CHAPTER 15

Biotechnology for Cassava Improvement: Genetic Modification and Clean-Seed Production

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In Memoriam

*Carlos Julio Herrera (r.i.p.) was co-author of this chapter for the first edition of this book, which was published in the Spanish language. The chapter was entitled "Biotecnología para el manejo de plagas en la producción de semilla limpia" [*Biotechnology for pest control in clean-seed production*]. Carlos was widely respected as an expert and teacher on insect pests of cassava. We therefore dedicate this English-language chapter to his memory.*

Carlos Julio Herrera leads a training session with a group from the Women Farmers' Association of Santa Ana (ASOPROSA)[7], Department of Cauca, Colombia. Themes were the production of planting materials and control of cassava pests and diseases. (Photo by R. Escobar.)

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7. For an explanation of this and other acronyms and abbreviations, see *Appendix 1: Acronyms, and Abbreviations, and Technical Terminology,* this volume.

Introduction

Genetic transformation technology, using *Agrobacterium*, has made possible the acquisition of transgenic varieties of the world's most important food crops. The varieties from first-generation transgenic plants are today cultivated on 134 million hectares. They contain genes that mostly confer resistance to herbicides and insect pests. The list of genetically modified crops includes soybean, maize, cotton, colza, alfalfa, sugar beet, tomato, and red pepper, among others. They are cultivated in 29 countries, including the EU countries of Czech Republic, Germany, Poland, Portugal, Romania, Slovakia, Spain, and Sweden; and the Central American countries of Costa Rica and Mexico (James 2011).

Genetic modification technology to improve world agriculture was also adopted for cassava in the 1980s. Somatic embryos of genetically modified cassava plants were first produced between 1993 and 1996 (Sarria et al. 1993, 1995; Schopke et al. 1996). Since then, innovative traits have been introduced, ranging from resistance to herbicides (Sarria et al. 2000), through reduced cyanide content in the plant (Siritunga and Sayre 2003; Siritunga et al. 2004), to modifications of the amount (Ihemere et al. 2006) and quality (Raemakers et al. 2005) of starch accumulated in roots. Jørgensen et al. (2005) recently demonstrated that silencing of specific genes for biosynthetic routes in cassava is possible. For example, the quantity of cyanogenic glycosides (cyanide) that the plant produces can be reduced, using RNA interference (RNAi) technology. We can now talk of a fast-approaching second generation of transgenic cassava by the introduction and expression of genes to improve the nutritional value of the roots, i.e., increasing carotene content (Welsch et al. 2011) and protein (Abhary et al. 2011), or by introducing into cassava genes that will help control the devastating disease caused by the African Cassava Mosaic Virus (Vanderschuren et al. 2007).

New genetic information has been introduced into cassava plants, essentially using *Agrobacterium tumefaciens* as a natural vector of the genes of interest. Commonly, two types of tissues are used to generate *de novo* plants: totipotent cells (able to differentiate and regenerate a complete organism) derived from somatic embryos, and also known as friable embryogenic calluses (FECs; Taylor et al. 1996); and the cotyledons of somatic embryos (Taylor et al. 1996). Transgenic plant regeneration is usually achieved by inducing or germinating embryos and/or inducing organs (organogenesis) directly from totipotent cells. Common agents for selecting transgenic cells include antibiotics, herbicides, fluorescence, and sugars such as mannose.

Cassava is conventionally propagated in the field by planting pieces of stems, known as stakes, obtained from plants of the previous crop. This system presents relative advantages, particularly the main one of lower production costs. However, under certain circumstances, the system incurs problems of transmission of pests and diseases (usually viral and/or bacterial in nature). Such problems hinder the exchange of germplasm between countries, create seed shortages during the year's production peaks in certain regions, delay distribution systems, and slow down the adaptation and/or adoption of new clones. These and other problems can be resolved by applying *in vitro* techniques.

Implementing multiplication systems that use *in vitro* tissue culture of planting materials enables the distribution of disease-free clones of interest to small farmers or industries. CIAT has defined the basic system for *in vitro* propagation and germplasm cleaning, thus facilitating the consolidation of the *in vitro* bank held at CIAT and the exchange of genetic materials among countries.

