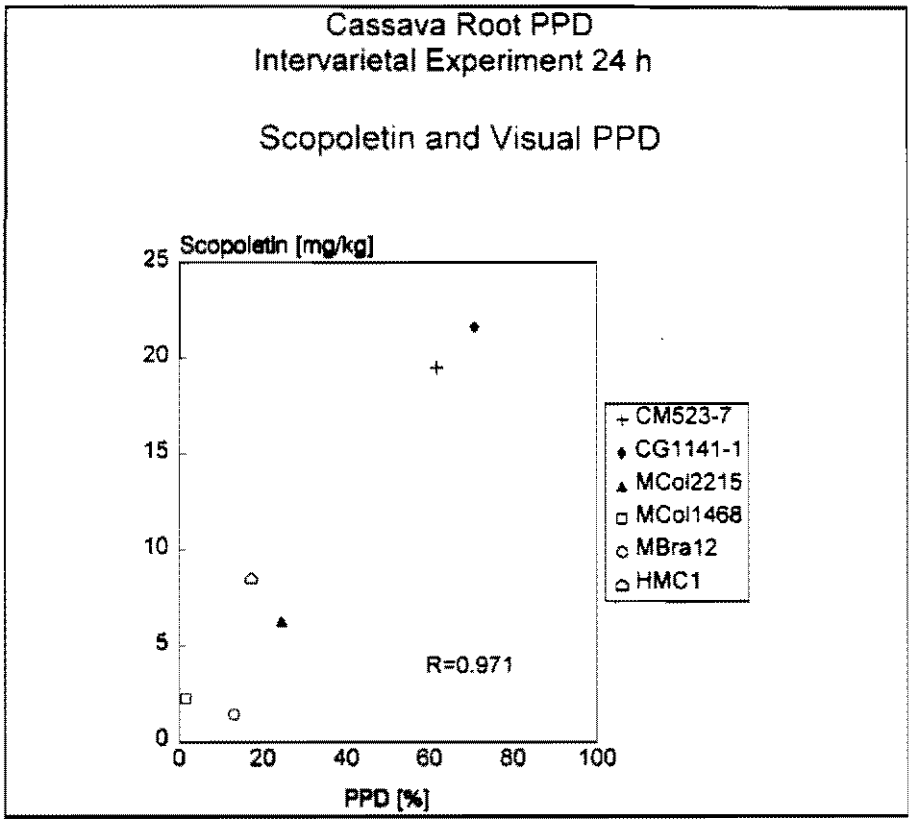


Fig. 2

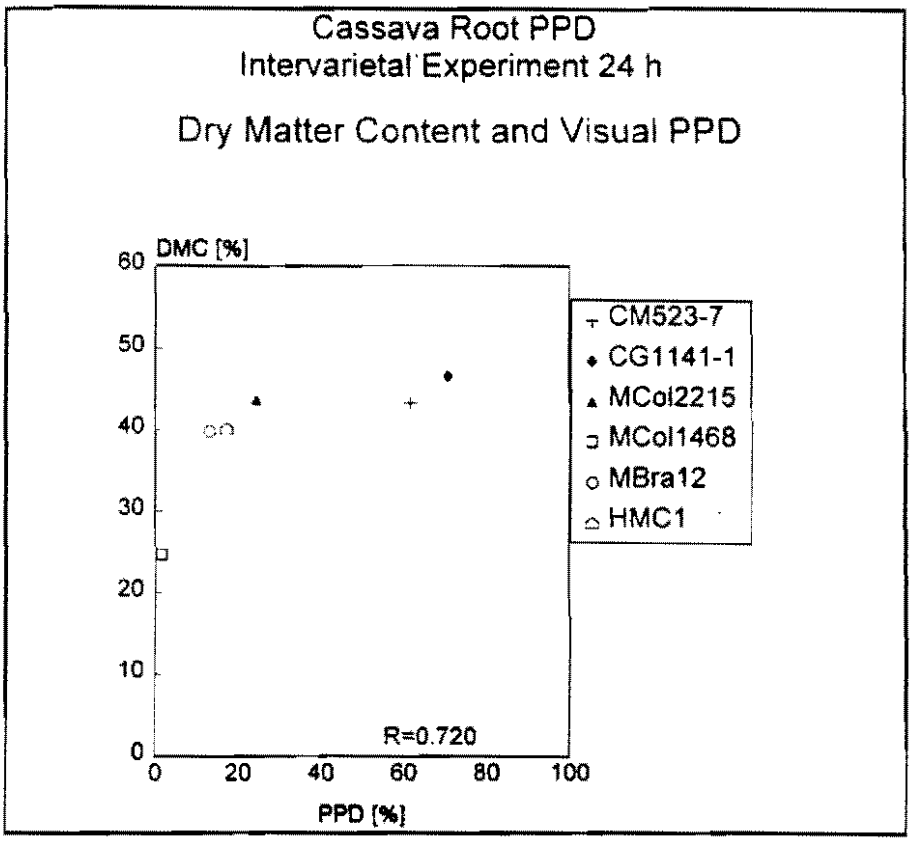


Fig. 3

Cassava root PPD and scopoletin levels in relation to wounding: root waxing experiment. The results (24 hours post-harvest) are shown in figures 4 and 5. Visible PPD and scopoletin content showed a remarkably close relationship, with the highest values for both parameters being produced at the wound, then decreasing from the wound along the root length. A lesser degree of correlation between PPD and DMC was also found.

In the non-waxed control root, on the contrary, there were considerable fluctuations and there was no apparent correlative tendency linking either scopoletin or DMC with PPD. The extreme differences between the waxed and the unwaxed wounded roots may perhaps be explained by considering the situations of the two roots. In the case of the waxed wounded root, there was only one large root wound. Moisture-loss from the root was by only one route of exit and all entry of air into the root was by only one route of entry. The cassava root's WHR, therefore, would be a coordinated, concerted process, centered on the wound. The levels of PPD and scopoletin reflect this. In the unwaxed wounded root, moisture loss and air entry would still be mostly via the large wound, but there would also be small wounds all along the root (perhaps also diffusion through the peel), and a more fragmented WHR would be induced. The root's WHR metabolism, considerably dispersed, would lose overall coordination, as indicated by the lack of correlation between PPD and both scopoletin content and DMC.

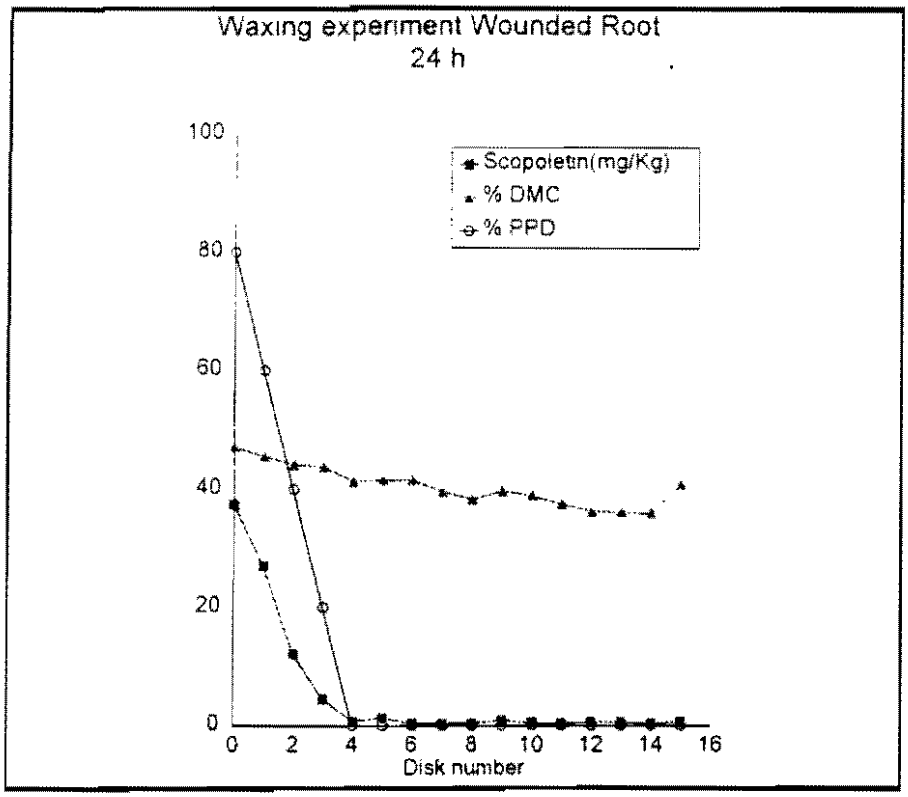


Figure 4.

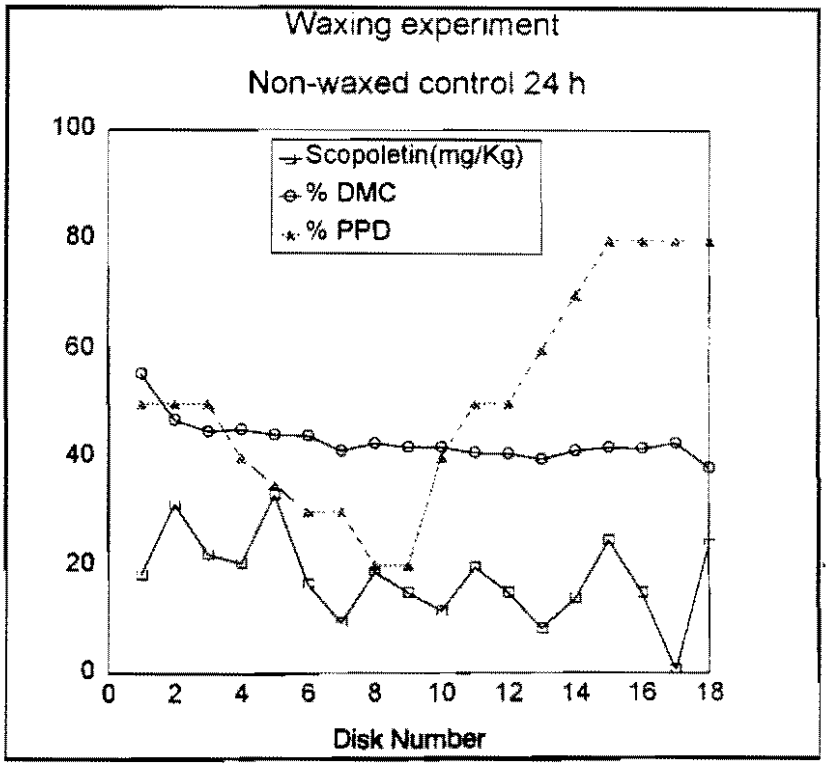


Figure 5.

Future plans

Additional experiments involving parenchymal tissue blocks are planned. These experiments would involve the addition of exogenous purified PAL and/or PAL inhibitor, purified CHS substrate, glifosate and trans-cinnamic acid. The effects upon visually-observed PHD, and upon measurable parameters such as flavonols, peroxidases, etc could be measured.

On the other hand, water activity (a_w) may be electronically measured. It is known that, depending on the extent to which wounded cassava parenchymal tissue dries out and is exposed to O2, PHD severity may vary. As a_w is a measure of far greater importance in enzyme interactions than DMC or water content, this parameter may be more helpful towards discovering/defining a critical cut-off point for the initiation/acceleration of PHD with far greater accuracy.

References

Beeching J.R., Dodge A.D., Moore K.G., Phillips H.M. and Wenham J.E. . (1994). Phisyological deterioration in cassava: possibilities for control. Trop. Sci. 34: 335-343.

Rickard J.E. (1985). Physiological deterioration of cassava roots. J. Sci. Food Agric. 36: 167-176.

Wheatley C.C. (1982). Studies on cassava root physiological deterioration. PhD thesis, Univ. of London.

Risner C.H. (1993). The quantification of hydroquinone, catechol, phenol, 3-methylcatechol, scopoletin, m,p,o-resol in indoor air samples by high performance liquid chromatography. J. Liquid chromatography. 16: 4117-4140.

2.3 Project: Exploring the genetic potential and stability of ß-carotene content in cassava roots

C. Iglesias[1], J. E. Mayer[2], A.L. Chaves[2] and F. Calle[2]
[1]Cassava Program and [2]Biotechnology Research Unit

Abstract

To determinate the genetic potential, stability and availability of pro-vitamin A compounds in cassava roots and derived products, we have screened 900 cassava landraces from CIAT's germplasm collection; we correlated ß-carotene content with root color, studied the genetics of ß-carotene accumulation in cassava roots and we have determined the ß-carotene losses and degradation in processed products.

Background

Due to its outstanding performance under marginal conditions, the cassava crop has been continuously pushed to more marginal environments with poorer soils. WHO and FAO have recognized that improving the micronutrient status of the global population is key to enhance the livelihood of millions of people deficient in vitamins and minerals. Therefore, enhancing the pro-vitamin A content of cassava could have a considerable impact in the nutritional status of rural and urban societies in those marginal areas.

Expected Outputs

(i) Characterization of genetic diversity of cassava with respect to pro-vitamin A content.

(ii) Determination of the stability of pro-vitamin A compounds in processed cassava and

(iii) Determination of the effects of environmental variation on total carotenes content in selected genotypes.

Progress Report

ß-carotene evaluation procedure. An extraction procedure was adjusted for cassava root taking as base the procedure outlined by Safo-Katanga et.al. (1984). The extraction protocol involves petroleum ether (35-65 °C). After extraction, the organic phase is dried with sodium sulfate and concentrated under vacuum by rotaevaporation at 40 °C. The extracts were redissolved in chloroform before analysis by the spectrophotometric method.

Spectrophotometric analysis was performed using a Shimadzu UV-VIS 160A Recording Spectrophometer. UV detection was at 455 nm. A calibration curve was determined for a range of ß-carotene concentrations between 1 and 20 µg/g.

Screening of cassava landraces from CIAT's germplasm collection. It was the first time that a systematic screening for root color and ß-carotene content was done in CIAT's germplasm collection. A total of 632 accessions were screened. Those accessions were pre-selected from a total of 5500, based on their previous characterization for root color.

The data shows a broad distribution of concentrations from less than 0.1 to 2.4 mg/100 of fresh roots. Table 1 presents the mean values and standard deviations for groups of accessions classified according to root color. Even those deep yellow roots showed a broad range of concentrations, from 0.6 to 2.4 mg/100 g fresh root.

Table 1. ß-carotene concentration in cassava roots, classified according to root color

Genotype	Common Name	Origin	Root color	ß-carotene (mg/100 g)
MBRA 516	Olho Verde	Manaus-Brazil	Deep yellow	2.55
MCOL 2285	Furetsikae	Guainia-Col.	Orange	2.40
MCOL 2109	Manaca	Amazonas-Col.	Orange	2.14
MCOL 2295	Bitsurikae	Guainia-Col.	Orange	2.12
MBRA 481	Baixota	Manaus-Brazil	Orange	2.04

Table 2 presents the accessions with the highest concentration of ß-carotene. It seems possible to select genotypes with 2 mg/g of ß-carotene out of the available genetic variability. Given that the daily requirement of vitamin A is around 3 mg, the consumption of 150 g of roots from genotypes with such high concentration can supply that, provided the ß-carotene is 100% available to the human organism. The results are expressed in fresh basis in order to have a direct idea on the potential of each accessions.

The 5 genotypes having the highest concentration were collected in the Amazonian region of brazil and Colombia, where yellow-root cultivars are preferred by farmers (Table 2). This group of top selected accessions for ß-carotene concentration represents the basis for the future recombination work. It is expected that transgressive segregants could be selected out of the recombinant progenies.

Table 2. Cassava genotypes with the highest concentrtion of ß-carotene in roots

Root color	Numerical scale	ß-carotene (mg/100 g)	Standard deviation
White	1	0.13	0.48
Cream	2	0.39	0.28
Yellow	3	0.58	0.28
Deep yellow	4	0.85	0.17
Orange	5	1.26	0.11

Correlation of root color with ß-carotene content. Based on the results from the ß-carotene evaluation in cassava germplasm accessions, and among segregating progenies it can be concluded that cream color is not exactly half-way between white and yellow, but it is shifted

towards yellow, meaning that yellow color has a degree of dominance, and genotypes with higher yellow intensity could be observed within the range of available variability.

Across a total of 632 samples, a significant correlation of 0.82 was determined. This means that 67% of the total variability in ß-carotene content can be explained by the variability in root color. It is possible to improve ß-carotene content by visual selection for color intensity, but there still some margin to improve the selection efficiency by quantitative evaluation of ß-carotene content.

Inheritance of ß-carotene content. A previous report by Hershey and Ocampo (1989) established that root color was determined by a partially dominant gene. Although that can be true depending on the parents used for the study, the inheritance of root color and therefore ß-carotene content, seems to be more complex.Our study include two contrasting parents, rated. Therefore, there is still room for more complex inheritance if we consider that parents with more pronounced root colors can be selected.

During the previous growing cycle and on single basis, the hypothesis of two genes with epistatic effects controlling root color, was developed. These genes were nominated as Y_1 with complete dominance, allowing for the transport of ß-carotene at high levels to the roots and the gene Y_2 with partial dominance allowing for the accumulation of ß-carotene in the roots. The genotype of the white root parent was estimated to be $y_1y_1Y_2y_2$ and the yellow root parents as $Y_1y_1Y_2Y_2$, according to the observed segregation in the F1. The expected segregation from those genotypes was 50% white, 25% cream and 25% yellow. The observed segregation has a probability between 80 and 90% of supporting the original hypothesis. The parent/progeniy performance for root color and ß-carotene content is presented in Table 3.

