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Development and application of transgenic technologies in cassava

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Abstract

The capacity to integrate transgenes into the tropical root crop cassava (*Manihot esculenta* Crantz) is now established and being utilized to generate plants expressing traits of agronomic interest. The tissue culture and gene transfer systems currently employed to produce these transgenic cassava have improved significantly over the past 5 years and are assessed and compared in this review. Programs are underway to develop cassava with enhanced resistance to viral diseases and insects pests, improved nutritional content, modified and increased starch metabolism and reduced cyanogenic content of processed roots. Each of these is described individually for the underlying biology the molecular strategies being employed and progress achieved towards the desired product. Important advances have occurred, with transgenic plants from several laboratories being prepared for field trails.

Introduction

A review of the current status of transgenic programs in cassava (*Manihot esculenta* Crantz) is timely. After a period of technology development in the 1990s, progress has been significant, with several research groups reporting the production of cassava plants genetically modified for enhanced value to producers and consumers. Field trials are being planned, and efforts directed at integrating desired traits into farmer-preferred germplasm intended for future commercial deployment. After a brief introduction to the crop, we will examine the important role that transgenic technologies can play in realizing the full potential of cassava as a staple food crop and commercial commodity in the 21st century. The gene transfer systems presently employed to integrate foreign DNA into the cassava genome will be outlined, followed by a fuller examination of the agronomic traits currently being addressed in genetic transformation programs around the world. Future directions of transgenic programs will be discussed as well as an assessment of progress being made in deploying transgenic cassava to the field.

As one of the few staple food crops not cultivated within the industrialized countries, the importance of cassava tends to be unknown or under-appreciated by much of the research community. However, cassava plays an important role underpinning food and economic security in many of the world's least developed and food-deficient countries (Thro *et al.*, 1999). Cassava is exploited primarily for its ability to accumulate and store starch within large swollen secondary root structures. The crop is grown by subsistence farmers for

on-farm consumption, as a cash crop for sale in local markets and by commercial operations on large scale farms for animal feed and for processing into starch to supply the growing food processing and chemical industries within developing countries. The FAO reported that in the year 2002, farmers in the world's tropical regions committed more than 16 million hectares of land to its cultivation, an area approaching that of the global total for *Solanum* potato (FAO). Presently, more than 600 million people consume products made from cassava on a daily basis, with the crop ranking behind rice and maize as the most important source of dietary energy in the tropics. Dependence on the crop will not diminish as production is projected to increase 60% by the year 2020 in response to needs of the growing population within these regions (Scott *et al*., 2000).

Lack of investment in cassava research and development prior to the 1980s has resulted in a significant portion of cassava's yield potential remaining untapped. Demonstrated yields of 80–100 metric tons (t) fresh weight of roots per hectare in controlled field trials contrast with a present world average of 10.7 t per hectare and 8.9 t per hectare in Africa (FAO). Unlocking this genetic potential in order to increase production and improve traits such as starch and nutritional quality, post-harvest characteristics and reduced cyanogenic content has become the focus of improvement programs.

Role of transgenic technologies in cassava improvement

Most cassava farmers are resource-poor, lacking the ability to purchase and apply agrochemicals on a regular basis. Development and deployment of enhanced germplasm therefore remains the most important method for ensuring improved cassava production. Conventional breeding programs at the Centro Internacional de Agricultura Tropical (CIAT), Colombia, and the International Institute for Tropical Agriculture (IITA), Nigeria, the two CGIAR Centers which hold the UN mandate for cassava, have been successful in developing and delivering cassava varieties with enhanced disease and insect resistance, dry matter content and improved processing qualities in Africa (Manyong *et al*., 2000; Nweke *et al*., 2002), Asia and the Americas (Jennings and Iglesias, 2002). While marker-assisted breeding will aid breeders and bring benefits to cassava producers (Fregene and Pounti-Kaerlas, 2002; Akano *et al*., 2002), it is recognized that traditional breeding will remain problematic and, on its own, unlikely to provide all solutions for improving the crop to suit the varying needs of small farmers and commercial production in the tropics (Jennings and Iglesias, 2002; Kawano, 2003). The application of transgenic technologies to integrate desired traits into farmer preferred cultivars and landraces as well as elite breeding lines is therefore highly attractive. Indeed, the ability to transfer new genetic materials into the cassava genome in this manner is essential if the crop is to fully benefit from major advances occurring in the genomic and post-genomic era. Transgenic technologies allow beneficial traits to be transferred from one cassava cultivar to another and from wild relatives to cultivated *Manihot*, circumventing species boundaries and the problems of outcrossing and inbreeding depression, inherent to this vegetatively propagated crop. In addition, integration of genetic material from exotic sources such as viruses for pathogen-derived resistance strategies, and bacterial genes for insect resistance become possible, and can be developed and deployed to benefit cassava farmers.