This chapter provides an overview of the advances made in (1) cassava transformation achieved recently, focusing on the use of genetically modified cassava to study genes and promoters, improve the nutritional quality of roots, study leaf retention, and alter starch content and quality to produce biocombustibles; and (2) multiplication systems for planting materials at small-farmer and industrial levels.

Cassava as a Model for Testing the Expression of Genes and Promoters

A major constraint to incorporating new traits into cassava through genetic modification comprises the proven availability of promoters—regulatory sequences for gene expression—in this plant's storage roots. Based on previous reports on the specific expression in cassava roots of a glutamic acid-rich protein (also known as Pt2L4; de Souza et al. 2009), the promoter region (CP2) of this gene was cloned in front of the *GUSPlus* reporter gene and expressed in genetically modified plants grown in the field (Beltrán et al. 2010).

The expression of the reporter gene led to an intense coloration of storage roots and stem vascular

tissues. Leaves showed a less intense expression, and the pith an absence of expression. Fluorometric analyses revealed that the promoter CP2 was equally active in root pulp and stems, although 3.5 times less active in leaves. These findings were corroborated by quantitative analyses of messenger RNA (mRNA) levels, using real-time PCR developed for transgenes in cassava (Beltrán et al. 2009).

A second, larger version of promoter CP2—promoter CP1—was also cloned in front of the *GUSPlus* gene and found expression in transgenic carrot plants, which also have roots that store carbohydrates. The transformation and regeneration system in carrot is much more expeditious than that of cassava. Promoter CP1 seems to preferentially express in secondary phloem and root vascular cambium, although, again, expression in vascular leaf tissue was less, by six times.

The results of these studies demonstrated that genetically modified cassava plants grown in the field provide a good model for testing genes and promoters of interest. They also enabled the isolation and characterization of regulatory sequences of cassava genes for use in directing preferential expression to the root, the most economically valuable plant organ.

Provitamin A Deficiency in Cassava and Prospects of Improving this Trait through Biotechnology

The cassava plant originates in South America, where, since ancestral times, it has been consumed as a food staple providing dietary energy. The roots contain large quantities of carbohydrates, hence its importance in nutrition. However, they contain little protein and few micronutrients (OECD 2009), compared with sweet potato, potato, bean, maize, or wheat. Nevertheless, it is widely consumed because of its ability to accumulate carbohydrates and to tolerate drought and acid soils (Kawano 2003). It is the principal dietary energy source for more than 600 million people, especially low-income populations in less developed countries, who often face food shortages (Thro et al. 1999a, 1999b). Cassava is therefore an appropriate crop for which to use biotechnology to produce varieties with higher levels of nutrients such as ß-carotene.

Beta-carotene is the fundamental source of vitamin A, essential for human and animal health, and best known as critical in the maintenance of ocular epithelia in, for example, the retina and cornea. Not so well known, but nevertheless equally important, is the role vitamin A plays in enabling T-lymphocytes to produce antibodies during infections, so that these natural killer cells carry out phagocytosis. Vitamin A is also attributed as having the roles of a hormone for cell development and gene expression, and of an anticarcinogen. Cell differentiation and growth are regulated by vitamin A. Today, we know that vitamins and minerals, together with other food components, significantly reduce chronic diseases such as cancer, cardiovascular problems, and degenerative diseases related to aging (Álvarez et al. 2004).

When consumed in a conventional diet (i.e., at 100 g of cassava per day), ß-carotene levels in cassava are insufficient to fulfill Required Daily Allowance (RDA) standards. Conventional improvement would help raise these levels, but procedures are complicated because of cassava's polyploidy, heterozygous nature, and slow multiplication. Furthermore, the accumulation of ß-carotene in cassava roots involves several genes, as to be expected of a multigene synthesis route. Hence, in the attempt to introduce all the "good" alleles of the relevant genes into a single variety, strategies for conventional improvement become even more complicated.