Table 3. **Segregation for root color and ß-carotene concentration in a cross between contrasting parents**

Genotypes	Number of individuals	ß-carotene (mg/100 g)
CM 2772-3 (yellow)		0.42
CG 1372-6 (white)		0.08
White	20	0.09
Cream	10	0.28
Yellow	9	0.38

Stability of ß-carotene after processing. Cassava is processed before comsumption. During processing, the product is exposed to some heat treatment, which can affect ß-carotene. In a group of 28 clones, the following products were analyzed for ß-carotene content: fresh root (FR), roots cocked over 30 minutes (CR), cassava flour dried in the oven (CFO) and cassava flour dried

under sun (CFS). The results in Table 4 showed a large and significant effect of processing, followed by the effect of varieties. In average, boiling reduced ß-carotene concentration the least, followed by CFO. Sun drying (CFS) reduced the ß-carotene concentration to the lowest level, according that the compound is photo-labile.

Table 4. Analysis of variance for the experiment involving different root processing and their effect on ß-carotene content.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	F values
Reps	1	0.05	0.05	0.1
Treatments	111	195.87	1.76	3.9
Varieties	27	85.88	3.18	7.0
Processing	3	80.56	26.85	59.2
V x P	81	29.43	0.36	0.8
Error	111	12.24	0.45	
Total	223	208.15		

Although the correlation among different processing across genotypes was significant, the relative magnitude indicates that the genotypes with the highest ß-carotene concentration for FR is not usually the one that results with the highest concentration after processing. Also, a test of stability after different processing has to be conducted.

An example is presented in Table 5. The genotype MBRA 473 seems to be very stable; in spite of not having one of the highest concentrations of ß-carotene in FR, it presents the highest for CR and CFO and the second highest for CFS. It is important to study how much of the ß-carotene left after processing is available to the human body, once the cassava product is consumed.

Table 5. Effect of root processing on the concentration of ß-carotene in different cassava genotypes

	ß-carotene(mg/100 g dry root)				Reduction with respect to fresh roots		
Genotype	FR	CR	CFO	CFS	CR	CFO	CFS
MBRA 473	3.33	3.25	2.10	1.15	2%	37%	65%
MBRA 476	4.50	1.68	1.60	0.56	63%	64%	88%
MBRA 502	3.14	1.67	0.69	0.66	47%	78%	79%
Trial mean	1.89	1.20	0.92	0.46	34%	44%	73%

Future plans

In order to study the interaction of genotypes by environmental growing conditions, a group of 14 genotypes selected for their high ß-carotene content on fresh roots, have been planted in 3 sites. during 1996 harvest, evaluation of ß-carotene content on fresh roots as well as in different processed products, will be studied.

References

Hershey, C.H. and Ocampo, C. (1989). Description of new genetic markers in cassava. Cassava Newsletter 13:1-5.

Safo-Katanga, O., Aboagye, P., Amartey, S.A., and Olaham, J.H. (1984). Studies on the content of yellow-pigmed cassava. In: Terry, E.R. et.al.(eds). tropical Root Crops Production and Uses in Africa. IDRC, Ottawa, Canada. pp. 103-104.

2.4 Project: Tolerance mechanisms to acid soils using the tropical forage *Brachiaria* as a model*

P. Wenzl[1], L.I. Mancilla[1], A.L. Chaves[1], C.A. Pineda[2], J.E. Mayer[1], I.M. Rao[3], R. Albert[4], E. Heberle-Bors[4]

[1] Biotechnology Research Unit (CIAT), [2] National Accelerator Centre (Faure, South Africa), [3] Tropical Forages Program (CIAT), [4] University of Vienna (Austria), * Donor: "Kommision für Entwicklungsfragen" of the Austrian Academy of Sciences

Abstract

Key aspects of acid soil adaptation including nutrient uptake mechanisms in the presence of toxic aluminum ions, accumulation of putatively phytotoxic phenolic compounds, and gene induction under stress conditions are being investigated using three *Brachiaria* species (*B. decumbens* cv. Basilisk, *B. brizantha* cv. Marandú and *B. ruziziensis* cv. Common). The so-called "acid soil syndrome", a combination of aluminum toxicity and low nutrient supply (N, P, K, Ca, Mg), is simulated by means of low ionic strength nutrient solutions designed with GEOCHEM 2.0 software. Plant growth in control and stress nutrient solutions was monitored by measuring a number of plant attributes including biomass production, leaf area, total root length, and number of root tips. Results indicate that *B. ruziziensis* is more susceptible to acid soil stress than the other two species.

The influence of acid soil stress on the activity of plasma membrane H^+-ATPase is being investigated because the proton gradient created by this key enzyme is used as driving force for nutrient uptake. The spatial distribution of nutrients like P, Ca, K, Fe, Mn, Cu, Zn, Si, S, Cl, and Al in cryosections of root tips is visualized by "proton induced x-ray emission" (PIXE) in order to reveal interspecific differences in nutrient uptake strategies. Phenolic compounds were extracted and analysed with reversed-phase HPLC. Two major compounds in roots of *B. ruziziensis* were identified that accumulate under acid soil stress. Seedling bioassays are being performed to test the level of phytotoxicity of root extracts containing different amounts of these compounds. For isolation of stress-induced genes, root tips of plants grown under stress conditions were used for mRNA extraction and a PCR-based method is currently being applied to construct cDNA libraries.

Background

Although *Brachiaria* species are the most widely planted commercial forage grasses in tropical America, virtually nothing is known about the molecular basis of their outstanding tolerance to acid soils. However, *B. ruziziensis* is less adapted than other *Brachiaria* species in terms of persistence as evaluated in field experiments. Field experience from various regions of tropical America has indicated that pastures based on *B. decumbens* are most persistent.

Interspecific crosses between the sexual *B. ruziziensis* and the apomictic *B. decumbens* and *B brizantha* are underway to combine desirable traits such as spittlebug resistance and forage quality with acid soil tolerance. An improved understanding of molecular mechanisms underlying the differences in acid soil adaptation among species will facilitate development of a simple and reliable screening methodology.

Expected Outputs

Short-term goals encompass (i) establishment of a nutrient solution technique for simulation of the "acid soil syndrome", (ii) description of stress response in terms of root and shoot attributes with special focus on root architecture, (iii) interspecific comparison of nutrient accumulation in root tips, in particular visualization of the effect of Al on the spatial distribution of nutrients, (iv) evaluation of the role of the plasma membrane H^+-ATPase in interspecific differences in acid soil adaptation, (v) evaluation of the production and exudation of toxic phenolic compounds under stress, (vi) isolation and expression/sequence analysis of genes induced under acid soil stress.

A major output of this research will be improved knowledge on acid soil tolerance mechanisms in *Brachiaria* species. This will help to develop screening procedures to evaluate acid soil adaptation in genetic recombinants of *Brachiaria*. Gaining insights into adaptation strategies in well adapted *Brachiaria* species could also contribute to similar investigations in other CIAT crops like rice or beans.

Progress Report

Growth in low ionic strength nutrient solutions. Nutrient solutions were designed with GEOCHEM 2.0 taking into account the following criteria: the basic composition was taken from Blamey et al. 1991. Precipitation of aluminum compounds, as indicated by GEOCHEM, was avoided. Aluminum was added as $AlCl_3$ rather than $Al_2(SO_4)_3$ because the soluble $AlSO_4^+$ complex which forms readily in the presence of SO_4^{2-} ions is not rhizotoxic [Kinraide and Parker 1987]. Main treatments were: a control solution (co-Al) without aluminum that contained sufficient amounts of all nutrients and a multiple stress solution (st+Al) that exhibited low levels of N, P, K, Ca, Mg in combination with a toxic concentration of aluminum ($\{Al^{3+}\} = 44\ \mu M$). To separate the effect of aluminum and low nutrient supply, an aluminum containing control solution (co+Al) and a low nutrient stress solution without aluminum (st-Al) were prepared also.

For every growth experiment, seeds were surface-sterilized and germinated for 7 days in Petri-dishes under sterile conditions. Twelve groups of three plants were transferred to plastic containers containing about 3 L of constantly aerated low ionic strength nutrient solution in a polyethylene bag and grown in a growth chamber with a 12h/12h day/night rhythm at 25°C. Nutrient solutions were freshly prepared and changed daily. At harvest, root/shoot fresh weight, leaf area, total root length, number of root tips, and root/shoot dry weight were measured.

The effectiveness of these solutions was tested on three rice varieties differing in acid soil tolerance. Root and shoot dry matter and particularly root elongation were affected severely in the st-Al treatment, even in the most tolerant variety.

Table 1: Root and shoot attributes of three *Brachiaria* species grown in a st-Al nutrient solution[1] as percentages of their respective values in a co-Al solution[2]. Letters a,b,c indicate significant differences among species at the 0.05 level.[3]

Parameter	Species		
	B. ruziziensis	***B. decumbens***	***B. brizantha***
shoot biomass	45[a]	71[b]	73[b]
leaf area	33[a]	61[b]	63[b]
root biomass	81[a]	139[b]	118[b]
root length	35[a]	100[c]	65[b]
no. root tips	24[a]	58[b]	61[b]

[1] composition in µM: 100 NO_3^-, 10 NH_4^+, 60 Ca^{2+}, 60 K^+, 30 Mg^{2+}, 1 $H_2PO_4^-$, 6 H_3BO_3, 1 Mn^{2+}, 1 Zn^{2+}, 0.2 Cu^{2+}, 0.001 MoO_4^{2-}, 5 Fe^{3+}, 5 $EDTA^{2-}$, 5 SiO_3^{2-}, 80 Na^+, 100 SO_4^{2-}, 268 398 Cl^-, 80 Al^{3+}, 54 68 HCl (pH=4.2)

[2] composition in µM: 500 NO_3^-, 50 NH_4^+, 300 Ca^{2+}, 300 K^+, 150 Mg^{2+}, 5 $H_2PO_4^-$, 6 H_3BO_3, 1 Mn^{2+}, 1 Zn^{2+}, 0.2 Cu^{2+}, 0.001 MoO_4^{2-}, 5 Fe^{3+}, 5 $EDTA^{2-}$, 5 SiO_3^{2-}, 80 Na^+, 286 SO_4^{2-}, 252 398 Cl^-, 67 75 HCl (pH=4.2)

[3] The mean percentages and their corresponding variances were estimated by means of jackknifing and subsequently compared with the Tukey-Kramer method (Sokal and Rohlf 1981, Buonaccorsi and Liebhold 1983)

In contrast, *Brachiaria* species, especially *B. decumbens*, were less susceptible to multiple stress conditions. Results obtained so far indicate that (i) all *Brachiaria* species were aluminum tolerant in terms of dry matter production because the co-Al nutrient solution almost did not inhibit growth (data not shown). (ii) *B. ruziziensis* was less adapted to combined aluminum and nutrient stress than the other species as judged by relative growth parameters (Table 1). Reduction in shoot and root dry matter, leaf area, total root length and the number of root tips was greater than in the other two species. (iii) In contrast to the other two species, *B. decumbens* maintained root elongation under multiple stress conditions. (iv) Low nutrient supply in the presence or absence of aluminum caused a shift in partitioning of biomass towards the roots. This "phenotypic plasticity" was more pronounced in *B. ruziziensis* and *B. decumbens* (data not shown).

This protocol developed to simulate acid soil stress is currently used to study aspects of acid soil adaptation including nutrient uptake mechanisms, production and exudation of phytotoxic phenolic compounds, and gene induction under acid soil stress.

Plasma membrane H^+-ATPase. The K^+-stimulated plasma membrane H^+-ATPase transports protons from the cytoplasm outwards into the intercellular space creating an electrochemical

proton gradient which drives the uptake of nutrients. It has been demonstrated that Al^{3+} can inhibit H^+-ATPase activity in vitro and that low levels of cations like K^+, Ca^{2+}, Mg^{2+} in vivo can modify its kinetic parameters. Experiments using plants grown under control and stress conditions were initiated to measure kinetic characteristics that could be important to maintain or even increase activity in the presence of Al and low nutrient levels at low pH in vivo. These parameters include specific activity, K_m, v_{max}, pH profile, extent and mode of inhibition of H^+-ATPase activity by Al^{3+} ions in vitro, and stimulation/inhibition by different in vitro levels of K^+, Ca^{2+}, and Mg^{2+}.

Table 2: Influence of various inhibitors upon ATPase activity of root plasma membranes isolated from plants grown in a co-Al nutrient solution. Values represent means of three replicates and are given as percentages with respect to standard assay conditions without inhibitor.

Inhibitor		Target	Species		
			B. ruziziensis	***B. decumbens***	***B. brizantha***
N_3^-	(1 mM)	mitrochondrial H^+-ATPase	97	93	97
MoO_4^{2-}	(1 mM)	phosphatases	93	94	94
NO_3^-	(50 mM)	tonoplast H^+-ATPase	95	89	102
erythrosin B	(200 nM)	plasma membrane Ca^{2+}-ATPase	91	93	102
erythrosin B	(100 µM)	plasma membrane H^+-ATPase	26	27	59
VO_4^{3-}	(100 µM)	"	47	29	68
	(500 µM)	"	29	25	51
DES[1]	(100 µM)	"	77	71	57
DCCD[2]	(100 µM)	"	79	60	61

[1] diethylstilbestrol
[2] dicyclohexylcarbodiimide

Plasma membranes were prepared applying a method which is based on differential partition of membrane types between two aqueous polymer phases according to their surface characteristics [Larsson 1983]. Protein content of the resulting vesicle suspension was determined by means of a modified Bradford method, suitable for quantification of membrane proteins [Esen 1978]. For measurement of the ATPase activity the vesicle suspension was incubated with ATP in the presence of Mg^{2+} and K^+ at pH 6.5 for 30 min, the reaction was stopped by addition of TCA, and the liberated phosphate was quantified by means of an ammonium molybdate antimony potassium tartrate method [Salinas and Garcia 1985].

In order to confirm the purity of the obtained membrane vesicles, ATPase activity was measured in the presence of various inhibitors, specific for ATPases of other membrane types and

unspecific phosphatases. Results indicated a high degree of purity (Table 2). We are thus currently evaluating kinetic characteristics in detail.

Spatial distribution of nutrients in root tips. Nutrient uptake is generally considered to be specially active in root tips. Differences in uptake mechanisms or preferences towards certain elements, e.g. in the context of the anion-cation balance, could result in differences in the levels and or distribution of nutrients in root tip tissue. Aluminum could displace cations like Ca^{2+} from cell walls and membranes or precipitate PO_4^{3-}. Published evidence indicates a role of Si accumulation in the context of aluminum tolerance in grasses. In collaboration with Carlos Pineda, National Accelerator Centre, Faure, South Africa, we initiated studies in order to apply "proton induced x-ray emission" (PIXE) to root tips, a technique which can measure nutrient concentrations with a spatial resolution of ca. 5 µm.

Two types of specimens were analysed: (i) root tips (3-5 mm) were excised, washed in deionized water, lyophilized, and flattened between two glass slides coated with formvar. A proton beam with a diameter of 5 µm was scanned along the root's long axis, collecting datapoints in distances of 50 µm. From each datapoint P, K, Ca, S, Cl, Fe, Si, Mn, Cu, and Zn concentrations were calculated in ppm assuming cellulose as the dominant matrix material. (ii) root tips were frozen in liquid nitrogen and embedded in Tissue Tek. Transverse sections were cut with a cryotome, mounted onto a thin formvar film spanned over a hole in a plexiglass support, and lyophilized. PIXE scans yielded a two-dimensional "concentration map" for each element.

Preliminary results demonstrated a good spatial resolution and sample to background ratio for the transverse sections. Longitudinal scans indicated accumulations of certain elements in the region of the root cap (P, Ca, K, S, Fe, Cu, Zn); differences among species seem to exist.

Phytotoxic phenolic compounds. Field studies carried out at EMBRAPA-CPAC, Planaltina, Brazil, suggested that persistence of legumes when associated with *Brachiaria* species (e.g., *B. brizantha* cv. Marandú) could be affected by allelophatic compounds produced by *Brachiaria*. These compounds could also cause autotoxicity to *Brachiaria* species leading to poor persistence under field conditions. A couple of phenolic compounds have been demonstrated to exhibit allelopathic activity. Thus, phenolic compounds were extracted in ethanol from roots shoots of plants grown hydroponically under control and stress conditions. Crude extracts were analysed directly by means of reversed-phased HPLC using an acetonitrile-water (0-55 %) gradient [Graham 1991].

In roots of *B. ruziziensis* two major compounds were detected at 280 nm that accumulate ten- to twenty-fold under stress conditions (Fig. 1). Small amounts of these compounds were also detected in *B. decumbens*, but not in *B. brizantha*. An attempt was made to identify them comparing their retention times and UV spectra with 23 phenolic standards. Umbelliferone was found to have the same retention time and exhibited a qualitatively similar UV spectra as the

peak at 14.2 min. However UV-absorbance maxima and fluorescence behavior were different. Molecular characterization is envisaged, but will depend on the availability of proper equipment outside CIAT.

