CASSAVA BIOTECHNOLOGY RESEARCH AT CIAT/COLOMBIA

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ABSTRACT

Cassava is probably the most efficient producer of carbohydrate per unit land area under tropical conditions. The high productivity of cassava makes it an attractive source of renewable industrial raw material, provided ways are found to reduce production costs and solve constraints. Cassava has a long growth cycle, anywhere from 8-24 months, which means that it is visited by many pests that may also transmit diseases. It is vegetatively propagated, and securing sufficient and healthy planting material can be a problem for many small farmers. Biotechnology can contribute to solutions of these problems and realize great benefits for cassava farmers. Since the 1980s CIAT has worked to realize the potential of biotechnology for cassava, especially to solve those problems that can not be dealt with effectively through conventional approaches. Cassava biotechnology research at CIAT falls into three broad areas, namely: genetic transformation, molecular marker development/marker-assisted breeding, and the rapid multiplication of healthy planting material.

Genetic transformation projects include the engineering of cassava with the bt gene for resistance to the cassava stem borer (*Chilomina clarkei*), and other pests susceptible to the bt protein; the production of herbicide resistant cassava, Round-up ready cassava; and the bio-engineering of cassava for the production of novel polymers.

The CIAT molecular genetic map of cassava --- the first such map to be constructed entirely at a CGIAR center --- is being applied to dissect complex traits, such as early bulking, and to realize earlier unachievable goals, such as breeding for resistance to the African Cassava Mosaic Disease (ACMD) in Latin America. ACMD is not only the most serious constraint of the crop in sub-Saharan Africa, but is also a potential threat in tropical America and Asia. The whitefly biotype that serves as the virus's vector has already been found in the Caribbean and in Brazil, and it is a matter of time before the virus appears as well. Simple sequence repeat (SSR) markers from the map have also been employed in the characterization of genetic diversity, towards a definition of heterotic patterns in cassava.

The rate of spread of a successful variety continues to remain slow. Rapid propagation of cassava, using the continuous media cycling method (RITA), is being tested to provide large quantities of disease-free material to farmers or to commercial producers of planting material. The CIAT cassava biotechnology team also works in partnership with the Latin American and Caribbean Cassava Consortium (CLAYUCA) to apply biotechnology to overcome constraints of cassava, and to make the crop more competitive, both as a source of food and as raw material for animal feed and other industrial uses. Such alliances between the public and private sectors to solve problems of mutual concern, are the best hope for increasing the income of millions of poor producers and consumers through cutting-edge science.

INTRODUCTION

Cassava is probably the most efficient producer of carbohydrate per unit land area under tropical and small farmer conditions. The high productivity of cassava makes it an attractive source of renewable industrial raw material. But cassava suffers from several production constraints, which can reduce yield considerably, and make the crop less profitable in the highly competitive carbohydrate market. Salient amongst the constraints are the long growth cycle, anywhere from 8 to 24 months, which means it is visited by

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many pests that may also transmit diseases. The long period to harvest also hinders the flexibility of availability, a trait required by an industrial crop, while it also lengthens considerably the gestation period for new improved varieties. Cassava is vegetatively propagated, and securing clean and healthy planting material can be a problem for poor farmers. Biotechnology can contribute to the solution of some of these constraints, and to realize great benefits for small farmers. Since the 1980s, CIAT has worked to realize the potential of biotechnology for cassava, especially to solve the problems that can not be dealt with effectively through conventional approaches. Cassava biotechnology at CIAT falls into three broad areas, namely: molecular markers for cassava breeding, genetic transformation for pest resistance and starch quality, and tissue culture for rapid multiplication of healthy planting material. This presentation is a brief overview of each area, going into more details with one example; the paper finally concludes on how these biotechnology tools can be applied to cassava research and development in Asia.

A. Molecular Markers for Cassava Breeding

1. An overview

Molecular markers have been employed in crop improvement primarily to make breeding more efficient, and thus reduce the cost and time required for the production of new varieties. Markers, on a genome wide basis, have also been used to characterize germplasm collections, to identify new sources of genetic variation for faster progress in breeding. Markers associated with traits of agronomic interest, have also been used to provide an accurate picture of the breeding value of genotypes, by eliminating the confounding influences on the phenotype of other deleterious loci and the environment. At CIAT genetic markers have been used to characterize genetic diversity of both the cultivated and wild relatives, and to identify new sources of genetic variation. Markers have also been used to map resistance genes, for use in negative marker-assisted selection of disease resistance in the absence of the pathogen (elimination of susceptible genotypes); markers are also the start-off point for the cloning of these resistance genes. Finally, associations between molecular markers and traits of agronomic interests, which are mostly quantitatively inherited, are being employed to elucidate the genetics of these traits.