Gene transfer technologies in cassava

As for all crop species, transgenic systems in cassava are reliant on the development of tissue culture systems capable of generating totipotent cells and tissues. These act as the target for transgene insertion, after which selection for the successful integration events takes place and fully transformed plants regenerated (Su, 2002). No reproducible system for shoot regeneration from mature tissues has been reported in cassava. However, early studies identified that somatic embryos could be induced and whole plants recovered from immature leaflobe explants (Stamp and Henshaw, 1987; Szabados *et al*., 1987) on a Murashige and Skoog (1962)-based medium supplemented with auxin. Plants regenerated in this manner are clones of the mature parent material, preserving all traits of the given germplasm. These multicellular embryogenic structures, and the secondary

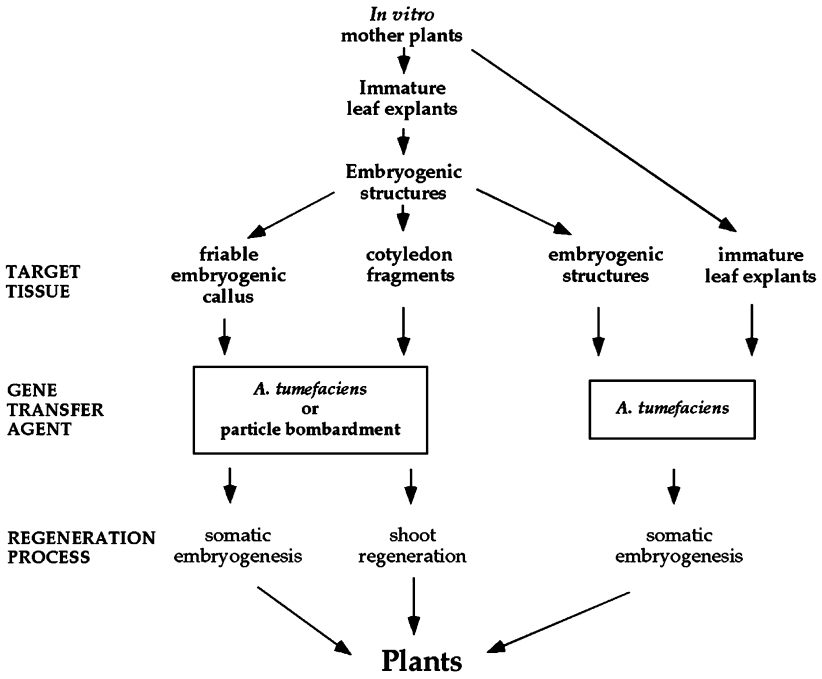
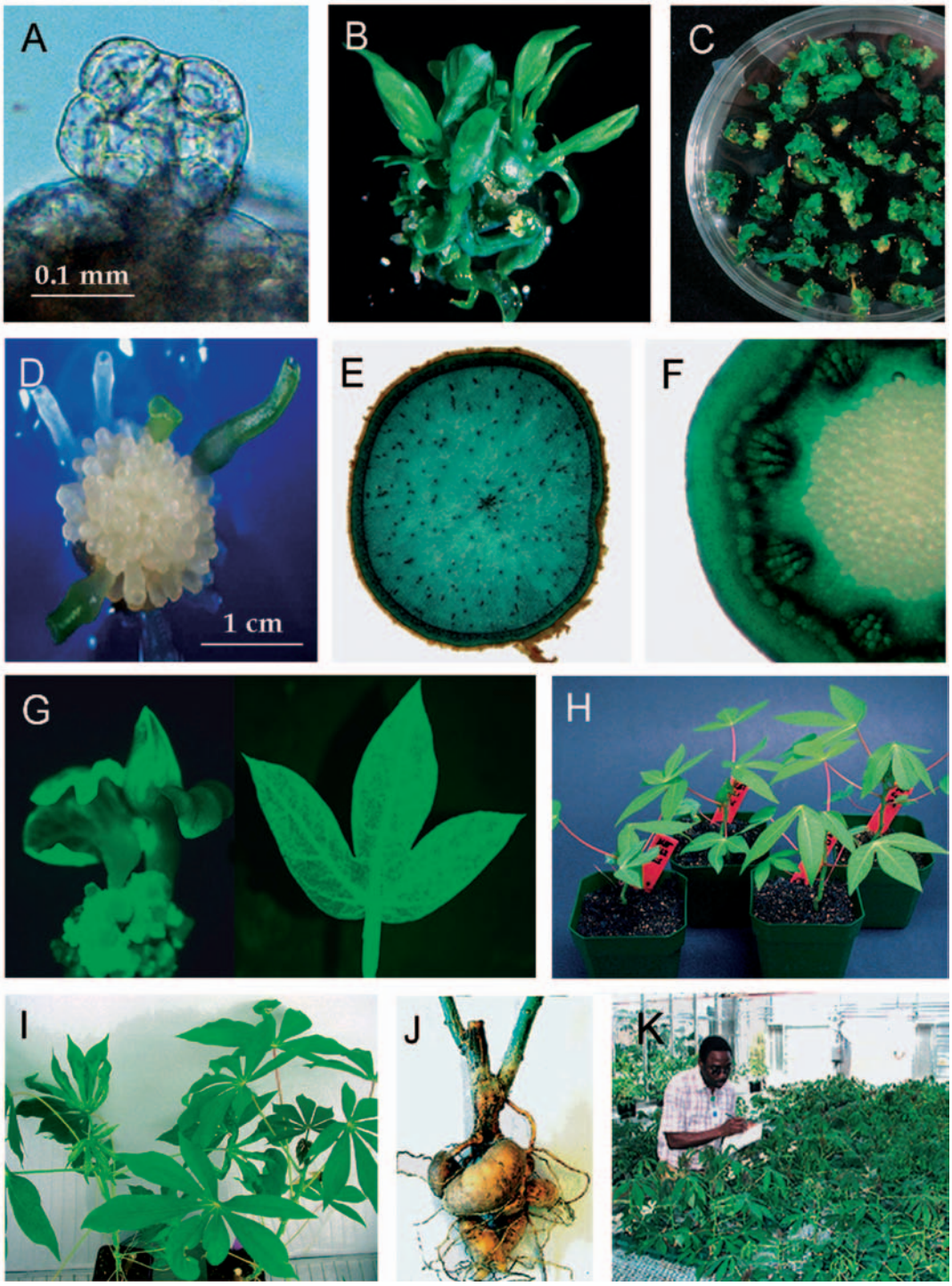