The international initiative HarvestPlus (www.harvestplus.org) involves interdisciplinary and interinstitutional research to reduce micronutrient malnutrition in humans. Within this initiative, CIAT collaborated with the University of Freiburg (Germany) to develop, through conventional improvement and/or biotechnology, cassava genotypes more able to produce and store ß-carotene in roots. This effort's first results demonstrated that cassava genotypes vary in accumulating carotenes in roots. Values ranged from 0.102 to 1.040 mg of total carotene per 100 g of fresh weight (Chávez et al. 2005).

In at least four examples of crops—rice, potato, tomato, and canola—ß-carotene content has been increased substantially by inserting genes of the carotene synthesis route, and which are directed by promoters to express in specific organs, or constitutively. The genetic transformation of rice, using genes of the carotene route (Ye et al. 2000; Paine et al. 2005), was successful in increasing total carotene content in the grain by as much as 27 times to a maximum 37 μg/g. More than 80% (>30 μg/g) corresponded to ß-carotene. In canola, ß-carotene was increased by 50 times (Shewmaker et al. 1999). More recently, in potato, Diretto et al. (2007) demonstrated that ß-carotene in the tuber can be increased by 3600 times, reaching 47 μg/g of dry weight. Hence, on a diet of 250 g of potato per day, half of the RDA

requirements would be supplied. Both in rice grains and potato tubers, the carotene synthesis route was sufficiently complemented to suggest that a similar strategy can be attempted for cassava.

The first significant result that HarvestPlus obtained from its biotechnological approach was the knowledge that the carotene synthesis route does operate in cassava roots. That is, the genes find expression in this organ (Arango-Mejía 2005), indicating that the necessary substrata for the activity of the route's enzymes are present. Without this background, designing an unconventional improvement strategy (i.e., genetic modification) to increase ß-carotene content in cassava roots, by inserting new gene combinations, would be more difficult.

The second significant result is that transgenic cassava plants were produced. They combined one or more genes from the carotene synthesis route with promoters that preferentially directed the expression of these to roots. These plants have already been field-tested (Welsch et al. 2010). The results of overexpression of a gene for phytoene synthetase of bacterial origin (*crt*B) in the root demonstrated that increasing total carotene content is possible. A white-root cassava (genotype 60444; transgenic event pCAS-Phyt-12) that normally carries ≤0.6 µg/g (dry weight) of carotenes contained about 21 µg/g (dry weight), that is, about 35 times as much. The ß-carotene content in this same, non-transgenic genotype increased proportionately from 0.4 to 6.7 µg/g (dry weight).

Thus, these results demonstrated that increasing carotene content in cassava roots is feasible. It can be done by inserting heterologous (foreign) genes under the control of promoters that preferentially express in storage-root tissues. Research is continuing with the genetic modification or transfer of transgenes to yellow-rooted genotypes that have higher carotene content. These genotypes include advanced breeding lines like GM905-21 and GM905-57.

Increasing ß-carotene content in cassava roots would bring additional advantages to the farmer, for example, higher "resistance" to postharvest physiological deterioration (PPD). Chávez et al. (2005) detected a trend in roots with higher carotene contents to delay the beginning of PPD. Possibly, in this type of "resistance", molecules of the type ß-ionone, derived from the catabolism of ß-carotene, are involved, as they play a role in the response to biotic stresses such as fungal infections that are characteristic of PPD (Bouvier et al. 2005). However, PPD is a highly variable characteristic. It is difficult to measure visually; it is influenced by the environment, and depends heavily on the storage conditions of harvested roots.

Leaf Retention

One way of increasing yields of crops such as cassava is to delay leaf senescence by increasing cytokinin levels in the leaves. Cytokinins are plant growth factors implicated in plant development, including leaf longevity. The phenomenon of holding back leaf senescence is known as "stay green" (Thomas and Howarth 2000). Millions of people use cassava as their principal source of carbohydrates, which is why the possibility of using genetic modification to increase root yield (i.e., starch) by increasing leaf longevity is being explored. The trait of "stay green" in cassava is of great commercial interest. Plants improved conventionally for foliage retention have already shown increases of dry matter content as high as 33% more (Lenis et al. 2006). Moreover, delayed senescence enables the plant to have more leaves at harvest, which then can be used as forage of excellent nutritional quality (Buitrago 1990).