2. A Molecular Genetic Map of Cassava

With funding from the Rockefeller Foundation, a molecular genetic map of cassava was constructed from an intra-specific cross between TMS30572, an improved line from IITA, Ibadan, Nigeria, and CM2177-2, an elite line from CIAT, Cali, Colombia. The F_1 mapping progeny consists of 150 individuals. Traits of agronomic interest present in the parents and expected to segregate in the cross include: resistance to African cassava mosaic disease (ACMD), resistance to cassava bacterial blight (CBB), and early harvestability in the female parent, TMS30572. In the male parent, traits include: good cooking quality, resistance to CBB, and a high photosynthetic rate. The map which was published in 1997 (Fregene *et al*.,1997) has a total of 300 RFLP, RAPD, SSR, and isozyme markers; more than 70% are RFLP markers. The 1997 map is estimated to cover 80% of the cassava genome and requires saturation. Efforts are currently geared to placing another 300-400 molecular markers on the map. To make the genetic map of cassava widely available, especially to cassava breeders and researchers in national agricultural research and

extension systems (NARES), it was decided that the new markers to be added should be easy to use, while maintaining the same level of information as RFLP markers.

With support from the Swiss International Center for Agriculture, and the participation of a NARES cassava breeder from Nigeria, a project was initiated to generate 300-400 simple sequence repeat (SSR) markers for the genetic map of cassava. SSR markers are simple motifs of di-, tri-, or tetra-nucleotides repeated several times. The regions flanking the repeat sequences are usually conserved, and suitable for the design of PCR primers. They are, therefore, PCR-based, meaning they are easy to use, and co-dominant markers, having the same level of information as RFLP markers. SSR markers also have the unique advantage of ease of automation. In cassava, SSR markers were developed using several genomic libraries enriched for SSR sequences, followed by the sequencing of more than 500 positive clones. About 450 primer pairs have been designed and 200 tested so far, while 90 SSR markers have been mapped. At the moment, the search for SSR markers has turned to looking in non-enriched cDNA, and small fragment genomic libraries, to reduce the high level of duplication found with enriched libraries, and to convert the RFLP markers to SSR ones using BAC (Bacterial Artificial Library) library clones.

3. Characterizing Genetic Diversity and Defining Useful Variation

Progress in crop improvement depends upon the skillful exploitation of crop genetic diversity. The success story of maize hybrid production, and the green revolution wheat and rice varieties are probably the best illustrations of this fact. Cassava breeding has existed at CIAT for the past 27 years, and a group of parents with excellent general combining ability has been identified from a large germplasm collection that represents land races from the crop's center of diversity. Sixty four SSR markers, with a broad coverage of the cassava genome, were employed in an automated fashion, to analyze the parental genotypes, including others from IITA, a collection from Tanzania, and a randomly selected set of land races from the world cassava collection at CIAT. A total of 315 genotypes were analyzed, resulting in a large data matrix of more than 20,000 data points. Principal component analysis (PCA) based on genetic distances was performed on the SSR allele data. Analysis reveal clustering in the cassava genotypes according to region but the high GCA formed did not form any clear cluster in relation to other genotypes. Like maize, cassava appears to have highly differentiated gene pools, and has a large percentage of dominant/recessive gene action loci, two key characteristics required for heterosis. Existing yield data, from crosses between individuals from certain clusters, suggests that this may be so, and molecular markers can be used to predict heterosis, but evidence for this is at the moment being confirmed.

Several studies have revealed that cassava was domesticated from populations of the wild *Manihot* species, *M. esculenta*, sub spp *flabellifolia*. Other studies have also shown that the amount of genetic variation present in the natural population of this wild species are significantly more than that found in cassava. These findings suggests a founder's effect, or a genetic bottle neck, at the domestication of cassava. If this is the case, useful alleles for yield, and yield components may yet exists in the cultivar primary gene pool. We have initiated an advanced back cross quantitative trait loci (QTL) mapping