Figure 1. A schematic representation of the four systems currently employed to recover genetically transformed cassava plants.

embryos which can be generated from them (Raemakers *et al.*, 1993), were used as the target for transgene insertion when programs for genetic engineering of cassava were initiated in the early 1990s. After a period of frustration, evidence for the production of transgenic embryos and plants was first provided in 1994 (Sarria *et al.*, 1995). This was followed by breakthroughs in plant regeneration from the embryogenic systems, leading to recovery of confirmed transgenic cassava plants expressing the *uidA* (Li *et al.*, 1996; Schöpke *et al.*, 1996) and luciferine marker (Raemakers *et al.*, 1996) genes. At the time of writing, four distinct transformation systems are employed to produce genetically transformed plants. These are illustrated schematically in Figure 1. For more detailed review of cassava tissue culture and regeneration systems the reader is directed to Raemakers *et al.* (1997), Fregene and Pounti-Kaerlas (2002) and Taylor *et al.* (2002).

All four transformation systems are reliant on the production of embryogenic tissues from *in vitro* leaf-lobe explants, but vary in the way in which these are subsequently manipulated and mated to the gene transfer systems (Figure 1). Molecular confirmation of transgene integration and expression has been confirmed in plants regenerated from each protocol. In the first case, friable embryogenic callus (FEC) is generated from the somatic embryogenic structures described above, in a manner analogous to the totipotent tissues generated from zygotic embryos in the cereals and crops such as soybean (Taylor *et al.*, 1996). Mature somatic embryos are produced from these sub-millimeter embryogenic units (Figure 2A) and plants germinated on a medium containing cytokinin. FEC, and the embryogenic suspensions established from them, have been successfully employed as target tissues for integration of transgenes via microparticle bombardment (Raemakers *et al.*, 1996; Schöpke *et al.*, 1996; Taylor *et al.*, 2001) and *Agrobacterium tumefaciens* (González *et al.*, 1998; Zhang and Pounti-Kaerlas, 2000; Schreuder *et al.*, 2001). In the second method, somatic embryos produced from the leaf-lobes are allowed to mature and produce green cotyledons. After excision from the embryo, plantlets are regenerated via shoot organogenesis from the cut surface of these foliose organs (Figure 2B). The initial report of transgenic plant recovery after *A. tumefaciens* inoculation of cotyledon pieces (Li *et al.*, 1996) was followed by confirmation that genetically transformed plants can also be produced by microparticle bombardment of cotyledon-derived callus tissues with the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particle inflow gun (Zhang *et al*., 2000a). More recently, two further protocols for the production of transgenic cassava plants were also reported. In these cases, *A. tumefaciens* is used to introduce transgenes directly into the embryogenic structures generated by the leaf-lobe explants. This can occur by direct inoculation of leaf-lobe explants to generate transgenic primary embryos (Arias-Garzón and Sayre, 2000), or by co-culture with the secondary embryos which can be proliferated from these initial embryogenic events (Sarria *et al*., 2000; Siritunga and Sayre, 2003). Subsequent culture on selection medium ensures proliferation of only transgenic tissues, followed by recovery mature embryos and germination to produce whole plants.