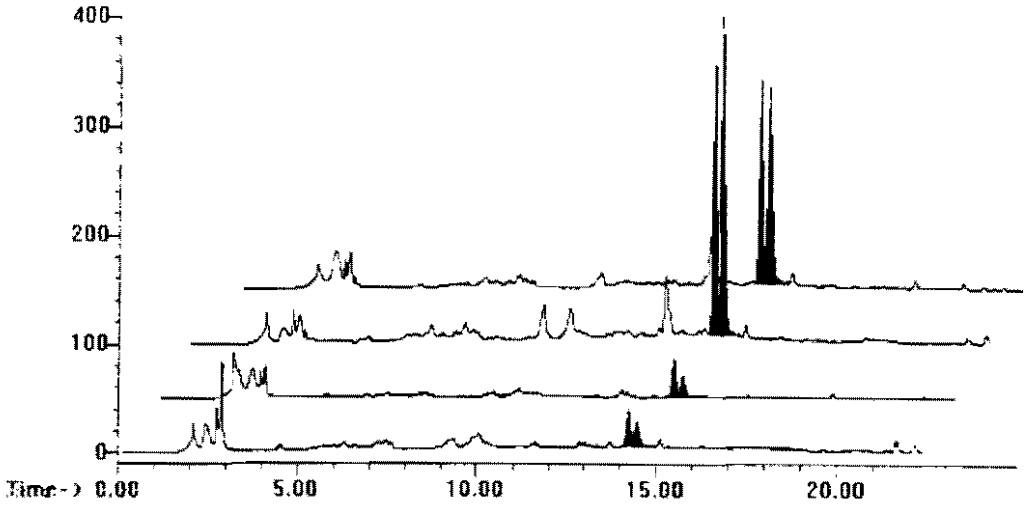


Fig. 1: Reversed-phase HPLC chromatograms (280 nm) of *B ruziziensis* root extracts. Treatments: from bottom to top: co-Al, co+Al, st-Al, st+Al

In order to evaluate the phytotoxic potential of the three *Brachiaria* species, seedling bioassays are being performed on *Brachiaria* and a number of legumes. Seeds are germinated for 8 days under sterile conditions in the presence of increasing amounts of root/leaf extracts. Root length and germination rate are used as indicators for phytotoxicity. By this, we attempt to correlate HPLC chromatograms with phytotoxic activities of root/leaf extracts which eventually could allow us to identify some candidate peaks with phytotoxic activity.

Gene induction under acid soil stress. Isolation of stress-induced genes provides an alternative approach to gain insights into acid soil adaptation. Comparison of DNA sequences of isolated genes with known genes in databases and evaluation of their inducibility by different stress factors could yield valuable information with respect to their role in putative tolerance mechanisms.

Experiments were initiated by isolation of mRNA from root tips of plants grown in a st+Al nutrient solution by means of oligo(dT) magnetic beads. First and second strand cDNA synthesis was performed on the beads and a non-phosphorylated adaptor that contained a Sal I restriction site was ligated to the blunt-ended cDNA coupled to the beads. The non-ligated strand of the adaptor was melted off and the 5' overhand was filled with Klenow polymerase. Then the whole second strand of the cDNA was melted off and converted into a double strand using Taq polymerase and a Not I site containing oligo(dT) primer-adaptor as primer. An aliquot of the ds-cDNA was then PCR amplified with Sal I adaptor and Not I adaptor sequences, digested with

Sal I and Not I, and is currently being cloned into plasmid vectors for cDNA library construction (Fig. 2).

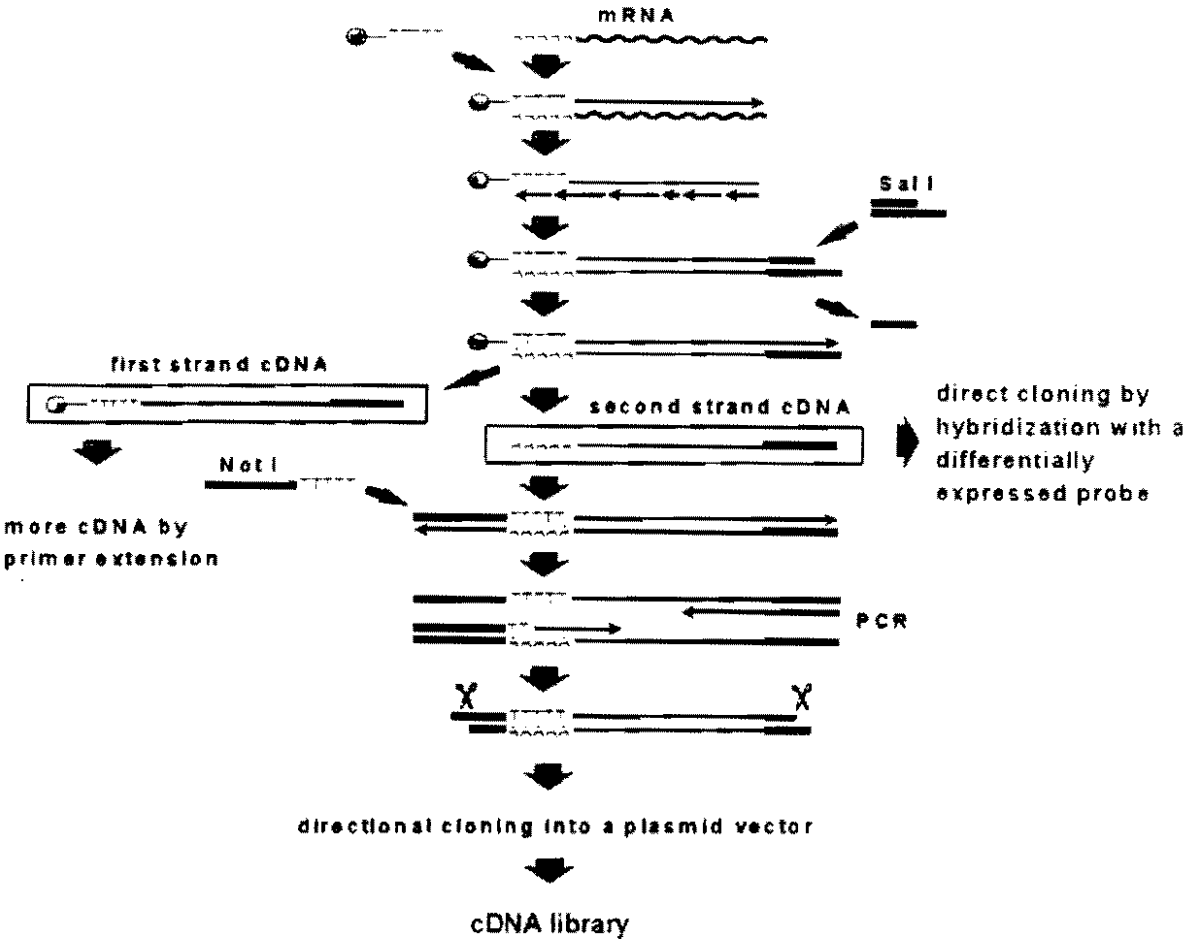


Fig. 2: Strategy applied for the preparation of cDNA libraries from small amounts of mRNA using magnetic beads and PCR

The resulting cDNA library will be screened with st+Al specific probes enriched by subtractive hybridization with driver cDNA derived from root tip mRNA of plants grown in a co-Al solution. As an alternative to subtractive hybridization, we currently intent to apply "amplified fragment length polymorphism" (AFLP) for the isolation of stress-induced genes [Zabeau and Vos 1992].

Future plans

Screening with low ionic strength nutrient solutions. The multiple stress solution designed to simulate acid soil conditions (st+Al) contains low levels of N, P, K, Ca, Mg, and S in combination with Al. In order to study interspecific differences in detail we will cultivate the

three species in corresponding single stress solutions and at different Al levels (with and without Si). For measuring total root length and number of root tips, image analysis with a flat-bed scanner will be applied. Root system architecture will eventually described by means of fractal dimension and or topological parameters [Fitter 1991]. It is further envisaged to determine tissue nutrient concentrations.

Organic acids. Organic acids presumably play a multiple role in acid soil adaptation. They (i) can complex and thereby detoxify Al ions, (ii) solubilize inorganic phosphorus from soils, and (iii) could also play a role as C-source for N-fixing bacteria in the rhizosphere. Thus, organic acids in roots, leaves, and root exudates of plants grown in the four basic solutions (co-Al, co+Al, st-Al, st+Al) will be HPLC-analysed.

PIXE analysis of root tips. Transverse sections and flattened root tips of plants from the four basic treatments will be scanned with the PIXE microprobe to analyse Al, P, Ca, and Si (eventually other elements too).

Plasma membrane H^+-ATPase. Plasma membrane H^+-ATPase activity of plant roots from the four basic treatments will be studied in detail evaluating the following characteristics: specific activity, K_m, v_{max} (Lineweaver-Burk plot), extent and mode of inhibition by Al (competitive/uncompetitive), stimulation/inhibition by K^+, Mg^{2+}, Ca^{2+}, substrate specifity (ATP, GTP, ITP), pH profile of activity, and influence of the temperature on activity in order to detect phase transition(s) of plasma membrane lipids (Arrhenius plot).

Phytotoxic phenolic compounds. Seedling bioassays will be performed using seeds of *Brachiaria* and several legumes to measure the level of phytotoxicity of root/leaf extracts from the three *Brachiaria* species grown under co+Al and st+Al conditions. Root extracts of *B. ruziziensis* grown in single stress solutions will be HPLC analysed with the aim to identify the stress factor which causes accumulation of the putatively phenolic compounds reported above. A molecular characterization is envisaged at the University of Vienna.

Stress-induced genes. We plan to construct cDNA libraries from root tips of all three species grown in st+Al solutions. Probes for differential screening will be enriched applying a PCR-based technique for subtractive hybridization. Once differentially expressed genes have been isolated, their expression in the four basic as well as in single stress solutions will be studied by means of northern blots or ribonuclease protection assays using whole roots, or with RT-PCR using mRNA prepared from root tips. Adaptation of AFLP for isolation of differentially expressed genes will be continued.

Bibliography

BLAMEY FPC, EDMEADES DC, ASHER CJ, EDWARDS DG, WHEELER DM (1991): Evaluation of solution culture techniques for studying aluminum toxicity in plants. In: Wright et al (eds.): Plant-soil interactions at low pH. Kluwer Academic Publ. Netherlands.

BUONACCORSI J.P., LIEBHOLD A.M. (1988): Statistical methods for estimating ratios and products in ecological studies. *Environ Entomol.* **17**: 572-580.

ESEN A. (1978): A simple method for quantitative, semiquantitative, and qualitative assay of protein. *Anal Biochem.* **89**: 264-273.

FITTER A.H. (1991): Characteristics and functions of root systems. In: Waisel Y., Eshel A., Kafkafi U. (eds.): Plant roots - the hidden half. Marcel Dekker, New York, USA.

GRAHAM T.L. (1991): A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. *Plant Physiol.* **95**: 584-593.

KINRAIDE T.B., PARKER D.R. (1987): Non-phytotoxicity of the aluminum sulfate ion, $AlSO_4^+$. *Physiol. Plant.* **71**: 207-212.

LARSSON C. (1983): Partition in aqueous polymer two-phase systems: a rapid method for separation of membrane particles according to their surface properties. In: Isolation of membranes. Academic Press. London, Great Britain.

SALINAS J.G., GARCÍA R. (1985): Métodos químicos para el análisis de suelos ácidos y plantas forrajeras. CIAT. Cali, Colombia.

SOKAL, R.R., ROHLF F.J. (1981): Biometry. The Principles and Practice of Statistics in Biological Research. 2nd edition. W.H. Freeman and Company. New York, USA.

ZABEAU M., VOS P. (1992): European patent application.

2.5 Project: Monitoring resistance to Propanil in *Echinochloa colona* populations

A.J. Fischer[1], J.E. Mayer[2], A.L. Chaves[2], H.V. Ramirez[1], and D.N. Varela[1]
[1] Rice Program and [2] Biotechnology Research Unit

Abstract

Intensive use of propanil (3,4-dichloropropionanilide) to selectively control junglerice (*Echinocloa colona* (L.) Link) in Latin American rice fields has caused the development of resistant (R) biotypes of this weed; safe use in rice, and poor control of R junglerice result from rapid hydrolysis of propanil by aryl acylamidase activity in those plants, as opposed to that in susceptible (S) junglerice. HPLC assays of plant extracts from 2 to 3-leaf-stage junglerice showed low propanil concentrations and a buildup of metabolites in R as opposed to S plants. Older R and S plants (30 days) showed no differential degradation of propanil 24 hour after herbicide application, confirming previous observations suggesting that a different mechanism of resistance may be present in older plants.

Background

Echinochloa colona (L.) Link, junglerice, is one of the most relevant weeds in tropical Latin American rice fields. The intensive use of post-emergence applications of propanil for more than 10 years to control selectively this weed in rice, has resulted in increasing abundance of propanil-resistant biotypes junglerice populations (Garro, et al. 1991; Fisher, et al. 1993). Aryl acylamidase activity is responsible for the degradation of propanil to DCA (3,4-dichloroaniline) in plants. This hydrolysis occurs much slower in E. colona (S), where propanil accumulates, than in rice or R *E.colona* biotypes. Organophosphate or carbamate insecticides can block this reaction resulting in rice and *E. colona* (R) injury (Yih, et al. 1968; Leah, et al. 1994) . Amidase activity and propanil metabolism in *E. colona* decreases with age, but resistance did not decreased as it would be expected, the presence of a different mechanism of resistance to propanil was suggested.

Identifying the fields where herbicide resistance has been developed, knowing the proportion of resistant individuals in those fields, as well as their levels of resistance, is key for weed management. The detection of resistance in the field often involves labour-intensive and time-consuming bioassay greenhouse studies. Data interpretation from such experiments usually requires non-linear regression interpretation to fit logistic equations which provide biologically realistic regression parameters (Streibig, et.al 1993; Seefeldt, et al. 1994). The use of ^{14}C-labelled propanil has been successful in studies of uptake and metabolism of propanil (Leah, et al. 1995). However the use of this technique is still somewhat under developed in Latin America. Therefore, a quicker and safe-to-use tool for detecting and quantifying levels of resistance, would be desirable for processing large amounts of plant samples collected in rice fields.

Expected Outputs

To test an alternative procedure for detecting and monitoring the evolution of resistance to propanil in junglerice populations: such assays should also serve to quantify the concentrations of propanil and its metabolites present in tissues of plants previously sprayed with a commercial rate of the herbicide, allowing for further studies on the fate of propanil in older junglerice plants.

Progress Report

Levels of resistance in *Echinochloa colona* accessions. In a previous study (Fisher et al., 1993), seven junglerice populations had been collected in Colombian rice fields with different histories of propanil use, and subjected to dose-response studies with propanil, where the dry matter accumulation by plants sprayed with a range of propanil dosages was recorded. Data from that study were re-interpreted using non-linear regression. GR_{50} values were estimated using a four parameter logistic model (Streibig et al., 1993; Seefeldt et al., 1994).

The accessions collected differed markedly in their response to propanil rates (Figure 1), as shown by the corresponding range of GR_{50} values (Table 1), out of which accessions 2 and 5 were the extremes. The non parallel dose response curves obtained with accessions 2 (S) and 5 (R) (Figure 1) would suggest according to Streibig et al., (1993) that resistance to propanil involved factors other than, or in addition to, a modification of the target site. The logistic model used, fitted some accessions better than others (Table 1). The poorer ones could partly be due to the theoretically heterogeneous nature of the individual genotypes within each accession (population), which may somewhat differ in their response to propanil.

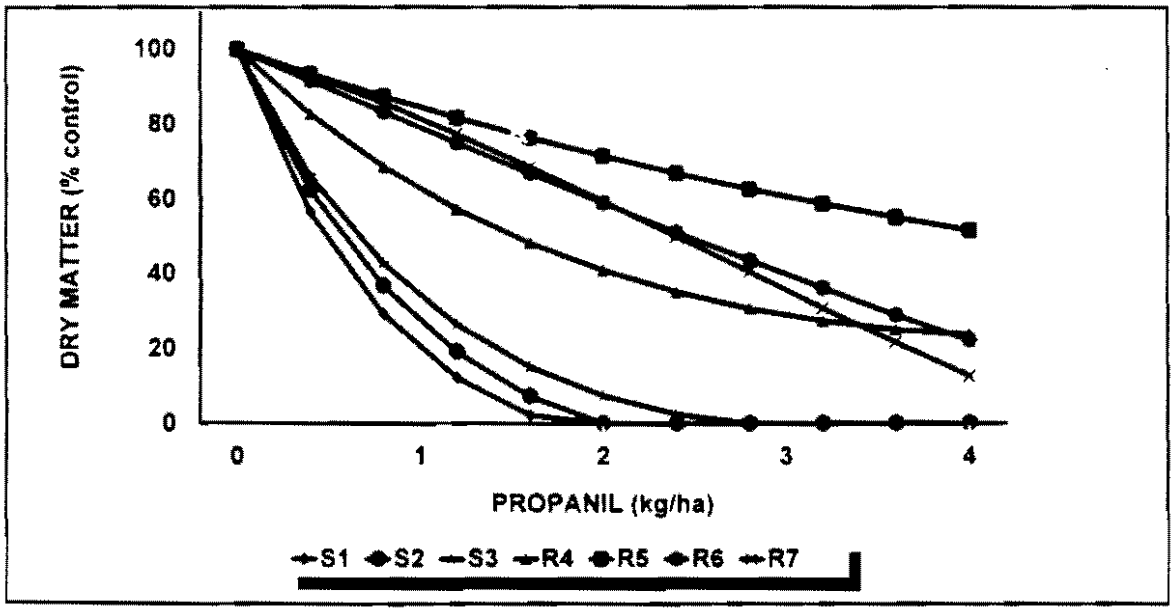


Figure 1. Response curves of seven Echinochloa colona accessions to propanil doses.