Thus, Zhang and Gruissem (2004) introduced the bacterial gene *ipt* into cassava. This gene codes for the enzyme isopentenyltransferase (Akiyoshi et al. 1984; Barry et al. 1984). It is active during leaf senescence, once it is activated by its promoter SAG12, itself derived from *Arabidopsis thaliana* (Lohman et al. 1994; Weaver et al. 1998). The transgenic cassava line 60444-529-28 was selected for field evaluation at CIAT in collaboration with the Institute of Plant Science (now the Institute of Agricultural Sciences) at ETH–Zurich, Switzerland) and the Department of Crop and Soil Sciences of Cornell University (Ithaca, NY, USA). Agromorphological traits were measured, together with levels of cytokinins, abscisic acid (ABA), glucose, sucrose, and starch, all indicators of gene *ipt* expression (López 2008).

Results showed that, effectively, cytokinin levels significantly increased in basal leaves, in tandem with an increase of ABA in apical leaves. Glucose and sucrose levels also increased in apical leaves, stems, and abscission areas. No positive impact on dry matter content was apparent in this particular trial, probably because of unexpected dry periods. Precipitation was also more abundant and erratic than normal, which probably also had an effect on root dry matter content. An alternative explanation is suggested through

experiments by Medford et al. (1989), who determined that increased cytokinin levels can generate changes in the root systems of tobacco and *Arabidopsis*. Changes related to these phenomena are not ruled out for cassava storage roots, despite the difficulty of observing them. They may well have contributed to the reduced dry matter in the transgenic line 60444-529-28.

Another explanation, which also requires experimental verification, is that dry matter was affected by changes related to sugar translocation. Basal leaves may have made higher demands as their longevity increased. However, the transgenic storage roots did not differ morphologically from those of the control.

Measuring sugars in the plant is important, as these usually increase during senescence (Wingler et al. 1998; Stessman et al. 2002). Increase can trigger symptoms associated with senescence such as the yellowing of leaves. Statistically, glucose and sucrose levels were clearly higher in the apical leaves, abscission areas, and stems of transgenic cassava plants. This suggests a pattern of free-sugar remobilization from the youngest leaves towards other plant organs, or the activation of sugar synthesis in younger leaves.

The relationships between altered cytokinin levels and source-sink proportions have been studied in tobacco and lettuce (McCabe et al. 2001; Cowan et al. 2005). Some nutrient deficiencies in young leaves relate to changes in cytokinin and sugar levels (Jordi et al. 2000). Indeed, Cowan et al. (2005) showed that the SAG12-*ipt* transgenic tobacco plants were slower than wild plants to increase the root-to-shoot ratio and the specific leaf area (SLA) after drought. This was interpreted as delayed capacity to remobilize nutrients from source organs (leaves) towards the sink (roots).

The protocol developed to characterize the performance of the transgenic cassava line 6044452928 under field conditions was sufficiently satisfactory and comprehensive for use as a model for future transgenic evaluations. This work also demonstrated that transgenesis is a viable alternative for modifying traits of agronomic importance in cassava such as leaf longevity. Observations made in our field experiments showed that the SAG12-*ipt* system did not obviously affect cassava yield, at least, not under our evaluation conditions in Colombia. Our findings were supported by the results of Zhang et al. (2010) who tested the same genotype in open fields in China discovering that transgenic cassava plants expressing SAG12-ipt did not improve root number, nor root weight when tested in the field. On the contrary, the non-transgenic wild types produced more and heavier roots (Tables 1 in Zhang's paper).