Considerable discussion has focused on which of the above systems is preferable (Fregene and Pounti-Kaerlas, 2002; Taylor *et al*., 2002). Criteria for assessment include, time to recover transgenic plants, labor intensiveness, capacity to adapt the procedure to a range of agronomically important cultivars and the likelihood of generating chimeras or somaclonal variants. All four systems have their strengths and potential weaknesses. Problems of somaclonal variation within plants regenerated from embryogenic suspensions have been addressed by switching to the use of young FEC cultures as the target for transgene insertion (Raemakers *et al*., 2001; Taylor *et al*., 2001). Improved procedures for the recovery of plants via shoot organogenesis from cotyledon fragments have also been reported (Figure 2C) (Zhang *et al*., 2001; Hankoua *et al*., 2003). In all cases preference has developed for using *A. tumificiens* as the gene transfer agent, and this is now the standard method for production of genetically transformed cassava plants. As in other species, use of *Agrobacterium* results in more consistent recovery of transgenic plants containing single-copy insertions of the transgene than is the case with direct gene transfer systems such as microparticle bombardment. Consensus from the laboratories producing transgenic cassava is that from about 75 transgenic plant lines analyzed by Southern blotting to date, 50% contain single insertion events when *Agrobacterium* is employed as the gene transferring agent, compared to only 10% when integration is achieved via microparticle bombardment.

A critical issue for the successful adoption of transgenic cassava by farmers in the tropics is the capacity to integrate transgenes coding for beneficial traits into the appropriate germplasm. Hundreds of genetically distinct varieties of the crop are known to exist. While it is envisioned that only a very small fraction of these will ever be targeted within genetic engineering programs, there is a need to develop capacities to transform the most important landraces, improved varieties and breeding lines for each of the major cassava growing regions (Taylor *et al*., 2002). This raises important technical challenges for the research groups engaged in these activities. Development of efficient, genotype-independent transformation systems is the ultimate aim for all transgenic crop programs, but remains elusive due to varying *in vitro* morphogenic response, between even closely related cultivars. To date transgenic plants have been recovered from nine cassava cultivars: Col 22, Col 1505, Col 2215, Per 183 and Ica-Negrita from South America; 60444, Bonoua Rouge and L2 from Africa and Adira-4 from Indonesia. As resources are dedicated to this effort, progress is being made in bringing more germplasm into the tissue culture and transformation systems. For example, more than 20 cultivars from Africa, Asia

Figure 2. Various stages of the culture systems and the transgenic tissues and plants generated within cassava genetic transformation programs. (A). Sub-millimeter embryogenic unit from an African cassava cultivar used as a target for transgene insertion by *A. tumifaciens* or microparticle bombardment. (B). Plantlets regenerating from somatic embryo-derived cotyledon tissues in cv. Col. 22. (C). Enhanced regeneration of shoots from cotyledon fragments on medium supplemented with silver nitrate. (D). Acyanogenic somatic embryos of cv. Col2215 developing on antibiotic selection medium. (E). GUS expression in a cassava storage root under control of the tissue-specific promoter p54/1.0. (F). GUS expression in the cassava petiole under the control of the tissue-specific promoter p15/1.5. (G). Somatic embryos and leaf from cv. 60444 expression an ER-targeted version of the green florescent protein under control of the enhanced CaMV 35S promoter. (H). Plants of the West African cv. Kataoli regenerated from friable embryogenic callus. (I). Cassava plant transgenic for the AC3 transgene from African cassava mosaic virus, displaying enhanced resistance to the pathogen. (J). Secondary roots of a cassava plant developing on a plant transgenic for modified starch. K. Resistance to challenge with the viruses which cause cassava mosaic disease are assessed in plants genetically transformed with the *AC1* gene from African cassava mosaic virus.

and the Americas have now been shown to produce FEC tissues (Raemakers *et al*., 2001; Taylor *et al*., 2001; Lopez *et al*., 2002). With such a range of diverse germplasm to work with, the availability of several transformation systems should be considered a strength of transgenic programs in cassava, increasing the possibility that in the near future, any given cultivar will be amenable to integration and expression of desired transgenes.