scheme to mine favorable alleles for root quality, canopy strength, harvest index, and pest and disease resistance, aimed at broadening the genetic base with exotic alleles. The advanced back cross scheme has been used successfully in tomato, rice and maize to transfer favorable alleles to cultivated germplasm. In cassava, an allogamous crop, the scheme has been modified to reflect this. Basically, it involves making F_1 crosses of about 100 individuals each between four genotypes of sub spp *flabellifolia*, that best represent genetic diversity, and eight elite lines representative of the CIAT cassava gene pools. All F_1 individuals that flower are back-crossed to the respective parents to produce BC_1 families. Negative selection is performed at the seedling stage on the BC_1 families, and remaining progenies are back crossed to produce the BC_2 families, which are clonally evaluated in replicated single row (6 plants) experiments. The best four BC_2, with the highest phenotypic variation for the traits in question will be evaluated in replicated trials and also genotyped with markers. QTL analysis should identify new alleles from the wild donor and provide a tool for further breeding. The best BC_2 lines are then tested in a marker-assisted scheme as parental genotypes for improving the selected traits. For a closely related species such as *M. esculenta*, sub spp *flabellifolia*, QTL mapping is performed at the F_1 stage and identified QTLs are used directly in breeding. This second scheme is being used to identify QTLs for high dry root yield and starch content for introgression into good Asian varieties such as KU50.

4. Marker-assisted Selection for Disease Resistance in the Absence of the Pathogen

CIAT has several gene tagging projects for resistance to pests and diseases; they include African cassava mosaic disease (ACMD), cassava white fly (*A. socialis*), cassava bacterial blight (CBB), and cassava root rot (*Phytophtora* spp) with an aim of improving the efficiency of breeding for pest and disease resistance. Only gene tagging for ACMD resistance is discussed here. ACMD is the number one production constraint in sub-Saharan Africa, and a potential risk to Latin America and Asia, given the recent accidental introduction of the vector, the B biotype of the white fly. ACMD also complicates the exchange of germplasm between endemic areas and other parts of the world.

Breeding for ACMD resistance at CIAT is limited by an absence of the pathogen in Latin America, and also by the need to breed for resistance to at least three different strains of the virus. With funding from the Rockefeller Foundation, a project to tag all known sources of resistance to ACMD was initiated by CIAT and IITA. The female parent of the cassava map population (TMS30572) has resistance to ACMD, and also represents the currently deployed source of resistance from the *M.glaziovii* source. A BC_1 mapping population was developed by back crossing five F_1 progeny to TMS30572; these progenies were established *in vitro* from embryo axes and shipped to IITA.

A second mapping population was developed at IITA involving the new source of resistance from TME 3, a Nigerian land race. The new source of resistance shows near immunity to the West and the East African strains of the virus. Classical genetic studies show that the currently deployed source is recessive and the new source is a single dominant gene in the heterozygous state. Both ACMD resistance mapping populations were evaluated over two seasons for disease resistance in replicated trails in two high disease incidence sites in the field; they were also genotyped with molecular markers from

the genetic map. A simple regression of disease response on marker genotypic classes revealed that a region of chromosome D explained about 50% of phenotypic variance for resistance from the *M.glaziovii* source, while a region on chromosome R explained more than 70% of phenotypic resistance of the new source of ACMD resistance.

A scheme has been initiated to use the marker in a marker-assisted scheme to breed for resistance to CMD in Latin America. At the same time a map-based cloning effort has also been initiated to clone the resistance gene for faster deployment via genetic transformation. The scheme involves fine mapping the gene, creating a contig of large DNA fragments around the gene, genetic transformation with candidate DNA fragments that carry the gene, and sequencing. Fine mapping of the region is ongoing, and a bacterial artificial chromosome (BAC) library of large DNA fragments has been constructed for contig mapping. The cassava BAC library which was constructed by CIAT scientists through a visit to the Clemson University Genome Institute (CUGI) in Clemson, South Carolina, has a total of 55,000 clones, of average size 80kb, and a 5X coverage of the cassava genome.

5. Dissection of the Genetics of Complex Traits

The map of cassava has also been employed to elucidate the genetics of agronomic traits that are quantitative in nature, with low broad sense heritability, such as dry matter yield, starch content, and early bulking (early harvestability). Only early bulking is discussed here; it is an important breeding objective in all cassava producing regions, and a key requirement for the transition of cassava from a traditional to an industrial crop. Combining a high starch yield, high dry root yield and early bulking is not an easy breeding objective. Identification of markers associated with the trait can be employed to eliminate inferior genotypes in a large number of breeding populations and thus increase selection efficiency for earliness.