Table 1. Propanil rates for 50% growth reduction of seven Echinocloa colona accessions, and the coefficients of determination of regresssions.

Accession #	GR_{50}	R^2
1	0.53 (0.02)[1]	0.99[*,2]
2	0.37 (0.02)	0.99**
3	0.78 (0.05)	0.98**
4	1.71 (0.25)	0.98**
5	>4[3]	0.89*
6	3.37 (2.62)	0.79*
7	2.22 (2.62)	0.79*

[1] Values in parenthesis are standard errors of the estimate.
[2] *, P<0.5; **, P.0.01
[3] Lies outside the range of dosages studied.

Propanil degradation: HPLC studies. HPLC assays were conducted with S and R junglerice plants (from accessions with the lowest and highest GR^{50} values, respectively) at the 2-leaf stage and just before flowering (45 d.a.e.), and 24 hours after they have been sprayed with 1.5 Kg propanil/ha (in 200 l/ha) in the greenhouse. The plant extracts were prepared from E. colona leaves using Tris-HCL (pH 7.0) as extraction buffer. The homogenized filtered and centrifugated material were analyzed immediately through HPLC. A calibration curve was prepared using propanil (pure compound).

The figure 2 (a.b.c and d) shows the results from the HPLC assays. When young (2-3-leaf stage) junglerice plants were treated with propanil and assayed 24 hours after, the propanil concentration in R was 80% lower than in S plants. Concomitantly, an increase in the levels of other substances, presumably propanil metabolites and their conjugated (Yih et al., 1968; Leah et al., 1994) could be clearly differentiated in R plants. Such peaks were notoriously lower in S plants (Fig. 2a). When older plants (just before flowering) were assayed, no differences in propanil contents could be observed between R and S plants 24 hours after propanil application (Fig. 2c y 2d). This agrees with Leih et al., (1994) who found slower rates of propanil metabolism and aryl acylamidase activity in 30 day-old plants with no decrease in resistance, suggesting that a different mechanism may be involved in conferring resistance to propanil at this stage.

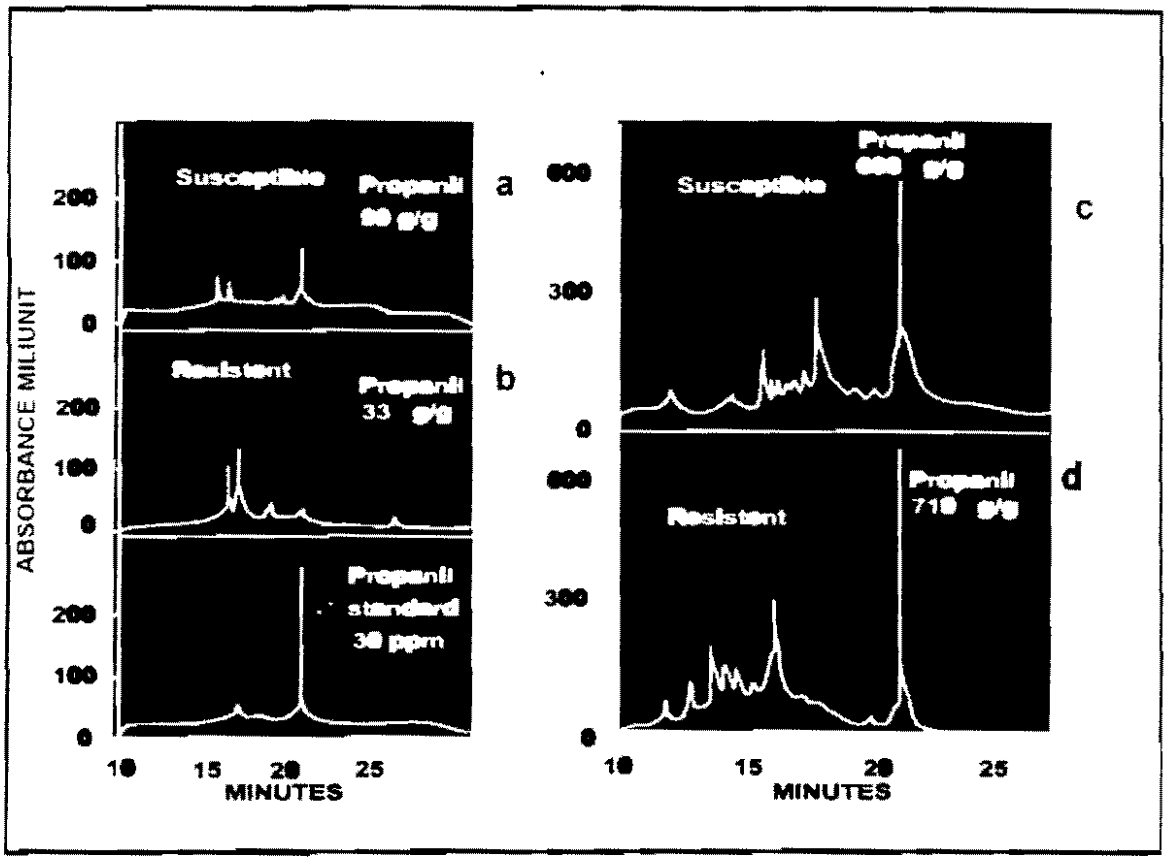


Figure 2 chromatograms of E. colona leaf extracts, after spraying young (a & b) and old (c & d) plants with propanil

Future plans

The continuous use of propanil must be avoided, and there are others herbicides that could be used alternatively However, given the mounting evidence of complex patterns of cross resistance emerging from diverse studies, there is no long-term certainty that alternating herbicides of different chemistry and mode of action would not eliminate the buildup of herbicide resistance Therefore, it is of paramount importance that plants escaping a herbicide treatment be removed with another herbicide or by other means, and that seed production by these plants be prevented Rotations with other crops can allow for the use of other herbicides that cannot be used selectively in rice However it is often difficult to grow other crops on the poorly-drained rice soils. More needs to be known in terms of the mechanisms and heredity of propanil resistance before more specific management options can be prescribed The extent and severity of propanil resistance in populations of E colona needs to be evaluated in Latin America, a rapid detection method would be very helpful for this purpose These preliminary experiments on resistance to propanil in populations of junglerice are part of an interinstitutional (CIAT and AgrEvo, Colombia) effort to develop a major project to address this problem

References

Fischer, A.J., E. Granados and D. Trujillo. 1993. Propanil tolerance in populations of junglerice. Weed Sci. XCI, 201-206.

Garro, J.E., de la R. de la Cruz, and P. J. Shannon. 1991. Propanil resistance in *Echinocloa colona* populations with different herbicide use histories. Proc. Brighton Crop Protection conference 8A-XI, 1079-1083.

Leah, J.M., J.C. Caseley, C.C Riches and B. Valverde. 1994. Association between elevated activity of aryl acylamidase and propanil resistance in jungle-rice, *Echinocloa colona*. Pestic. Sci. XCII, 281-289

Seefeldt, S.S., J.E. Jensen and E.P. Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Technology. IX, 218-227.

Seefelt, S.S., D.R. Gealy, B. Brewster and E.P. Fuerst. 1994. Cross-resistance of several diclofop-resistant wild oat (Avena fatua) biotypes from the Willamette Valley of Oregon. Weed Sci. XCII, 430-437

Streibig, J.C., M. Rudemo and J.E. Jensen. 1993. Dose-response curves and statistical models. p. 30-55 in J.C. Streibig and P. Kudsk, eds. herbicide Bioassays. CRC Press, Boca Raton, Fl.

Yih, R.Y., H. McRae and H.H. Wilson. 1968. Mechanism of selective action of 3,4 dichloropropionanilide. Plant Physiol. XCV, 1291-6.

3. PROJECT AREA: GENE TRANSFER AND CONSERVATION OF GENETIC DIVERSITY

3.1 Project: Insterspecific hybridization assisted by embryo rescue and molecular markers in *Phaseolus*

A. Mejia[1], P. Herrera[1], A. Criollo[2], S.P. Singh[2], W. Roca[1]
[1]Biotechnology Research Unit; [2]Bean Program

Background

Using a methodology developed earlier in this project large numbers of *Phaseolus vulgaris* x *P. acutifolius* hybrid lines have been produced and their selfed progeny evaluated in the field for the expression of bacterial blight resistance and other agronomic traits of interest.

Over 50 advanced lines have been selected which express high resistance to bacterial blight and maintain all agronomic traits. Several interspecific hybrid lines have now moved to regional trials.

This year, we have initiated a new cycle of congruity backcrossing involving in it new tepary bean lines.

Progress Report

(i) Selection of advanced recurrent hybrid lines continued under field conditions for resistance to bacterial blight and adaptation to poor soils. Out of 50 lines, and one razer blode inoculations, 10 have finally been selected with very high bacterial blight resistance and good adoption to poor soils.

Table 1 shows the new common bean x tepary crosses designed to introgress *Empoasca* tolerance traits to the common bean. We have reached the eighth congruity backcross.

As in previous work, fertility (seed production) was gained when the last parent was *P. vulgaris* and, conversely fertility was significantly diminished when the last parent was *P. acutifolius*.

The initial cross, with tepary parent G40001, a facilitator of hybridization, served as a bridging to reduce incompatibilities in subsequent crosses with other tepary genotypes.

Seed obtained from these crosses (Table 1) has been grown for seed increases and future use in agronomic trials.

Table 1. New congruity backcrosses between *P. vulgaris* and *P. acutifolius* using embryo rescue

Cross	---	*vulgaris: acutifolius* ratios	---	Seed production
A		**IP x G40001** (1 *vul*: 1 *acu*)		**NO**
B	----------	**x IP** (2 *vul*: 1 *acu*)		**YES**
C	----------	**x G40102** (2 *vul*: 2 *acu*)		**NO**
D	----------	**x IP** (3 *vul*: 2 *acu*)		**YES**
E	----------	**x G40102** (3 *vul*: 3 *acu*)		**NO**
F	----------	**x IP** (4 *vul*: 3 *acu*)		**YES**
G	----------	**x G40102** (4 vul: 4 *acu*)		**NO**

IP : ICA Pijao (*P. vulgaris* parent)

G4001 and G40102 (*P. acutifolius* parents)

3.2 Project: Genetic Transformation of Cassava, Beans and *Brachiaria*

3.2.1 Agrobacterium - Mediated Transformation of Cassava

E. Torres, N. Balcazar, F. Angel . W.M. Roca
Biotechnology Research Unit.

Background

Earlier work in this unit resulted in: (i) selection of an agropine/mannopine (CIAT 1182) wild A. tumefaciens. strain and the M-Perf 183 cassava variety as the best bacterium-variety combination: (ii) a plasmid for our transformation experiments (pGV1040) which contains the following chimeric genes: neomycin phosphotransferase II (nptII); ° glucorinidase (gus) and phosphinotricin acetyltransferase (bar) genes; (iii) infection of cotyledonary leaves from somatic embryos (cv. M. Perf) with the CIAT 1182 strain harboring pGV1040, and the induction of somatic embryons in a medium supplemented with 16-32 mg/L of Basta; (iv) several Basta-resistant plantlet were obtained and screened by PCR using specific primers for the gus and nptII genes. and the products were analyzed by Southern blot using the 6 Kb plasmid T-DNA as probe; (v) only the lines which gave positive signas for both genes were transferred to soil and taken to the greenhouse. This year our efforts focused on the following: (i) demostration of the insertion and expression of the transgenic in cassava regenerated plants by molecular and resistance assays; (ii) the construction of vector for the transformation - resistance against the cassava stem borer; (iii) initiation of work to isolate and clone a starch branching enzymed.

Progress Report

1. Demostration of cassava transformation
In order to demostrate insertion and expression of introduced genes in regenerated plants after transformation, three assays were performed:

(i) First assay

- Resistance to Basta, analysis for expresion of the bar gene was made in vitro with leaf discs and different concentrations of Basta (2, 4, 6, 8 mg/L). Only young leaves were used for this test.

- Transformed plants displayed different leves of tolerance to Basta; only those displaying tolerance to 8 mg/L Basta were selected: 15 plants out of 230 (6.5%).

(ii) Second assay

- Fifteen plants, selected in the first screening were used for this assay. Six leaves from each plant were analyzed for °- glucoronidase activity, Basta resistance and transgene insertion using PCR.

- Gus Test. Histological tests were performed to detect expression of the gus gene, using whole shoot tips from transgenic plants. Fourteen plants out of 15 plants tested were gus positive. Results obtained suggest that the expression of gus gene could be related to the state of development and the type of tissue tested, with expression of gus decreasing as shoot tip tissues differentiate and mature.

- Basta and PCR tests. Plants preselected with 8 mg/L Basta in the first screening were also positive for PCR. For the second PCR test, a specific primer for gus was used. Amplified products were transfered to Hybond N+ by Southern blot and hybridized with the 6 Kb probe.

- Four plants out of 15 analyzed in the second screening, were positive for both Basta resistance and gene insertion using PCR. The plants were: 55 2.1, 108 2.3, 137 5.1 and 207.

(iii) Third assay

- Genomic Southern hybridization. The four plants selected from the second assay were used for Southern blot. Total DNA from each plant was digested with Bgl II was hybridized with different probes. One probe was the 6 Kb fragment obtained from pGV1040, Bgl II-digested, plasmid. This 6 Kb fragment contains the three chimeric genes npt I I, gus and bar. Clear positive signal was obtained wiht the transformed plant 55 2.1. Weaker signals were observed with plants 108 2.3 and 207. When thegus gene alone was used as probe, the same plants found positive with the 6 Kb probe were also positive, but displaying a stronger hybridization signal. However, in this case, a cross hybridization with a repetitive band was also detected in all plants tested. To determine the number of different insertions in transformed plants, Sca I restriction enzyme cutting inside 6 kb region was used. After hybridization with bar gene as probe, three bands were evident in plant 108-2.3, corresponding to 10, 6 and 4 kb, indicating that three different iinsertions are present.

  In conclusion, this year we have been able to present conclusive of evidence of the generation of transgenic cassava plants.

Future plans

- Additional genomic southern of cassava with the transformed plants, using different probes.

- Digestions with different restriction enzymes, cutting inside the 6 Kb region insert, will be carried out to determinated the number of different insertions present in all transformed plants.

3.2.1.1. Construction of a specific vector to be use in *Agrobacterium*-mediated transformation of cassava

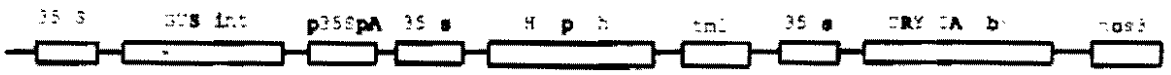
Norman Balcazar, Fernando Angel, W. Roca
Biotechnology Research Unit

Background

The Cry I A (b), gene isolated from Bacillus thuringiensis, coding for a delta-endotoxin inhibiting lepidopters, coleopters and dipters, has been largely used to transform tobaco, tomato cotton, maize and rice, in order to obtain resistant plants to mentioned pests. The lepidopter stemborer Chilomina clarkei is found attacking cassava in several areas of Colombia and others countries. Damage produced by the larval instars occurs in the stems, weaking the plant and often causing breakage; yield reduction of 40-60% due to stemborer has been reported.
Due to the difficulty to control stemborers and given the increase of infestation in cassava growing areas and because of the present abscence of host resistance, we have initiated this year a proyect to develop a transgenic aproach using the Cry IA (b) gene.

Progress Report

The Cry IA(b) gene was obtained from Plantek Co, Japan, by means of a collaborative effort with IRRI and CIMMYT. We have constructed the vector pBGHC1 to be used for Agrobacterium-mediated transformation of cassava. This vector contains three genes. They are: the gene cry I A (b), hygromicine phosphotransferase gene (Hph gene) and GUS-intron gene. Each gene them is under the control of specific promotors and terminal signals of transcription.



The pBGHC1 vector is derived from the pBIN19 plasmid. Both plasmids will be used to transform cassava through a binary sistem of transformation. 35 S and 35 SpA regions represent promotors and terminal sequences corresponding to those isolated from the cauliflower mosaic virus. Tml (tumor morphology) and nos 3' (nopaline synthase) represent transcription terminal sequences isolated from Agrobacterium.

Sequences used in the construction of pBGHC1 vector have been used in other constructs and are non tissue specific; this will allow us to detect expression of foreign genes at different developmental stages and in different tissues of transformed plant.

Future plans

In 1996, the pBGHC1 construct will be used to transform cassava via Agrobacterium using the derivated binary system.

3.2.1.2 Isolation of genes coding for a starch branching enzyme

N. Balcazar, F. Angel, W. Roca
Biotechnology Research Unit

Background

The starch branching enzyme plays an important role in determining the quality and amount of biosynthesized starch. This enzyme is involved in the synthesis of amylopectine. Cassava is an important starch source in the tropics and an important source of calories. Our objectives is to construct a gene vector, including genes coding for the branching enzyme to transform cassava via particle gun and/or Agrobacterium-mediated. The goal is to generate cassava lines with highly different amylase/amylopectin ratios for different end uses.

Progress Report. The gene coding for the branching enzyme was isolated and cloned from a potato cDNA library using as probe a PCR-product of 600 bp. Primers for amplification were based on the sequence published previously (Salehuzzaman et al. 1992).

After sequential hybridizations and several isolation steps, the gene has been isolated and cloned in pBSKS (Bluescript KS) Sequencing and cloning in a suitable vector for transformation of cassava are future activities.

References

Salehuzzaman S I N M, Jacobsen E, Visser R G F. 1992. Cloning, partial sequencing and expression of a cDNA coding for branching enzyme in cassava. Plant Mol Biol 20: 809-819.