Marker genes

A range of selectable and visual marker genes have been tested and developed for use in cassava transformation systems. The *nptII* gene, which imparts resistance to the amyloglycoside antibiotics, is used routinely by several laboratories, with selection for successful transformation events possible by culturing tissues on medium containing kanamycin, paramomycin or geneticin. Efficacy of the hygromycin resistance gene (*hpt*) has also been demonstrated, but is reported to suppress the frequency of plant recovery compared to transgenic tissues selected by expression of *nptII* (Raemakers *et al*., 2001; Schreuder *et al*., 2001; Hankoua, 2003). Use of the *bar* gene to impart resistance to the herbicide phosphinothricin acetyltransferase is utilized both as a gene of agronomic interest and as a selection system. In order to develop protocols for the recovery of transgenic plants without antibiotic or herbicide resistance genes, use of the *Escherichia coli* phosphomannose isomerase (*pmi*) gene was used to transform FEC and cotyledon explants and procedures for the effective recovery of transgenic tissues and plants using this positive selection system have been developed (Zhang and Pounti-Kaerlas, 2000; Zhang *et al*., 2000a). In addition, all three visual marker systems, GUS, luciferase and GFP (Figure 2G), have been successfully established and are routinely employed as tools for developing transgenic systems in cassava and investigating transgene expression patterns (Li *et al*., 1996; Raemakers *et al*., 1996; Schöpke *et al*., 1996 and Figure 2G).

Application of transgenic technologies in cassava

Since the late 1990s, the technologies outlined above have been utilized to produce genetically transformed cassava expressing traits with agronomic value to cassava producers and consumers. Progress in each of these major research areas is described below.

Reduced cyanogenic content

All cassava tissues accumulate cyanogenic compounds that can release cyanide into the body after ingestion. Biochemical studies have shown that the cyanogenic glycosides linamarin (95%) and lotaustralin (5%) accumulate in the vacuole while enzymes for their degradation are located in the cell wall. When tissues are crushed during processing or mastication these compounds are brought together and hydrolyzed to generate acetone cyanohydrin and glucose. Acetone cyanohydrin is then broken down spontaneously (at pH greater than five or temperatures above 35 °C) or by hydroxynitrile lyase (HNL) to produce acetone and hydrogen cyanide (White *et al*., 1998). Cyanogenic content is under strong genetic control and influenced by environmental conditions such as drought, varying between cultivars from 10 to 500 mg CN equivalents/kg dry weight in the storage roots (O'Brien *et al*., 1991). As even the lower end of this range exceeds FAO recommended levels for food derived cyanide exposure, proper processing prior to consumption is essential if the consumer is not to develop cyanide-induced disorders such as hyperthryroidism and ataxic neuropathy. While it should be kept in mind that cassava has been utilized as a human food for millennia and that the benefits derived from its consumption far outweigh any detrimental effects (Rosling, 1996), there is a desire to develop very low or even acyanogenic cassava. Decreased cyanogenic content in farmer-preferred cultivars would reduce the danger of exposure to cyanide by consumers and have potentially significant impact on commercial-scale cassava production. Processing large quantities of cassava roots in a single location requires time and resources and often generates toxic effluents. Overcoming such problems would improve cassava's economic competitiveness, helping it develop as an industrial crop and provide increased income for cassava farmers. Recent research activities at Ohio State University, USA and KVL University, Denmark, have made significant progress towards the production of acyanogenic cassava.

Two approaches have been taken. In the first, genes encoding a small (CYP79DI and CYP79D2) family of cytochrome P450s that catalyze the first dedicated step in linamarin and lotaustralin synthesis (Anderson *et al*., 2000) were expressed in an antisense orientation in transgenic cassava of cv. Col 2215 (Figure 2D). Transgenes were each independently under the control of the *Arabidopsis* Cab1 leaf specific promoter. Analysis of *in vitro* grown transgenic plants confirmed linamarin content of leaf tissues to be decreased between 34% and 94% compared to controls depending on the transgenic event studied. More strikingly, in all cases linamarin levels in transgenic roots were reduced to only 1% of that recorded for control plants (Siritunga and Sayre, 2003). While establishment of these lines in the greenhouse and analysis of mature leaves and storage roots is required to confirm this data, the results are most promising. Why a leaf-specific promoter should impact accumulation of cyanogenic compounds to a greater degree in roots than in shoot tissues is unknown, but may be due to reduced transport of cyanogens from leaves to the root system (Siritunga and Sayre, this issue). Further studies are underway to confirm this hypothesis.