However, as was expected, the SAG12-*ipt* system increased cytokinin levels exclusively in mature leaves. The resulting delayed senescence led to extended leaf life. The retarded senescence of the mature leaves caused changes in source–sink relationships, as reflected by the physiological symptoms associated with senescence (increases in sugars and ABA in apical leaves). However, these changes did not seem to be reflected in the phenotype of young leaves. Larger lobes and petioles in mature leaves could be related to the increase in cytokinins because of their participation in processes of growth and cellular division. Evaluation of the ability of the transgenic cassava line 60444-529-28 to tolerate drought, while minimally affecting yield, may reveal the true potential of taking advantage of the SAG12-*ipt* system in cassava.

Rapid Propagation of Certified Seed

The lack of technology for producing planting materials in sufficient quantities, and in optimal health conditions, becomes an obstacle for the commercial development of cassava. Conventional plant propagation—use of stakes—favors, among other things, the spread of diseases, thus affecting the quality and quantity of "seed" for planting and, hence, the expected yield per production cycle.

CIAT, in collaboration with various actors and agents in development, has implemented and adjusted several multiplication systems at different scales. Thus, with assistance from the DGIS (Netherlands), the Center implemented mass propagation, using bioreactors of the type RITA® (French acronym; Temporary Immersion System for Plant Tissue Culture; Teisson and Alvard 1995). This efficient multiplication system reduces unit costs and propagation time by about 50%, compared with the conventional stake systems and *in vitro* multiplication in solid media.

In the RITA® system, during the immersion cycles, tissues are bathed in a liquid medium that contains nutrients and hormonal regulators (mainly of the cytokinin type). The cycles alternate with dry rest periods and no aeration. The growth of roots, stems (new explants), and leaves is accelerated, and a large quantity of buds is produced. The propagation rate, using this system, is thus increased considerably and can be used for the next micropropagation cycles.

With this methodology, multiplication rates of more than 1:10 can be reached, depending on the variety (Escobar et al. 2001). RITA® has also been successfully used for the proliferation of somatic embryos of coffee (Berthouly and Etienne 1999), banana (Alvard et al. 1993), rubber (Etienne et al. 1997), sugarcane (Lorenzo et al. 1998), and other crops.

The successful use of bioreactors for mass propagation depends on the response of the variety to management under *in vitro* conditions in liquid media. At CIAT, this system was tested successfully for multiplying industrial cassava clones. Experimentally, it was also successful with yam, lulo, sugarcane, potato, tree tomato, sweet potato, and other crops. It was also successful for embryogenesis in cassava, rice, and *Brachiaria*.

The Cassava Biotechnology Network (CBN) conducted a survey of small farmers in different countries (Thro et al. 1997). It found that, in every region, the principal limitation for farmers is access to good-quality planting materials of local and/or improved clones in sufficient quantities at planting times. Thus, CIAT, with support from the CBN and the Participatory Research and Gender Analysis (PRGA) Program, established a low-cost *in vitro* multiplication system at farm level (Thro et al. 1999a; Escobar et al. 2006), to enable rural associations to directly access planting materials through biotechnological techniques. This system permitted the establishment of rural laboratories in the Department of Cauca (CIAT 1999), and the Atlantic Coast (CIAT 2008), Colombia. The associations had been selected for their interest and capacity to manage and implement participatory laboratory processes to benefit their communities and production systems.

The strategy on which the rural laboratories were based was the adaptation to local conditions, using infrastructure and low-cost inputs that are easily obtained from local markets. Examples of successful rural laboratories are those of the associations ASOPROSA, Department of Cauca; the San Jacinto Small Farmers' Municipal Association for Sustainable Development (ASOMUDEPAS), Department of Bolívar; and the Empresa Comunitaria San Rafael in Ovejas, Department of Sucre, all in Colombia.

Alternative systems of producing planting materials can therefore by developed by combining low-cost systems with mass-propagation systems and/or conventional, solid-phase, *in vitro* systems. Such systems would benefit countries that have problems accessing planting materials, but possess potential for development and have end users interested in creating "local banks" of pathogen-free, planting materials (Escobar 2009).

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To save space, the acronym "CIAT" is used instead of "Centro Internacional de Agricultura Tropical".

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