3.2.2 Development of a methodology for continuous production of Cassava somatic embryos

J.G.Cobo[1], E. Torres[1], W.M Roca[1]
[1]Biotechnology Research Unit

Background

Somatic embryogesis in cassava is considered as the only efficient way for regeneration. Up to date, somatic embryos can be induced from other explants, such as immature leaves, leaf lobes, somatic cotiledonary leaves and meristems. Auxins such as PICLORAM and DICAMBA have been tested with good results.

Somatic embryogenesis can be influenced by any factors involved in the process, e.g. explant type, origin of mother plants, hormonal content, and other media component. Using the potencial of somatic embryos to generate new embryos (secundary embryogenesis), we expect to find a simple methodology that will increase the amount of embrygenic material and develop a continuous production of embryos.

Progress report

Primary induction: Due to diferences founded in the induction media of MATHEWS *et al* when compared with CIAT media, several cooper levels in the form of CusSo4 were tested. As Table 1 shows, all cooper levels produced more responding explants (R.E.) and embryos per responding explant (E.R.E.) than the control. This results were similar with media containig PICLORAM at 8 mg/. Because CIAT induction media includes cassein hidrolizate (C.H.) the effect of these substance was evaluated. Table 2 shows the effect of various levels of casein hidrolizate the higher concentration of C.H. (control media) produced the same amount of friable callus as the free C.H. media (data no shown). The production of friable callus was not in agreement with previous reported results.

Proliferation phase: Fragmentation of large structures (25-60 mm^2) into smaller clusters (15-25 mm^2) increased proliferation efficiency (Table 3a) The clusters <15 mm^2 produced more friable callus (F.C.) and aparently decreased the ratio of final embryos produced per initial embryos (F.E./I.E.). When these groups were transfered to germination media, the smaller explants produced less R.E. with germinated embryos. The compactation grade of clusters was very important: groups that were chosen as compact produced almost twice F.E./I.E., more R.E. and less F.C. than not compacted groups (Table 3b). Aditional cooper used increased the efficiency of the process. Table 3c shows that all cooper treatments have more F.E./I.E. than control media (MS4). As for primary induction, C.H. increase the efficiency. Amount of friable did not respond to the treatment (Table 3d).

Auxin type and concentration were evaluated too. 2,4-D and PICLORAM were evaluated at 4 and 8 mg/l; the initials explants responded as shown in Table 3e. All treatments produced almost the same F.E./I.E. althought R.E. differed; the higher F.C.

amount was produced in PICLORAM media. ew have also evaluated the MS8 used for embryo proliferation in four cycles MS8-fragmentation-MS8. Figure 1 shows an increase between cycles interms of F.E/I.E (A), final fragments per initial fragments, F.F./I.F. (B), and the ratio A/B.

Liquid media was evaluated (Table 4) using proliferated embryos wich were transfered to germination media without regulators. Solid media embryos were cultured during 30 days and liquid media embryos, during 60 days. Liquid media affected the germination and elongation process, with an increased R.E. and F.E./I.E. of proliferated embryos but a decreased of the F.C. amount.

Cyclic embryogenesis: Cotiledonary leaves, embryogenic axis, not elongated embryo fragments and remainder tissues were utilized as explants (Annual Report, 1994). The effect of C.H. on embryo induction from cotiledonary leaves is shown in Table 5. Aparently the use of 50 mg/l decreased the E.R.E and increased the F.C. amount; 0 and 25 mg/l were similar. The adition of cooper resulted in better efficiency as is shown Tabla 6. In PICLORAM medium, all the levels of cooper gave better results than the control.

Disection: Cotiledonary leaves from germinated somatic embryos were dissected out. The intact axis was transfered to elongation media and the cotyledonary leaves were used as primary explants for cyclic embryogenesis. Table 7 shows the elongation and cicatrization frecuencie for dissected embryos and control embryos after two months in the elongation phase.

Future Plans

Although we have obtained a high amount of somatic embryos in juvenile stage, germination embryo and plant elongation must be improved in order to obtain higher number plants per initial explant. Use of activated charcoal, and ABA, and embryo desiccation will be important for this purpouse.

Refferences

Mathews, H. *et al.* 1993. Plant cell reports 12: 328-333
Raemakers, C.J.J.M. *et al.* 1993. Annals of botany 71:289-294
Raemakers, C.J.J.M. *et al.* 1993. Plant cell reports 12:226-229
Schopke, C. *et al.* 1992. in: proc. of the 1st sci. meet. CBN, Cartagena, Colombia. Ab. p:140
Stamp, J.A. and Henshaw, G.G. 1982. Z.Pflanzenphysiologie 105: 183-187
Stamp, J.A. and Henshaw, G.G. 1987. Plant cell and organ culture 10: 227-233
Stamp, J.A. and Henshaw, G.G. 1987. Annals of botany 59: 415-450
Sudarmonowati, E. and Henshaw, G.G. 1992. in: proc. of the 1st sci. meet. CBN, Cartagena, Colombia. Ab. p:128
Szabados, L. *et al.* 1987. Plant cell reports 6:248-251

Table 1: Effect of different levels of cooper on embryo induction using greenhouse grown meristems.

COOPER (µM)	R.E. (%)	E.R.E.
0	71.4	19.60
1	89.2	32.15
2	89.2	28.88
4	92.2	33.80
8	85.7	28.45

R.E.: Responding explants E.R.E.: Embryos per R.E.

Table 2: Effect of cassein hidrolizate on embryo induction from meristems obtained from greenhouse and in vitro plants

C.H. (mg/l)	Greenhouse plants		In vitro plants	
	R.E. (%)	E.R.E.	R.E. (%)	E.R.E.
50	82	21.58	81.8	9.71
25	82	16.46	74.4	9.70
0	84.4	14.44	69.7	6.59

Table 3: Effect of different factors on the proliferation phase

EXPERIMENT	TREATMENT	R.E. (%)	F.E./I.E.	F.C.
Fragmentation	Fragmented	92.8	2.38	no data
(a)	Control	82.1	1.68	no data
Compactation	Compacted	89.6	1.98	**
(b)	Not compacted	52.2	1.05	***
Cooper	0 (µM)	89.4	1.50	**
(c)	1 (µM)	90.0	2.02	***
	2 (µM)	85.0	1.75	***
	4 (µM)	90.0	1.90	***
	8 (µM)	90.4	1.83	****
Cassein hidrolizate	50 mg/l	89.3	1.78	***
(d)	25 mg/l	87.7	1.60	***
	0 mg/l	72.0	1.46	***
Hormone	4 mg/l 2,4-D	71.4	1.70	**
(e)	8 mg/l 2,4-D	76.1	1.73	**
	4 mg/l PICLORAM	80.9	1.76	***
	8 mg/l PICLORAM	71.4	1.78	****

F.E./I.E.: Final embryos per inicial embryos F.C.: Friable callus

** Small *** Medium **** High

Table 4: Effect of proliferation media type on embryo regeneration

	PROLIFERATION		GERMINATION PHASE			ELONGATION PHASE		
Media type	R.E.(%)	F.E./I.E.	R.E.(%)	G.G.I.E.	G.E.R.E.	R.E.(%)	E.E.I.E.	E.E.R.E.
Solid MS4	89.3	1.60	82.1	3.82	4.65	17.8	0.28	1.60
Liquid MS4	100	1.90	64.2	1.61	2.50	10.7	0.18	1.66

Table 5: Effect of casein hidrolizate on embryo induction from cotyledonary leaves

C.H. (mg/l)	R.E. (%)	E.R.E
50	100	34.81
25	100	50.30
0	100	48.18

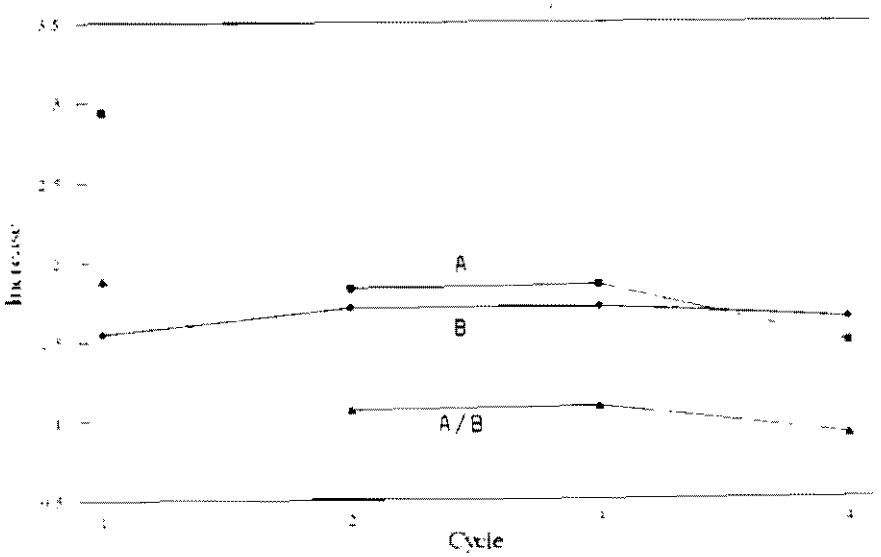
Table 6: Effect of cooper on embryo induction from cotiledonary leaves

Cooper	8 mg/l 2,4-D		8 mg/l PICLORAM	
(µM)	R.E.(%)	E.R.E.	R.E.(%)	E.R.E.
0	90.4	29.62	90.4	20.75
1	95.2	18.70	95.2	24.99
2	85.0	23.17	95.2	27.89
4	90.4	33.94	95.2	30.14
8	90.4	40.65	100	37.86

Table 7: Effect of dissection on elongation phase

Treatament	% Cicatrizat.	% Elongation
Control	4.8	17.0
Disection	12.1	21.9

Figure 1: Effect of MS8-fragmentation cycles on proliferation



3.2.3 Development of common bean genetic transformation protocol

A. Mejía , A.M. Gómez[1], H. J. Jacobsen[2], F. Wolter[2], E. Heinz[2] and WM. Roca[1]
Biotechnology Research Unit, CIAT[1] , The Universities of Hannover[2] and Hamburg[2]

Background

The genetic transformation of bean has been achieved by introducing DNA directly into the meristems present in embryo axis of mature seeds, using the biolistic approach. This methodology that avoids the need of protocols for plant regeneration from somatic cells or tissues, has however only been applied successfully to few genotypes, with very low rates of transformation (Russell et al. 1993). We (in 1994) and others (Sato et al. 1993) have used this methodology without success.

In 1994 we developed a methodology to induce organogenic callus tissue in bean, which we developed further during 1995 to achieve routinely the regeneration of whole plants from this tissues. We have used also during this year intensively the organogenic calli as a target in genetic transformation experiments with the particle gun. From the many shoots that we have been able to select in vitro after particle bombardment, and that we are now regenerating, we expect to get our first transgenic plants.

Expected outputs

(i) The development of a methodology for the production of transgenic bean plants. (ii) The production of transgenic bean plants expressing a gene for a selective glycerol-3-phosphate-acetyl-transferase (GPAT) enzyme of pea and an antisense DNA for the GPAT gene from the common bean. This should confer tolerance to low temperatures to the transformed plants (Wolter et al. 1992 and 1993).

Methodology

Using the PDS1000/He, the helium driven particle delivery device of Biorad, we bombarded organogenic calli of 4 bean genotypes: Bayo madero, A295, ICA pijao and C20, with two separate plasmids: 1) pUC9PG (Jacobsen, Hannover) carrying the genes GUS-Int (GUS gene containing an intron, Willmitzer, Berlin) and PAT (phosphinotricin-N-acetyl-transferase, Broer, Bielefeld, Germany) and 2) pBS Hyg (Iván Lozano, CIAT-VRU) carrying the gene hpt (hygromycin phosophotransferase). The genes PAT and HPT code for enzymes that confer the cells expresing them resistace to the antibiotical compounds phosphinotricin (glufosinate) and hygromycin-B respectively. Tissues are bombarded twice, at 1100 and 1300 psi.

Before or after bombardment the organogenic calli were subjected to different treatments in order to increase the frequency of integration or expression of the introduced genes. The treatments

included: 1) culture of the calli 3 weeks before and after the bombardment in media with auxins, as a way to increase cell divisions; 2) irradiation of the calli with UV-B light immediately after bombardment (see annual report 1994); 3) culture of the calli in medium with 5mg/l 5-Aza-citidine (Mandal et al. 1993) for 3 weeks, 2 weeks after bombardment; 4) cutting transversely the organogenic calli and bombarding the cut surface, in order to expose different cell-layers of the tissue to the projectiles.

After bombardment the calli are allowed to grow for two weeks without selection pressure, and then the putatively transformed calli are selected by sub culturing them at least three times, in three weeks intervals, on media containing 2 mg/l phosphinothricin or 20 mg/l hygromycin.

After this the calli are further cultured on shoot differentiation medium without selective agents, to allow further callus growth and shoot differentiation. Following the differentiated shoots are grafted in vitro onto hypocotyls of in vitro germinated seeds of the genotype Bayo madero, to induce shoot elongation and whole plant regeneration. During the first days after grafting the buds are maintained with the hypocotyls in tight contact with the help of eppendorf tips. Once the shoots develop the first trifoliate leaves, they are transferred to pots in a specially adapted greenhouse.

Progress report

The particle bombardment of embryo axis of mature seeds, as a method to obtain transgenic plants in beans, methodology that we used intensively in 1994 , didn't result in our hands in the generation of stable bean transformants. With a frequency of 2.5% we could detect GUS expressing buds in the greenhouse, but these showed to be chimeras that didn't generate transgenic seeds. Similar bad results with this methodology have been reported by Sato et al. (1993) in soybean.

This is the reason why in 1995 all our efforts were concentrated in the bombardment of organogenic calli. The advantages of the use of this tissue are: 1) more meristematic cells are exposed to the microprojectils and 2) an in vitro selection step can be applied more efficiently.

For this purpose, from the initially six genotypes chosen at the beginning of the project, only the genotypes that showed the best response to our organogenic callus induction and plant regeneration conditions, were chosen: Bayo madero, A295, ICA pijao and C20.

As one of the most important problems in the genetic transformation by particle bombardment is the very low rate of foreign DNA integration we subjected the calli to different treatments before or after the bombardments in order to increase the events of stable integration. The results of these experiments can only be analyzed if the transformed status of the selected tissues is cleared.

Between the end of 1994 and October 1995 we have bombarded in 29 sessions 275 petri dishes, from which we have been able to select approximately 500 clones that have resisted the culture on selective media. Because too high hygromycin or phosphinothricin concentrations in the selective

media could kill chimeric shoots that could be valuable to our objectives, we decided not to apply too strong selective conditions. Thus after the selection we expect to get also some escapes. We are now retesting the putative Hyg or PPT resistant phenotype, with higher concentration of the antibiotics (30 and 5 mg/l respectively), in order to eliminate escapes.

Due to the well known difficulty to elongate and root in vitro cultured bean shoots, we developed a micro grafting methodology to regenerate mature plants from the selected, putatively transformed shoots.

The percentage of micro grafts that grow further into plantlets is of about 50% with the actual methodology, that is good and easy enough to be used routinely. From each selected clone three or more micro grafts can be made, and micro grafts that fail to develop, can be rescued and, after a short culture phase in a maintenance medium, micro grafted again. Thus it can be said, that all selected clones are regenerable to plants.

At the moment 55 plantlets derived from micro grafted shoots are in the acclimatization phase in our biosafety greenhouse. Of which that have reached the flowering stage, all have shown to be fertile. The transformed status of the regenerated plants is to be confirmed.

Future plans

Using molecular detection techniques like PCR and RFLPs, the presence of the marker genes in the regenerated plants and in its progeny will be analyzed. If the stable integration of the foreign genes is confirmed, genetic transformation with the genes for a selective glycerol-3-phosphate-acetyl-transferase (GPAT) enzyme of pea and an antisense DNA for the GPAT gene from the common bean (see Wolter et al. 1992 and 1993) and other genes of agronomic value will be initiated.

References

Mandal A, Lang V, Orczyk W and Palva E T (1993). Theor. Appl. Genet 86, 621-628.

Russell D R, Wallace K M, Bathe,J H, Martinell B J and McCabe D E (1993). Plant Cell Rep. Vol 165-169.

Sato. S., et al. Plant Cell Reports (1993)12, 408-413.

Wolter, F, Schmidt, R and Heinz E, 1992. EMBO 11, 4685-4692.

Wolter et al. Proceedings of the Phaseous Beans Advanced Biotechnology Network(1993) Roca et al. eds; CIAT, Cali, Colombia, 431p.

3.2.4 Development of *Brachiaria* genetic transformation

S. Lenis, W. M. Roca
Biotechnology Research Unit

Background

Genetic transformation of *Brachiaria* is a basic prerequisite for undertaking genetic studies with homologous (e.g. apomixis) and heterologous (e.g. insect resistance), traits which might not be accessible for *Brachiaria* breeding.

In earlier work of the Unit and the TFP, we have developed an effective plant regeneration methodology with five *Brachiaria* spp. using isolated mature embryos as explants. In our studies, we have shown that plant regeneration is mediated by a proliferation phase of embryo scutelum cells, and occurs though somatic embryo differentiation.
This year we have initiated work towards developing a transformation protocol by particle bombardment of scutellar tissue.

Expected output

Our goal is to generate transgenic *Brachiaria* plants using marker genes and the bombardment technique developed in the Unit for rice.