The second approach capitalized on earlier research from the same laboratory and has also met with success. White *et al*. (1998) reported that levels of HNL in cassava storage roots were only 6% of that detected in leaves of the same plant, and commented that high residual acetone cyanohydrin in roots tissues could be decreased by overexpressing HNL in these tissues, thereby accelerating the detoxification process and protecting consumers. To achieve this, cDNA from a cassava hydroxynitrile lyase gene was cloned between a double CaMV 35S promoter and the pea rubisco terminator sequence and integrated into cv. Col 2215 (Siritunga *et al*., 2004). Analysis of greenhouse grown plants confirmed a 40–135% increase in HCL activity in leaf extracts for transgenic plants compared to controls, and between 800% and 1300% increase of HNL in storage root tissues compared to non-transgenic plants of the same cultivar. While, overall levels of linamarin and linamarase remained unchanged in the transgenic plants, elevated HNL in the storage organs was shown to reduce the amount of residual acetone cyanohydrin in homogenized roots by as much as a factor of three. This indicates that although overall levels of cyanogenic compounds have not been affected in transgenic plants, capacity for endogenously driven detoxification after harvesting has been significantly up-regulated. If confirmed under field conditions this technology offers potential to protect consumers while maintaining levels of cyanogens in the plant during its growth cycle.

Insect resistance

Cassava requires between 9 and 18 months to reach harvest depending on the cultivar, environment and the needs of the farmer. This extended period makes the crop vulnerable to serious infestation with insect pests. Important pests in Africa include mealybugs (*Phenacocuccus manihotii* and *P. herreni*) and cassava green mite (*Monoychellus tanajoa*), while in Latin America the major threat comes from the hornworm (*Erinnyis ello*), whitefly (*Aleurotrachelus socialis*) and stem borers, most especially *Chilomina clarkei* (Bellotti, 2002). The cassava stem borer is a significant and growing problem in Colombia and Venezuela. In northern Colombia, as many as 85% of fields have *C. clarkei* damage, where yield losses can be as high as 60% (Bellotti, 2002). The problem is compounded by the fact that the larval stages feed within the plant stem, infecting and damaging stem cuttings used to establish the next crop cycle. Chemical applications are economically impractical for most farmers and are only partially effective as the insect is protected within the plant tissue. No genetic resistance to the pest has been identified among more than 2000 cassava clones screened to date, indicating that traditional approaches alone are unlikely to provide farmers with resistant planting materials. In such cases transgenic approaches are attractive and have been successfully deployed in developing countries in crops such as cotton and maize (Qaim and Zilberman, 2003).

A program for transgenic control of stem borer and hornworm has been initiated at CIAT, in which *cry* genes are being integrated into economically important Colombian cassava cultivars. To date transgenic plants expressing the *cry1*Ab gene under control of the CaMV 35S promoter have been generated from economically important cultivars CM 3306-4 and SM 1219-9, in addition to the model African cv. 60444. CRY1Ab expression levels in transgenic lines of cv. 60444 have been determined (Ladino *et al*., 2002), and testing

of plants for resistance to stem borer and hornworm infestations is ongoing in the greenhouse. Early results against hornworm are encouraging. In the case of stem borer, it is considered that stronger transgene expression will be necessary in the stem tissues if full protection is to be generated. This may require re-engineering of the constructs with alternative *cry* genes under control of a tissue specific promoter to direct expression of the Bt protein to the stem.

A longer term, but exciting prospect for insect pest control is offered by mapping and genomic projects underway in cassava (other papers in this issue) which may facilitate identification of naturally occurring resistance genes within the cassava genome. A new cassava variety, Nataima-31, with resistance to the whitefly (*Aleurotachelus socialis*) was recently released by CIAT CORPOICA in Colombia (Vargas Bonilla *et al*., 2002). Progress in identifying host resistance to whitefly, which is becoming an increasing threat to production in Colombia (Bellotti and Arias, 2001), provides an example of how future transgenic programs will involve transfer of beneficial traits between resistant and susceptible cassava varieties, bringing the products of genomic research to benefit small farmers in the tropics.