One of the parents of the cassava map population, TMS30572, is an early bulking genotype: 80-90% of maximum yield is attained at eight months, making the cassava map population an excellent one for gene tagging for early bulking. Sixty plants of the 40 best and 40 worst genotypes (from two years of multi-locational replicated trials and harvest) for early bulking were planted in 6x10 m plots, with two replications at CIAT, Cali, Colombia; four internal plants were harvested every three weeks, beginning at six weeks after planting (WAP), until 30WAP. The dry root yield, dry foliage yield, harvest index, number of roots, and size of roots (diameter) where measured. A very early bulking clone from Brazil was included as control. A multiple regression analysis showed that dry foliage weight, and harvest index (HI), were the most important yield components in this experiment. A simple regression of dry foliage weight, and HI across the period of the experiment, on marker genotype class revealed QTLs that explained between 18-35% of phenotypic variance. Marker fidelity studies to confirm the use of these markers in breeding continues.

B. Genetic Transformation for Pest Resistance and Root Quality

CIAT has developed robust protocols for the regeneration and genetic transformation of cassava. The method is based on the *Agrobacterium tumefaciens*

transformation of friable embryogenic callus. Transformation efficiencies of 10-15% have been obtained. The transformation protocol is being employed to engineer resistance to an important pest of cassava, the cassava stem borer (*Chilomima clarkei*), which is endemic in the Colombian North Coast. The stem borer can cause losses of 50-100% of cassava stakes and result in a severe shortage of planting material. Resistance is being created by the insertion of a construct containing the Bt gene, pBIGCry, and two reporter genes, the gus and npt II genes.

A second transformation project, which is about to begin, is the genetic engineering of cassava to produce waxy starch in cassava (100% amylopectin) This is via the down regulation of the granule bound starch synthate gene (GBSSII), via anti-sense down regulation of the gene. The gene has been cloned from cassava, in collaboration with the Wageningen Agricultural University (WAU) in the Netherlands; constructs for transformation will soon be available. Another project, awaiting funding, is the genetic engineering of cassava for biodegradable polymers, polyhydroxy alkanoates (PHA). The genes for the production of PHAs have been cloned from bacteria and shown to express specifically to organs of plants with the key fatty acid biosynthesis pathway, required to produce the polymers. PHAs have been successfully produced in seeds of arabidopsis, and soybean. The genes required are ketothiolase, polyhydroxylase B, and polyhydroxylase C.

C. Tissue Culture for Rapid Multiplication of Healthy Planting Material

Cassava yields can be affected considerably by diseased, or poor quality planting material. Securing clean and healthy stakes can be a problem for small farmers. For the same reason, the rate of spread of successful varieties continues to remain slow; rapid propagation of cassava by small farmers in rudimentary conditions have been proposed as a means of increasing the rate of spread in Colombia. CIAT has joined hands with a NGO in southern Colombia to help farmers acquire the rapid propagation technique, via the use of simple and widely available materials. The continuous media cycling technique (RITA) is also being tested by CIAT for the production of cassava planting material on a larger scale, by bigger companies and by NARES labs.

CONCLUSION

Cassava biotechnology research at CIAT can contribute to cassava research and development in Asia. Expertise in tissue culture has been transferred to NARES in the region in the past. But maybe the greatest potential for impact exists in cassava breeding. Success in cassava breeding relies heavily on:

1. Parental genotypes: crosses between different pairs of genotypes have a varying degree of success, and good genotypes do not always give good progenies
2. Size of progenies: between 1983 and 1997 the Thai-CIAT breeding program released three improved genotypes selected from 327,000 genotypes from 4130 crosses (Kawano *et al*., 1998)
3. Selection scheme: selection at the second cycle of evaluation, the single row trial (SRT) is the most crucial for success; more than 95% of the progenies are eliminated at this stage.

Markers can be used to:

1. Increase selection efficiency by a marker-assisted negative selection for root dry matter content, disease and pest resistance, and harvest index, at the seedling trial stage to accurately eliminate inferior genotypes and increase the selection efficiency at the single row trial (SRT) stage. Potential for increasing the selection efficiency is greatest at that stage.
2. Choice of parental genotypes: Molecular markers can provide a quantitative estimate of genetic variability, and help in choosing parents that maximize genetic variation. Markers associated with traits of interest can also be employed to identify parents with highest breeding value.

Application of marker technology to plant breeding has become economically feasible, thanks to high through-put technologies, but marker development and application requires considerable investment of resources to begin. Marker development and application for Asian cassava breeding is best achieved through a regional network of labs with funding from a regional donor. An Asian cassava biotechnology network, modeled on the successful Asian rice and maize networks should be considered. A project to test the application of markers in Asian cassava breeding is a worthy venture, given the potential of such technologies, and should be pursued.

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