Progress report

(i) As a first step, we have determined the sensitivity level of *Brachiaria* embryos to the antibiotic Higromycin which will be used for the selection of transformed cells. Table 1 shows that the threshold concentration of Hygromycin, at which callus induction of three *Brachiaria* genotypes is arrested, is 8 mg/l.

Table 1. Effect of Hygromycin B on % callus induction from isolated embryos of three *Brachiaria* genotypes.

Hygromicin B mg / l	*B. brizantha* 6780	*B. ruziziensis* 16551	*B. ruziziensis* 443
0	70	55	40
2	68	45	28
4	59	35	19
6	42	13	5
8	1	0	0
10	0	0	0

Embryos were exposed to Hygromycin in medium M1 and callus growth was assessed after 30 days

This indicates that transformed *Brachiaria* cells can be effectively selected by placing bombarded embryos on a callus induction medium supplemented with 8mg/l Higromycin. Since the plasmid construct for transformation will harbor the hgr resistance gene, only cell which have-integrated the gene into their genome should be capable of growing on the Hygromycin medium.

(ii) Availability of a reporter gene for early assessment of transformation events has been a common tool with most transformation systems. We have tested the usefulness of the B-glucuronidase enzyme activity as an indicator of transient gene expression.

Table 2 shows that an average of 0.8-1.1 blue spots per embryo can be detected in two *Brachiaria* genotypes after 48 hours of bombardment with the plasmid ACT-1D harboring the gus gene.

Table. 2 Transient expression of gus gene in Brachiaria scutellun after 48 hours of bombardment with the ACT-1D plasmid using on He-driven particle accelerator.

Genotype	B - glucuronidase activity		
	No. embryos	No. blue Spots Total	No. Blue Spots per embryo
B. brizantha	280	306	1.1
B. ruziziensis	300	240	0.8

Bombardment with gold particles carrying the ACT-1D plasmid from 6cm distance under 1100psi negative pressure

This rate of expression is considered low if effective stable transformation is to be achieved.

(iii) Recently we have bombarded isolated embryos with the plasmid ACT-1D harboring the Hygromycin gene, and selected resistant callus with 6 mg/l Hygromycin. The callus was transferred to regeneration medium in two steps, contain 10 and 20 mg/l Hygromycin. Out of 40 embryogenic clusters only one developed into green shoots under selection stress. One plant has been grown and transferred to soil for further analysis.

Future Work

(i) Molecular and genetic assays will be carried out on the putative *B. ruziziensis* transformed plant to determine the insertion and expression of the transgene.

(ii) Bombardment of *Brachiaria* embryos will be continued and increased with a view to generate transformed plants.

3.3 Project: Genetic Transformation of Rice

3.3.1 Inheritance and stability of hph and gus genes in transgenic rice.

Z. Lentini[1], E. Tabares[2], F. Angel[2], W.Roca[2]
[1] Rice Genetics, [2] Biotechnology Research Unit.

Background

In spite of the successes in obtaining various transgenic monocot plants, very little is known about the inheritance and stability of foreign genes in transgenic cereal plants, which is of outmost importance for determining the values and application of genetically engineered cereal crops in agriculture. Mendelian inheritance of transgenes in rice has been reported. However, in most of the studies, data were presented on transmission of transgenes to T1 progeny plants only (Peng et al 1995). More recently, Goto et al (1993) reported both Mendelian and non-Mendelian inheritance of transgenes in rice co-transformed via electroporation. Following, we report the inheritance and stability of the GUS and hph genes in T1 progeny plants of ten T0 transgenic rice lines, and in T2 plants of two T0 lines.

Expected outputs

To determine the inheritance and stability of gene expression in rice transformed by particle bombardment, for determining the value of this biotechnique in broadening the genetic base.

Progress report

Co-transformation experiments were conducted using equal amounts of the pAct1D construct (kindly provided by Dr. R. Wu, Cornell University) containing the GUS reporter gene under the control of the rice actin-1 promoter- actin-1 intron, and a construct containing the hph selective gene encoding for hygromycin resistance (Hyg ^{r}) driven by the 35S CaMV promoter (Figure 1). The direct deliver of genes into immature embryos or immature panicle-derived calli was conducted using DNA-coated gold particles accelerated by the PDS-1000/He system. The tropical irrigated Latin American *indica* variety Cica 8, and the tropical upland japonica lines CT 6241-17-1-5-1 were used. The putative transgenic events were recovered using a step-wise selection on culture medium containing 30 mg/l hygromycin B (hyg B) followed by 50 mg/l hyg B throughout plant regeneration (Li et al., 1993). B-glucoronidase expression was determined on 1-week-old seedlings germinated in vitro. The segregation of the hph gene among offspring of the T0 and T1 transgenic plants was demonstrated by germinating T1 and T2 seeds on medium containing 50 mg/l hyg B.

A segregation of 3:1 was noted among offspring of four T0 transgenic plants from ten T0 lines evaluated (Table 1), indicating Mendelian inheritance from single genetic locus of a functional hph gene. On the other hand, the other six transgenic T0 plants showed a skewed segregation pattern, in which the number of Hyg^r offspring was significantly lower than the expected 3:1 (Table 1). The inheritance of the GUS gene was also evaluated on selfed-progeny from five T0

transgenic plants showing high level of GUS expression (Table 2). In this case, one plant (CM-16) showed a Mendelian segregation 3:1, and the line CM-17 showed a segregation corresponding to a homozygous transformation. CM-12 line showed a lower number of offspring with GUS expression than the expected, and the level of the B-glucoronidase expression was diminished respect to the original T0 transgenic plant. Plants CM-12 and CM-17 were co-transformed with both genes (Table 1 and 2).

Table 1. Inheritance of hph gene in the T1 generation

Transgenic TO rice line	T1 seeds germinated	Well-grown seedlings	Dear Seedlings	ratio	X^2	Probably
CT6241-17-1-5-1						
CM-1	47	35	12	3:1	0	1.00
CM-3	32	19	13	2:1	0.55	0.46
CM-11	25	10	15	1:1	0.64	0.42
CM-12	21	14	7	3:1	1.05	0.31
CM-17	26	19	7	3:1	0.21	0.64
CICA 8						
IM-1	363	155	208	1:1	0.10	0.75
IM-2	175	58	117	1:2	0	1.00
IM-3	70	44	26	2:1	0.58	0.45
IM-5	275	194	81	3:1	0.07	0.79
IM-7	363	155	208	1:1	0.10	0.75

Data was collected 2 weeks after the seeds were germinated in the light on byg (50 mg l) containing medium. All control seedlings were dead.

Table 2. Inheritance of the uidA gene in the T1 generation

Transgenic TO rice line	T1 seeds germinated	GUS + seedlings	GUS- seedlings	ratio	X^2	Probably
CT6241-1⁻-1-5-1						
CM-1	30	0	30	0:1	---	---
CM-3	25	0	25	---	---	---
CM-12	45	20⁻	25	1:1	0.56	0.46
CM-16	16	14⁻⁻	2	3:1	1.33	0.25
CM-17	25	25⁻⁻⁻	0	1:0	---	---

Data was collected 5 days after the seeds were germinated on MS medium without hormone in the light. All control seedlings were GUS⁻.

ß-glucoronidase expression: ⁻fain blue, ⁻⁻light blue, and ⁻⁻⁻ dark blue, respectively, was noted on roots, seed endosperm and primary leaves.

Six T1 plants from the line CM-12, and eight T1 plants from CM-17 with hygromycin resistance and GUS expression were selected to continue the inheritance study of these genes into the following (T2) generation. The two transgenes were stably transmitted into the T2 generation from the CM-17 line. In this case, all the T1 plants analyzed had integrated the hph and GUS genes into the genome (Figures 2 and 3) and showed a segregation pattern similar to the original T0 plant (Tables 3 and 4). In contrast, the hph gene was not stably inherited into the T2 generation from CM-12 line (Table 3), although the T1 plants contained the hph gene (Figure 2). The T1 plants from CM-12 had integrated the GUS gene into their genome (Figure 3), and four of the six T1 plants showed increased number of T2 progeny plants with GUS expression respect to the T1 generation (Table 4). Moreover, the lines CM-12-1 and CM-12-7 had higher level of B-glucoronidase expression (Table 4). Line CM-12-2 did not inherited any of the genes (Figures 2 and 3, Tables 3 and 4).

These results suggest that the reduction in GUS expression observed in the T1 and T2 generations of CM-12 may be due to inactivation of the transgene by methylation. Southern blot analysis of non-GUS expressing plants should be performed in order to evaluate this hypothesis. Besides transgene inactivation, a significantly lower number of offspring than the expected 3:1 could also be due to the linkage of the transgene with semidominant or dominant lethal mutations, and/or excision of the transgene from the genome (Hayakawa et al., 1992).

Table 3. Inheritance of hph gene in the T2 generation

Transgenic T1 rice line	T2 seeds germinated	Wel-grown seedling	Dead seedlings	ratio	X^2	Probably
CT6241-17-1-5-1						
CM-17-1	30	23	7	3:1	0.44	0.83
CM-17-2	26	16	10	2:1	0.29	0.59
CT-17-3	30	18	12	2:1	0.67	0.40
CT-17-4	25	18	7	3:1	0.12	0.73
CT-17-5	30	21	9	3:1	0.40	0.53
CT-17-6	30	25	5	3:1	1.11	0.29
CT-17-7	30	24	6	3:1	0.40	0.53
CT-17-8	60	47	13	3:1	0.93	0.53
CM-12-1	27	1	26	---	---	---
CM-12-2	30	1	29	---	---	---
CM-12-3	30	11	19	1:2	0.18	0.67
CM-12-4	30	0	30	---	---	---
CM-12-5	30	0	30	---	---	---
CM-12-6	30	0	30	---	---	---

Data was collected 2 weeks after the seeds were germinated in the light on hyg B (50mg/l) containing medium. All control seedlings were dead.

Table 4. Inheritance of the uidA gene in the T2 generation

Trangenic T1 rice line	T2 seeds germinated	GUS⁺ seedlings	GUS⁻ seedlings	ratio	X^2	Probably
CT6241-17-1-5-1						
CM-17-1	30	30***	0	1:0	---	---
CM-17-2	30	30***	0	1:0	---	---
CM-17-3	30	30***	0	1:0	---	---
CM-17-4	30	30***	0	1:0	---	---
CM-17-5	30	30***	0	1:0	---	---
CM-17-6	30	30***	0	1:0	---	---
CM-17-7	30	30***	0	1:0	---	---
CM-17-8	30	28***	2	1:0	---	---
CM-12-1	30	30**	0	1:0	---	---
CM-12-2	30	0	30	---	---	---
CM-12-3	30	15*	15	1:1	0	1.00
CM-12-4	30	28*	2	15:1	0.006	0.94
CM-12-5	30	24*	6	3:1	0.40	0.53
CM-12-7	30	28**	2	15:1	0.006	0.94

Data was collected 5 days after the seed were germinated on MS medium without hormone in the light. All control seedlings were GUS ß-glucoronidase expression. *fain blue, ** light blue, and *** dark blue, respectively, was noted on roots, seed endosperm and primary leaves

Future Plans

The inheritance and molecular analyses of the two trangenes into the T3 generation will be conducted. Other transgenic lines from a different variety will be included to determine the effect of the genotype on the inheritance of the hph and GUS genes.

References

Hayakawa, T., Zhu Y., Itoh K. ,Kimura Y., Izawa T. , Shimamoto K., Toriyama S. 1992. Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. PNAS 89: 9865-9869.

Li L., Qu R. , de Kochko A. , Fauquet C. , Beachy R.N. 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12: 250-255.

Peng J., Wen F., Lister R.L., Hodges T.K. 1995. Inheritance of gus and neo genes in transgenic rice. Plant Mol. Biol. 27: 91-104.

Goto F., Toki S., Uchimiya H. 1993. Inheritance of a co-transferred foreign gene in the progenies of transgenic rice plants. TRansgenic Res. 2: 302-305.

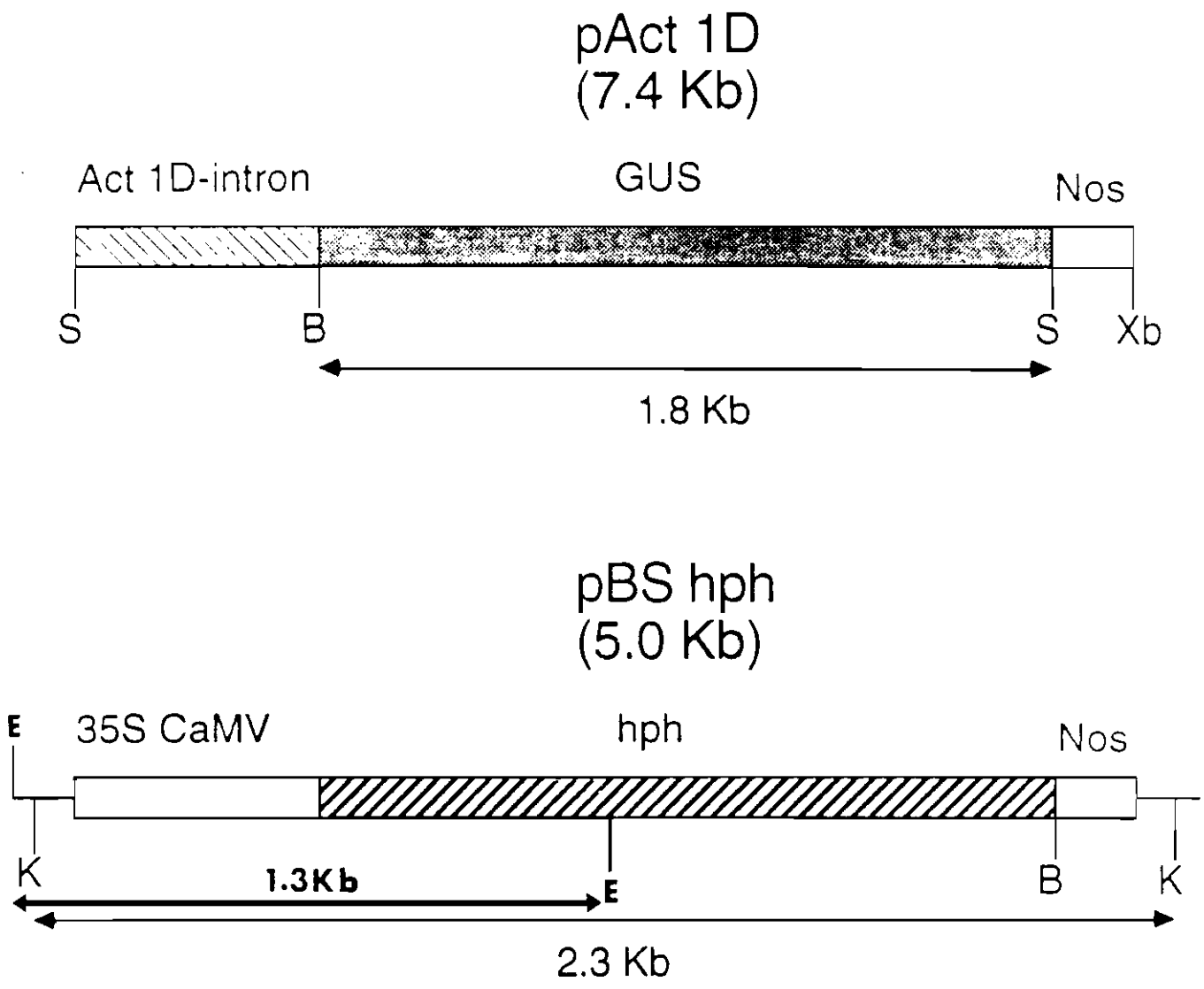


Figure 1. Gene constructs used in co-transformation of rice.
S = SstI, B = BamHI, xb = xba, K=KpN, E- Eco RI

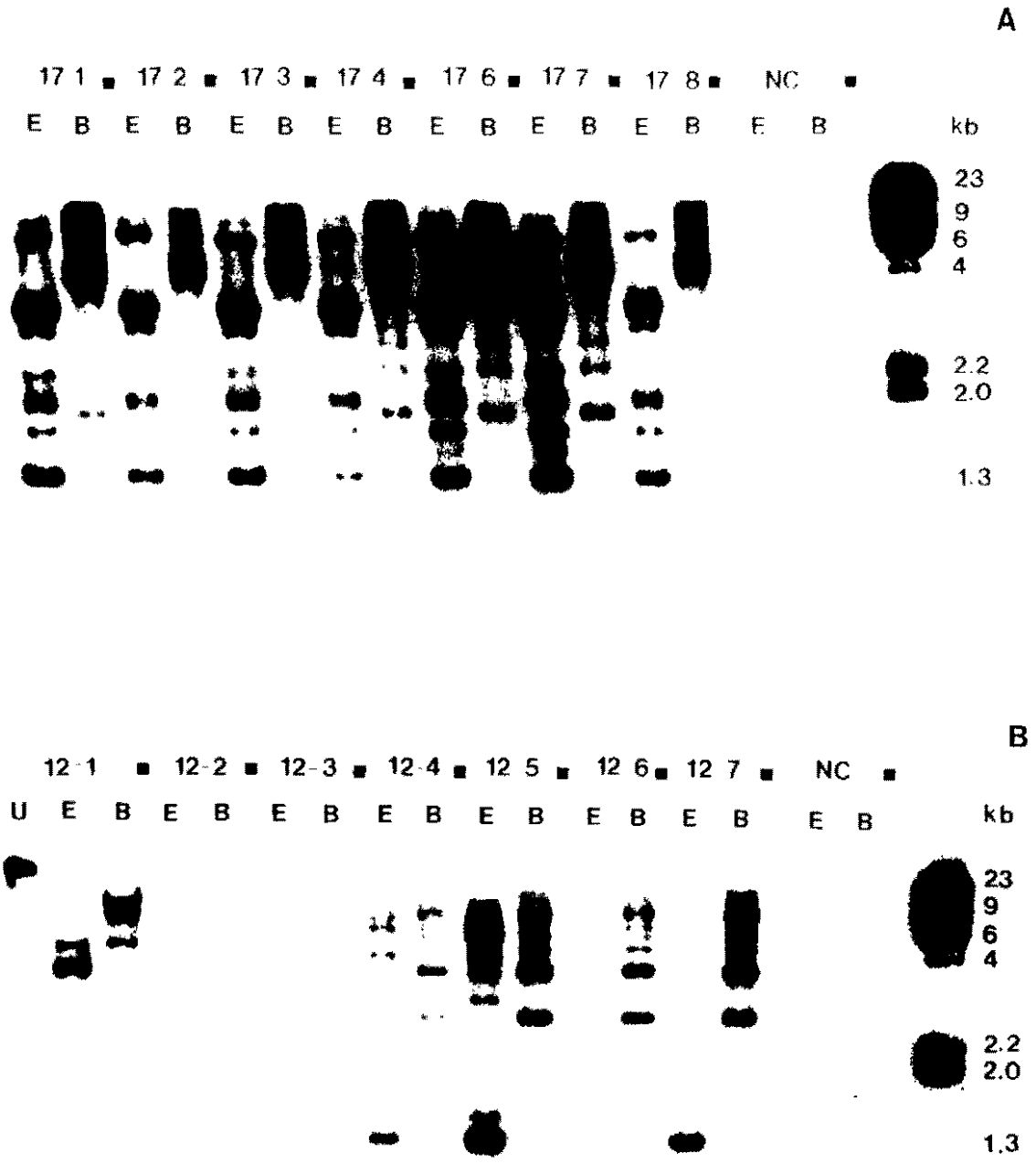


Figure 2 Southern blot analysis of genomic DNA from T1 progeny plants of CM-17 (A) and CM-12 (B) for the hph gene. Five ug DNA were loaded in each lane following digestion with (E) Eco R[illegible]ph gene fragment 1.3 (B) Bam HI (linearized plasmid). (U) undigested DNA. (NC) non-transgenic control.