Virus resistance

Cassava mosaic disease

Cassava mosaic disease (CMD) is the single most important disease of cassava. The causal agents are whitefly-transmitted geminiviruses belonging to the family Geminiviridae, genus *Begomovirus*. Eight distinct species of these bipartite, ssDNA nuclear replicating viruses are known to infect the crop in Africa and the Indian sub-continent (Fauquet and Stanley, 2003). Use of infected stem cuttings perpetuates the disease through subsequent cropping cycles, while synergistic interaction between geminivirus species simultaneously infecting the same plant can lead to very severe yield reductions and even crop failure (Pita *et al*., 2001; Calvert and Thresh, 2002). Although not all regions are equally impacted, in sub-Saharan Africa as a whole, CMD is estimated to be responsible for a 30–40% loss in harvested roots, equivalent to as much as 23 t of food annually. Valued conservatively at USD 53 per ton fresh weight (Scott *et al*., 2000), this translates to greater than USD 1 billion worth of lost productivity each year.

Conventional breeding programs at IITA have been successful in producing improved varieties with high level resistance to CMD, with more than 200 reported releases to farmers in the major cassava-growing regions of Africa (Manyong *et al*., 2000; Nweke *et al*., 2002). It is considered, however, that transgenic strategies also have an important role to play in combating CMD by introducing new sources of resistance into cassava germplasm. This can occur through integration of resistance genes directly into virus susceptible farmer-preferred landraces, by stacking transgenically derived resistance into existing conventionally improved varieties, and through genetic transformation of breeding materials. Two research institutes, the Danforth Plant Science Center (DPSC) in the USA and ETH, Switzerland, have been engaged in this effort, with both groups employing pathogen-derived resistance (PDR) strategies.

Studies by Stanley and co-workers in the model species *Nicotiana benthamiana* demonstrated that integration of a defective interfering (DI) sequence derived from a deleted B component of a Kenyan strain of African cassava mosaic virus (ACMV) could impart elevated resistance within transgenic plants when challenged with the homologous virus species (Frischmuth and Stanley, 1991). In an alternative approach, production of *N. benthamiana* transgenic for the *AC1* gene, which encodes the replication associated protein required to ensure replication of both viral genomic components, resulted in plants with significantly enhanced resistance to ACMV (Hong and Stanley, 1996; Sangaré *et al*., 1999). With the development of reliable transformation systems for cassava, these promising strategies were transferred to cassava by workers at ILTAB/DPSC.

Transgenic plants were recovered from the West African cv. 60444 containing integrated copies of a complete dimer of the DI sequence from ACMV-Kenya (Taylor *et al*., 2003). Challenge of this highly CMD susceptible cultivar with ACMV was shown to induce release of DI particles from the integrated dimer sequence via homologous recombination and promote their replication to levels which competed with the wild-type virus B component. While levels of imparted resistance against the pathogen were statistically

enhanced compared to controls, these were not dramatic and did not persist (Taylor *et al*., 2003).

In contrast, efficacy of resistance induced by integration of the *AC1* gene from ACMV-Kenya was found to be significantly more effective. This sequence was cloned between the cassava vein mosaic virus (CsVMV) promoter (Verdaguer *et al*., 1996) and the E9 terminator sequence and inserted into the genome of cv. 60444 by particle bombardment. Transgenic plants were found to have significantly enhanced resistance to CMD when challenged with ACMV, and other heterologous geminiviruses (Chellappan *et al*., this issue). Most importantly, imparted resistance was also confirmed against simultaneous inoculation with more than one geminivirus species, which when acting in synergistic combination often cause very severe disease in farmer's fields in Africa (Pita *et al*., 2001). Such strong cross-protection imparted by the *AC1* transgene from ACMV against different non-homologous geminivirus species was not expected, but has importance for field deployment of this transgenic strategy. Cassava plants in farmers' fields in Africa are exposed infection by diverse combinations of strains and species of CMD-inducing viruses. Any resistance strategy, conventional, transgenic or a combination of both, must therefore provide protection against this varied and evolving challenge. All virus inoculation in the above studies was achieved by particle bombardment of experimental plants with infectious viral clones (Pita *et al*., 2001) and plants maintained under greenhouse conditions (Figure 2K). Working in collaboration with the Kenyan Agricultural Research Institute (KARI), regulatory clearance to test the most resistant of these lines has been obtained from the National Biosafety Committee of that country. *In vitro* plantlets were transported to Western Kenya and established in soil in a biosafety screenhouse. Experiments to test transgenically imparted resistance under pressure from the natural whitefly vector were initiated in April 2004.