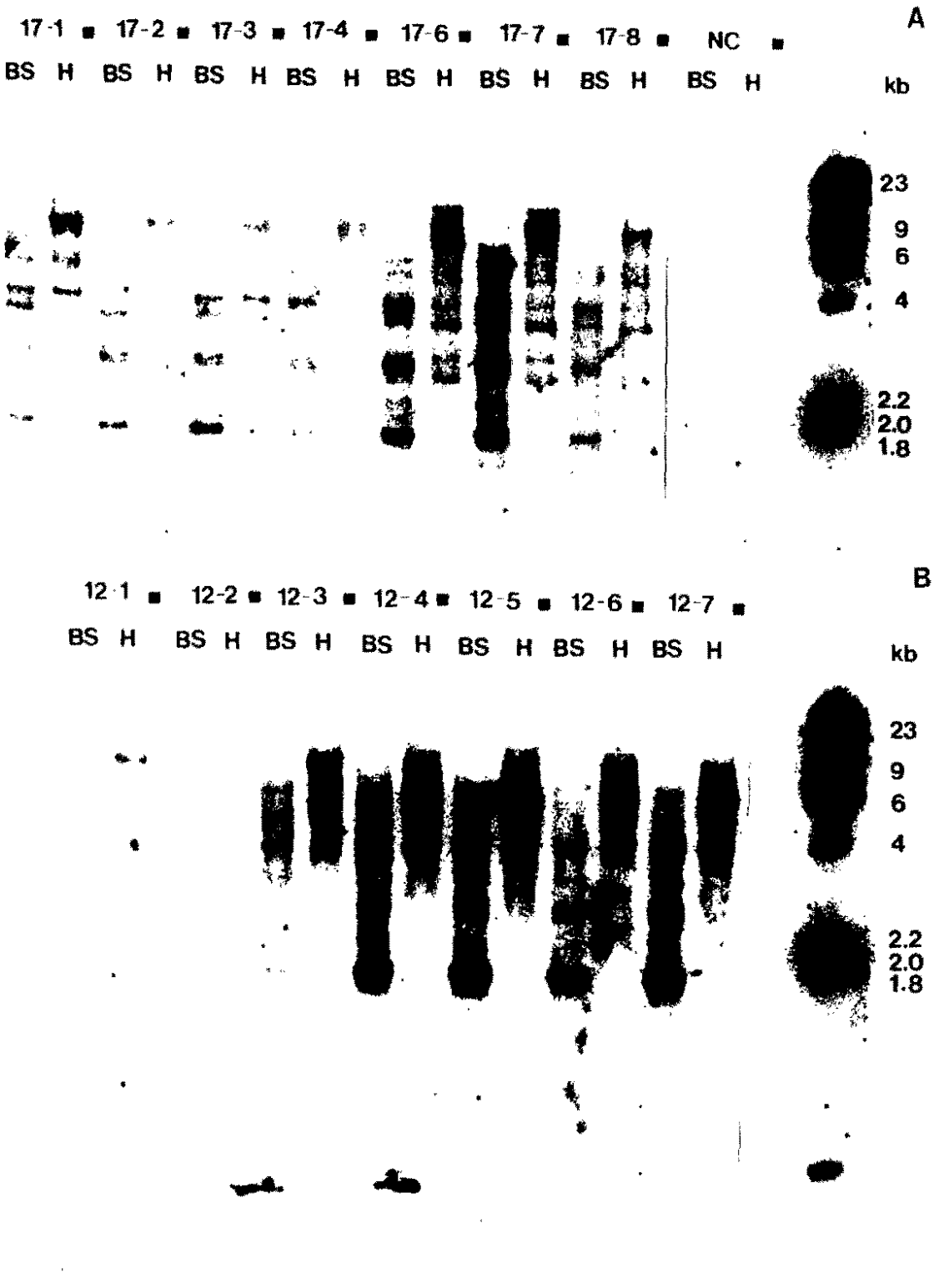


Figure 3. Southern blot analysis of genomic DNA from T1 progeny plants of CM-17 (A) and CM-12 (B) for the uidA gene. Five ug DNA were loaded in each lane following digestion with (BS) Bam HI/SsT I (uidA gene fragment 1.8 kb) or (H) Hind III (linearized plasmid). (NC) non-transgenic control.

3.3.2 Control of RHBV through Coat Protein Mediated Cross Protection and Anti-sense RNA Strategies.

Z. Lentini[1,2], L. Calvert[2], E. Tabares[2], I. Lozano[3], M.Cuervo[3], W.Roca[2].
[1] Rice Genetics, [2] Biotechnology Research Unit, [3] Virology Unit.

Background

Rice hoja blanca virus (RHBV) causes severe recurrent epidemics in the Andean, Central American, and Caribbean countries of tropical Latin America (Morales and Niessen, 1983). The planthopper insect Tagosodes oryzicola (Muir) is a serious pest of rice that causes direct damage and is also the vector of RHBV. This virus has the potential to cause severe yield losses (up to 100%) since there is limited distribution of the varieties that are resistant to both the vector and the virus. Some very popular varieties are resistance to the vector but are susceptible to the virus. The uncertainty of epidemics induces farmers to spray up to 5-6 times to control this planthopper vector of RHBV. There is a need to incorporate additional sources of resistance into improved germplasm to ensure stable and durable resistance, since the resistance is from a single resistant source. Moreover, the source of resistance does not confer immunity in most commercial varieties. Therefore, farmers start applying insecticides even when about 10% of the plants from resistant cultivars start showing RHBV symptoms.

RHBV is a member of the tenuivirus group that consist of 3-8 nm-wide filaments that may adopt helical or loose configurations (Morales and Niessen, 1985). The genome of RHBV consist of four ssRNA species (Ramirez et al., 1992). The nucleotide sequence of RHBV RNA4 is known and the genome encodes two genes in ambisense manner (Ramirez et al., 1993). The major NS4 protein is encoded by the viral (v) RNA strand. The molecular characterization of RHBV and the preparation of cDNA libraries has led to the design of novel virus-resistant strategies to genetically engineer commercially-grown rice cultivar. Two different strategies are being attempted: a) the nucleocapside (NC) cross protection and b) the antisense-gene down regulation of the major NS4 protein. The NC-mediated cross protection has been successful for the tenuivirus RStV (Hayakawa et al., 1992). The strategy for the expression of the RNA4 is to determine the function of the major NS4 protein and study the potential for a different method of producing viral resistant plants. The down regulation of this protein may be a novel mrthod of producing virus-resistant plants by breaking the cycle of transmission.

Expected outputs

(i) To transform rice with novel gene(s) for RHBV resistance; (ii) to incorporate these genes into Latin American commercial varieties or into genotypes to be used as parents in breeding.

Progress report

The direct deliver of genes into immature embryos or immature panicle-derived calli is conducted using DNA-coated gold particles accelerated by the PDS-1000/He system. The tropical irrigated Latin American *indicas* varieties Oryzica 1, Cica 8, and Inti and the tropical upland japonica line CT 6241-17-1-5-1 are used as targets. Constructs containing the RHBV-NC or the antisense RHBV-NS4 genes driven by the 35S CaMV promoter are being used. The 35S CaMV - hph gene is used as the selective marker. The putative transgenic events are recovered using a step-wise selection on culture medium containing 30 mg/l hygromycin B (hyg B) followed by 50 mg/l hyg B throughout plant regeneration (Li et al., 1993).

Work was conducted to determine the most efficient method to isolate rice DNA. Analyses indicated that although 2.5 ug rice DNA are theoretically sufficient to detect a single gene copy, in practice between 10 to 15 ug DNA are needed to obtain a strong signal in the autoradiograms during a short period of exposure (overnight to 48 hr)(data not shown). Thus, the availability of DNA becomes a limiting factor for replicating Southern blots. Analyses of various methods commonly used to isolate rice DNA including CTAB's, Dellaporta's, and various methods using urea, indicate that the method by Gilbertson (1991) is the most efficient. An average of 48 ug DNA/ gr fresh tissue from 71-day-old plants is obtained with Gilbertson's method in contrast to 16 ug for Dellaporta's and below 10 ug for CTAB's and urea's methods. The DNA yield increases to 70 ug DNA/ gr fresh tissue when using 41 day-old-plants.

After the complete step-wise selection process throughout plant regeneration on 50 mg l hyg B, a total of 165 plants from the antisense RHBV-NS4 and 187 plants from the RHBV-NC bombardments had been recovered. Preliminary analyses by Southern blot of genomic DNA and Northern blot of 38 plants recovered from the antisense RHBV-NS4 bombardments indicated that 2 of these plants (5.3%) contain and express the antisense-RNA4 gene (Figure 1). The identification of transgenic plants that express the RHBV antisense allows for the analysis of the affect of the major non-structural gene and to determine the down regulation of this viral gene confers resistance to RHBV.

Twenty one of 31 plants analyzed from RHBV-NC experiments contain the RHBV gene. In all cases, larger NC fragments than the expected length were visualized on the Southern blots. Apparently, a variety of integration patterns had been obtained in other works when circular plasmid is used (Hayakawa et al., 1992). Therefore, future experiments will include the linearization of the expression vector before bombardment. These plants will be analyzed by Northern and Western analysis to determine if the NC-gene is being expressed correctly. Recently PCR analyses, which were optimized with transgenic rice containing the hph gene (Figure 3), indicate that twenty five plants recovered from the RHBV-NC bombardments contain the hygromycin resistance gene (Figure 4). Analyses of these plants to confirm the integrative transformation of the RHBV-NC gene is underway.

Future plans

The analyses by PCR and Southern blot of the plants recovered from the hygromycin continues. Those plants containing the RHBV gene(s) will be characterized by Northern and Western (RHBV-NC only) analyses. Inheritance studies of progeny from the already identified promising plants is in progress to confirm stability of the integrative transformation and expression for the RHBV-NC and the antisense RHBV-NS4 genes. Following local regulations, the progeny from transgenic plants will be tested in the greenhouse under biosafety conditions for resistance to RHBV using viruliferous plant hoppers.

References

Gilbertson R.J..Rojas M.R. . Russell D.R. , Maxwell D.P. 1991. Use of the asymetric polymerase chain reaction and DNA sequencing to determine genetic variability of bean golden mosaic geminivirus in the Dominican Republic. J. Gen. Virology 72: 1-6.

Hayakawa, T., Zhu Y., Itoh K. ,Kimura Y., Izawa T. , Shimamoto K., Toriyama S. 1992. Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. PNAS 89: 9865-9869.

Li L., Qu R. , de Kochko A. , Fauquet C. , Beachy R.N. 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12: 250-255.

Morales, F.J., Niessen A.I. 1983. Association of speiral filaamentous viruslike particles with rice hoja blanca. Phytopathology 73: 971-974.

Morales, F.J., Niessen A.I. 1985. Rice hoja blanca virus. AAB Descriptions of Plant Viruses, No. 299.

Ramirez, B.C., Macaya G., Calvert L.A., Haenni A.L., 1992. Rice hoja blanca virus genome characterization and expression in vitro. J. Gen. Virology 73: 1457-1464.

Ramirez, B.C. , Lozano I., Constantino L.M., Haenni A.L., Calvert L.A. 1993. Complete nucleotide sequence and coding strategy of rice hoja blanca virus RNA4. J. Gen. Virology 74: 2463-2468.

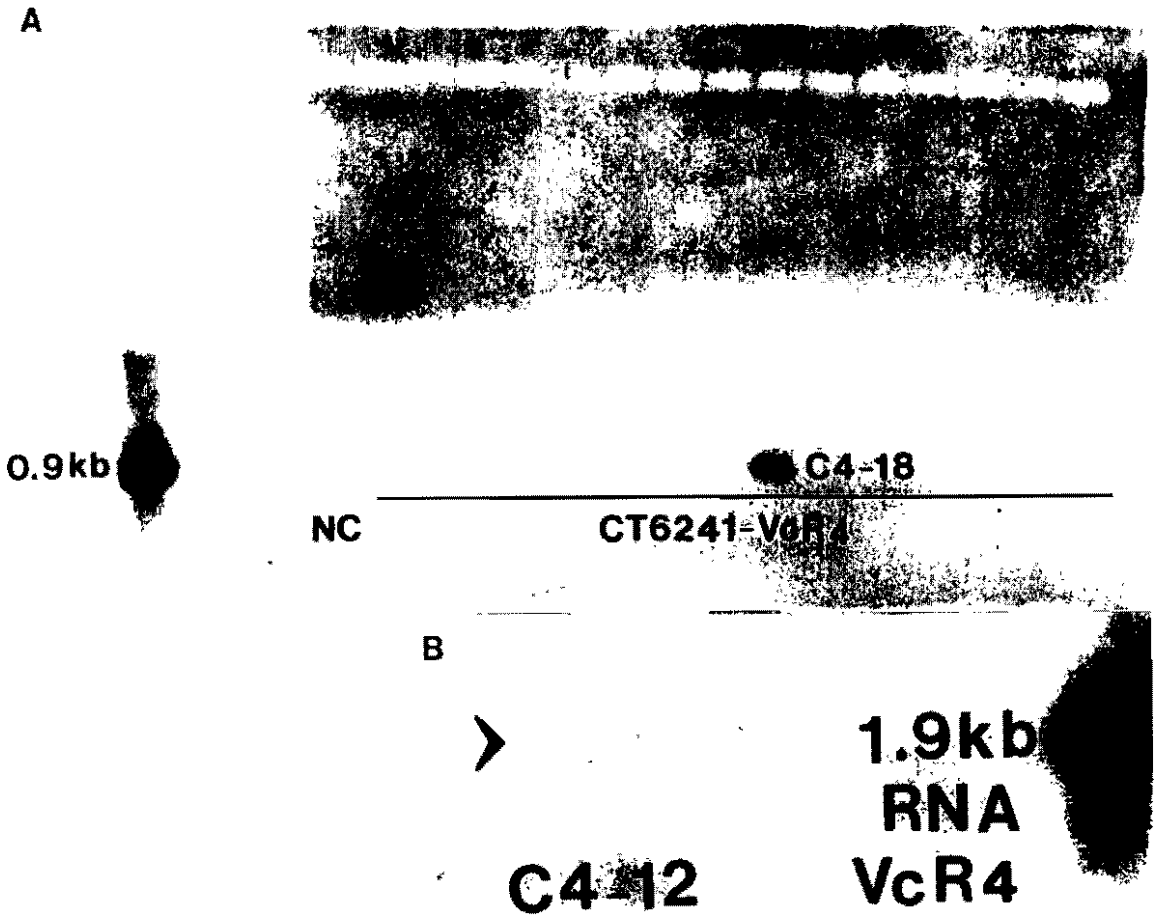


Figure 1. (A) Southern and (B) Northern blot analyses of plants recovered from RHBV-NS4 bombardments.

Figure 2. Southern blot ananlyses of putative transgenic plants containing the RHBV-NC gene.

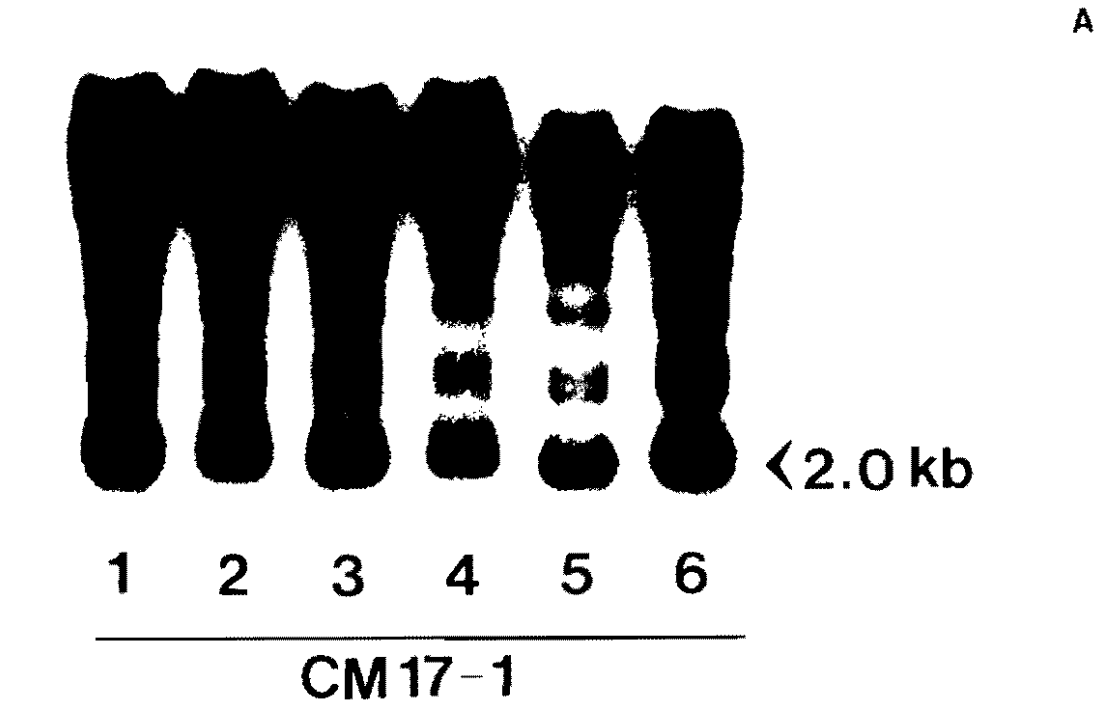


Figure 3. Hygromycin (hph) resistance gene detected in transgenic rice by Southern blot analysis of (A) genomic DNA and (B) PCR amplified gene fragment.

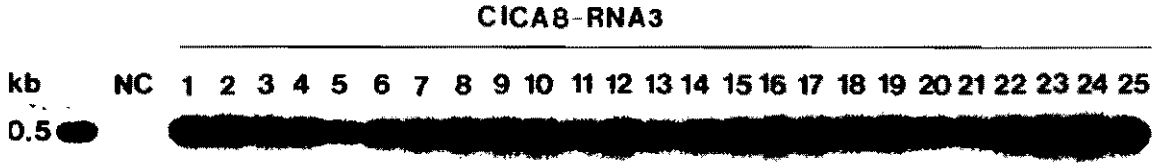


Figure 4. Southern blot analysis of hph PCR-amplified fragment of putative transgenic plants recovered from RHBV-NC bombardments.

3.3.3 Incorporation of Protection to Rice Sheath Blight through Genetic Transformation.

Z. Lentini [1], E. Tabares[2], N. Balcazar[2], W.Roca[2].
[1] Rice Genetics, [2] Biotechnology Research Unit.

Background

Plant resistance to pathogenic fungi involves multiple response pathways including the accumulation of defensive enzymes. These include B-1,3-glucanases, chitinases, thaumatin-like proteins, protease inhibitors, and ribosome-inactivating proteins (Collinge et al 1987; Linthorst 1991). The development of gene transfer systems has enabled testing the effects of expression of candidate anti-fungal genes on the course of plant infection by pathogens. Transgenic tobacco expressing a barley ribosome inactivating protein (RIP) showed an increased protection against Rhizoctonia solani (Logemann et al 1992), a major causal agent of sheath blight in the Americas. RIP's are N-glycosidases which cleave the N-glycosidic bond of adenine in a specific ribosomal RNA sequence. This barley RIP is a single chain protein (type 1 RIP) which inhibits protein synthesis in target cells by specific RNA N-glycosidase modification of 28S rRNA. RIP's do not inactivate self ribosomes, but show varying degrees of activity towards ribosomes of distantly related species, including fungal ribosomes (Logemann et al 1992).

Sheath blight is causing important crop losses in the Southern Cone of America and increasing spreads had been reported in Colombia, Mexico and Venezuela. All varieties are susceptible and there is not known source of resistance in rice. Biological control has not been successful either. At present, the control depends on heavy use of fungicides.

Expected outputs

We are interested in transforming indica rice with the barley RIP gene to confer increased protection against Rhizoctonia solani., which is found in tropical America; and to determine if the transgenic plants carrying the RIP are also protected against to Rhizoctonia oryzae and/or R. oryzae sativae , which are the species more commonly found in Argentina, Southern Brazil, Chile, and Uruguay. Such transgenics might be economically and environmentally desirable by leading to a reduction in the application of pesticides.

Progress report

A construct containing the RIP gene driven by the 35S promoter and the 35S CaMV -hph (hygromycin resistance) gene as the selective marker are being used. In order to ensure a constitutive expression of the RIP protein in the whole rice plant, constructions were made encoding for the RIP gene under the control of the CaMV 35S promoter. The RIP encoding gene was contained in the plasmid pPR69 (J. Logemann). A ca.1 Kb EcoRI fragment

containing the full length cDNA was subcloned in pUC19 to yield pRIP19. From the RIP sequence found in the GeneBank (accession BLSCRIP3) it was observed the presence of an NcoI restriction site encompassing the first ATG codon, and an AccI site 12 bases dowstream of the termination codon. It was decided to use these flanking sites for further manipulation of the RIP gene. A cassette 35S-OCS3' contained in the pA8 plasmid was subcloned into pUC19 as a ca.740 bp EcoRI-HIII to obtain pUCA8. The 864 bp NcoI-AccI fragment from pRIP19 was blunted with Klenow enzyme and subcloned into the SmaI site of pUCA8 to yield the plasmid p35SRIP. Subsequently, the cassette 35S-Hyg-tm13' from pTRA151 (a gift of N.Murai, Louisiana State University, USA) was spliced into the HindIII site of p35SRIP to finally obtain p35SRIPHy1 and p35SRIPHy2. The numbers indicate the relative orientation of the hygromycin cassette (clockwise and counterclockwise, respectively) to the RIP transcriptional fusion.

The direct gene transfer of the RIP gene is being attempted using immature embryos or immature derived-callus of the indica varieties Cica 8, Inti, and BR-IRGA 409, and the upland line CT6241-17-1-5-1. Gene delivery is performed using DNA-coated gold particles accelerated by the PDS-1000/He system. So far, a total of 183 plants with hygromycin resistance have been recovered after the complete step-wise selection process throughout plant regeneration on 50 mg/l hygromycin. RNA was isolated from 10 plants, and cDNA's were obtained and amplified by RT-PCR. This preliminary analysis suggest that 7 of these 10 plants are putative transgenics which contain and express the RIP gene.

Future plans

The integrative transformation of these plants is being confirmed by Southern blot hybridizations and inheritance studies. The expression of the RIP gene will be analyzed Northern and Western blots. The complete analyses of all plants recovered from the hygromycin selection process will be conducted. Following local regulations, putative transgenic plants will be tested in the greenhouse for resistance to sheath blight.

References

Collinge D.B and A.J. Slusarenko. 1987. Plant gene expression in response to pathogens. Plant Mol Biol 9: 389-410.

Linthorst H. 1991. Pathogenesis-related proteins of plants. Critical Reviews in Plant Sciences 10: 123-150.

Logemann J., G. Jach, H. Tommerup, J. Mundy and J. Schell. 1992. Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. Biotechnology 10: 305-308.

3.4 Project: Conservation of Genetic Diversity.

3.4.1 Cryopreservation of Cassava shoot tips in liquid nitrogen (L.N.).

R. Escobar [1], W. M. Roca [1] and C. Guevara [2].
[1]Biotechnology Research Unit and [2] Genetic Resources Unit.

Background

We have developed a basic protocol to recover viable cassava plants from shoot tips frozen in L.N., with var. MCOL 22 as a model, and thereafter reproduced the protocol with several cultivars, representing wide geographic distribution.

Several factors contributed to successful cassava cryopreservation, being critical: tissue desiccation, preculture and cryoprotection with sorbitol and DMSO. This year we have concentrated on the composition of the post-freezing media for obtaining viable tissue and plant regeneration.
We have also initiated experiments to develop an encapsulation technique of shoot tips as subject for cryopreservation. In addition, we have initiated work to extend cryopreservation to cassava cvs. that normally showed a low response to the current protocol.

Expected Outputs

An implemented methodology to establish an in vitro cassava base collection in L.N. (IVBG) at CIAT.

Progress Report

I. Post-freezing (reculture) recovery conditions. Table 1 shows that low concentration (i.e. 0.04mg/l) of BAP yielded high shoot regeneration from cryopreserved shoot tips, but as BAP concentration increased a drastic detrimental effect on shoot recovery was evident. On the other hand, shoot regeneration increased with increasing Kinetin (Kin) concentration, as also observed using 2iP. No shoots were generally regenerated when recovery media was supplemented with Adenine (A) or Thidiazuron (TDZ).

The general gradient of response to cytokinins in the recovery medium of cryopreserved cassava shoot tips would be:
Kin>2iP>BAP>TDZ>A.

Table 1. Effect of Cytokinins on plant recovery from cassava shoot tips cryopreserved in LN. (cv. MCOL 22)

Concent.	0.04 mg/l		0.3 mg/l		0.5 mg/l	
Cytokinin	% Viab.	% Shoot	% Viab.	% Shoot.	% Viab.	% Shoot
2IP	63	0	96	44	100	75
BAP	84.6	57.7	88	44	19	0
KIN	54.2	4	100	59	100	80
A	0	0	52	0	24	0
TDZ	64	24	87.5	0	100	0

II. Shoot tip encapsulation. Encapsulation of shoot tips in a soft matrix such as sodium alginate is expected to provide a practical and rapid way of cryopreservation in L.N. We have tested the effect of sucrose concentration and time of treatment on the viability and shoot regeneration from beads (alginate-encapsulated shoot tips) without freezing (Table 2).

The osmotic effect of sucrose and its related conditioning affect on encapsulated shoot tips needs to be assessed during the preculture phase as these are prepared for freezing.

Sucrose at 0.5M provided the best condition for viability and shoot formation throughout 1-7 days preculture. Shoot formation decreased drastically with sucrose concentration, and slightly with preculture time at 0.5M. However, at 0.75M and even more at 1M sucrose, shoot formation also decreased drastically between 1-5 days of preculture.

Sequential treatment with variable sucrose concentrations, and duration of treatment, can also exert differential response in terms of shoot regeneration.

Treatment with 0.5M sucrose for one day, followed by 0.75M for two days, clearly allowed higher shoot recovery without freezing than more extended sequential sucrose treatments or single sucrose concentration treatments (Table 3).

The effect of tissue water content, as modified by desiccation, will be tested in future experiments or it interacts with the sucrose concentration.

Table 2. Effect of sucrose concentration and preculture time on shoot tip recovery from alginate beads without freezing.

Days of precult	1		3		5		7	
Sucrose concent	Viab. %	Shoot %	Viab. %	Shoot %	Viab. %	Shoot %	Viab. %	Shoot %
0.5 M	100	100	100	83	100	70	100	90
0.75	100	10	100	10	100	0	100	0
1M	100	0	100	0	100	0	90	0

Table 3. Effect of sucrose concentration and treatment time on viability and shoot recovery from encapsulated shoot tips without freezing. (cv MCOL 22)

Treatment	% Viability	% Shoots
0.5-0.75-1M/1 day each	100	60
0.5 1d-0.75M/2 days	100	80
0.5M/3 days	100	62
0.75M/3 days	100	0

Future Plans

I. Most of our efforts in 1996 will be dedicated to extend the current cryopreservation protocol to a wider range of cassava genotypes. We will initiate the screening of varietal response with the core collection.

II. Identify recalcitrant (i.e. non-responding) cassava genotypes and carry out experiments designed to adjust the protocol to these materials.

III. Continue experiments using encapsulated shoot tips for freezing under the slow and rapid freezing protocols.

4. OTHER ACTIVITIES IN BIOTECHNOLOGY

4.1 Institutional development

In 1995, the BRU staff continued to dedicate significant efforts towards meeting CIAT's bridging role in biotechnology through cooperation with developing and developed country institutions/scientists.

(i) For a second year, a training course was offered to 18 scientists from 9 Latin American countries on "biotechnology for the conservation of agrobiodiversity". The course comprised lectures, lab. exercises and round table discussions on: molecular and *in vitro* culture technologies for studying agrobiodiversity from gene to the ecosystem level, evolution and taxonomy; the course focused on genetic resources conservation and enhancement-utilization applications using biotechnology tools and geographic information system.

The course also included discussions on critical topics such as access/exchange of genetic resources, property issues and biosafety. Funding: OEA, ICETEX, COLCIENCIAS. This years' courses involved the cooperation of CIP.

(ii) In view of the BRU's advances in the implementation and applications of the AFLP technique for genetic fingerprinting and gene tagging, the PROCISUR requested CIAT to organize a one-week workshop for 10 scientists from the NARIs of Brazil, Argentina, Uruguay and Chile. The Workshop will include the participation of Life Technologies Inc. (LTI) with an instructor and some supplies for the workshop.

(iii) In 1995, the BRU hosted 25 internship by developing country scientists, mostly Latin American and Caribbean, on research/training in molecular and cellular applications to agrobiodiversity and crop improvement.

(iv) Cooperation with CORPOICA. Through an agreement between CIAT and CORPOICA, a scientist from CORPOICA's Genetic Resources Program has been hosted in the BRU to conduct research on the molecular characterization of Colombian passifloras. This cooperation involves IPGRI-Las Americas, and is supported by a grant from IDB.

(v) In 1995, BRU staff has actively participated with paper presentations in the following international and regional scientific events: International Plant Molecular Biology Congress, The Netherlands; Genome Congress, San Diego, CA; Latin American Plant Biotechnology Congress, Iguazu, Argentina; Apomixis Congress, Texas; biosafety Meeting of the Biodiversity Convention, Madrid, Spain; Rice Genetics Congress. The

Philippines; Cassava Biotechnology Steering Committee Meetings, The Netherlands; Organization of the Chilean Biotechnology Program, Santiago, Chile; Peruvian Genetics Congress.

(vi) Cooperation with developed country institutions: BRU scientists visited the Biotechnology Group of CIBA GEIGY, in Releight, N.C. and Du Pont Co. Delaware, to discuss recent advances in genetic fingerprinting, gene tagging and marker assisted selection, and transformation; Univ. of Georgia, Athens, in relation to the cassava molecular mapping project; Univ . of Bath, U.K.; cassava somatic embryogenesis.

Visits to CIAT: Dr. H.J. Jacobsen, Univ. of Hannover, Germany (bean transformation project); I. Potrykus, ETH, Zurich (cassava transformation project); S. McCouch, Cornell Univ.; Ithaca (beans and rice transgressive segregation project); J. Steffens, Cornell Univ., Ithaca (molecular biochemistry sabbatic in the BRU); M. Levy, Purdu, Univ. West Lafayette, USA (Rice blast project); P. Chavarriaga, Univ. of Georgia, Athens, USA (cassava microsatellites project).

4.2 Publications

(i) In refereed journals

- **Chaves, A.L.; Vergara, C.; Mayer, J.** 1995. Dichloromethane as an economic alternative to chloroform in the extraction of DNA from plant tissues. Plant Mol. Biol. Reporter 13:13-25.

- **Destefano - Beltran, L.** Caeneghem, W.U.; Gielen, J.; Richards, L. van Montagu, M. van der Stracten, D. 1995. Characterization of three members of the Accsynthase gene family in *Solanum tuberosum* L. Mol. Gen. Genet. 246:496-508.

- **Lentini, Z.**; Reyes, P.; Martinez, C.; **Roca, W.** 1995. Androgenesis of highly recalcitrhan rice genotypes with maltose and silver nitrate....

- Welsh, W. Bushuk, W.; **Roca, W.**; Singh, S.P. 1995. Characterization of agronomic traits markers of recombinant inbred lines from intra-and inter-racial populations of *Phaseolus vulgaris* L. Theor. Appl. Genet.

- **J. Tohme**, O. Toro, **J. Vargas** and D. Debouck. 1995. Variability in Andean Nuña Common Beans (*Phaseolus vulgaris*, Fabaceae). Economic Botany 49(1):78-95.

- A. Gonzalez, J. Lynch, **J. Tohme**, S.E. Beebe, and R.E. Macchieavelli. 1995. Characters Related to Leaf Photosynthesis in Wild Populations and Landraces of Common Bean. Crop. Sci. 35:1468-1479.

(ii) Lab. Manuals, Articles in books

- **J. Tohme, N. Palacios, S. Lenis and W.Roca**. 1995. Applications of Biotechnology to Brachiaria. In: *Brachiaria* Monographs (in press).

- **D.O. Gonzalez, N. Palacios, y J. Tohme**. (comp., eds). 1995. Protocolos para marcadores moleculares. Unidad de Investigacion en biotecnologia, Centro Internacional de Agricultura Tropical (CIAT), Cali - Colombia. 78p.