Increased ACMV resistance in transgenic cassava has also been developed using antisense-RNA technology at ETH, Zurich. Three constructs were designed for expression of viral antisense-RNAs as 3′-untranslated regions (3′-UTR) of a selectable hygromycin phosphotransferase (*hph*) gene, driven by the CaMV 35S promoter. Targets for antisense-RNA interference were the mRNAs of the *AC1*, *AC2* and *AC3* genes from the A-component of the viral genome, which play key roles in ACMV replication and transcriptional regulation. DNA and RNA blot analyses confirmed integration and stable expression of the three antisense-RNA genes for 25–40 (depending on the construct) independent transgenic plant lines of the CMD-susceptible cultivar 60444. Results from viral accumulation assays in isolated leaves confirmed that replication of two ACMV isolates was strongly reduced or inhibited in transgenic plants. Compared to wild-type plants, delayed symptom developments and attenuated symptoms were observed in transgenic plants after ACMV-N*Og* infection under the greenhouse conditions and was correlated with reduced viral DNA accumulation in infected leaves. The results demonstrate the feasibility of imparting resistance to ACMV (Figure 2I) by expressing viral antisense RNAs targeted against viral mRNAs specifying essential non-structural proteins. Transgenic plants generated via the above strategies are now available for field testing.

In a parallel study at the same institute, an alternative resistance strategy has been tested in which a hypersensitive reaction is mimicked with the bacterial *barnase* and *barstar* genes from *Bacillus amyloliquefaciens* under control of the ACMV DNA-A bidirectional promoter. In non-induced conditions, this promoter has a low basal activity in both directions. Upon infection, the virion-sense (AV) promoter is up-regulated by the ACMV TrAP protein, while the complementary sense (AC) promoter is down-regulated. The gene for the ribonuclease *barnase* was cloned under the control of the AV promoter (*AVp*). Simultaneously, the gene for the *barnase* inhibitor *barstar* is expressed from the AC promoter (*ACp*) to counteract any basal expression of *barnase*. In order to adjust the expression level of *barnase*, constructs with different additional short open reading frames proximal to the *barnase* gene were designed and used for transforming cassava via microparticle bombardment. In 6 out of 60 transgenic plant lines recovered, integration of both *barnase* and *bastar* genes was detected by PCR and Southern analyses. Expression of *barnase* and *barstar* at the RNA level was detected by RT-PCR in five of these lines. Upon viral infection, the ratio of *barnase*/*barstar* would be expected to shift in favor of *barnase* due to the up-regulation of *AVp* by viral protein TrAP, resulting

in local cell death before the virus can spread to adjacent cells. A viral replication assay with leaves of untransformed and transgenic plants showed a reduction of viral replication activity in transgenic leaves by 86–99% (Zhang *et al*., 2003b). These transgenic plant lines have been transferred to the greenhouse where they are currently subjected to virus infection to verify increased resistance to ACMV.

Cassava brown streak disease

In addition to CMD, cultivation of cassava in Africa is also impacted by cassava brown streak disease (CBSD), which affects production mostly in the coastal regions of East Africa (Calvert and Thresh, 2002). Although known since the 1930s, CBSD is causing increasing concern due to its spread via planting of infected cuttings and increasing loss of food and income from necrosis of the storage roots induced by the disease. The natural vector remains unknown at this time, but there is growing evidence that the disease is caused by a virus of the genus *Ipomovirus*, family Potyviridae (Monger *et al*., 2001). To date no resistance has been found among African or American cassava cultivars screened against the virus, generating interest that transgenic strategies, such as coat protein or siRNA-mediated resistance, may be an effective way to secure cassava production in the infected regions. At the time of writing, research programs at IITA, Nigeria, are being initiated to generate germplasm with enhanced resistance to CBSD.

Herbicide resistance

Failure to control weeds in cassava fields can result in yield reductions of up to 50% (Leihner, 2002). Weed removal is required until an effective canopy is established by the new cassava crop and can take place by hand or chemical means, or a combination of both. Herbicide application is common on large cassava farms in Asia and the Americas where it reduces production costs, increases yields and alleviates problems of soil erosion associated with mechanical weeding. Loss of top soil after weed removal can be severe on sloping land with up to 100 t/ha washed into the river systems in one cassava cultivation cycle (Ca-david, 2002). Deployment of herbicide tolerant transgenic soybean, maize and cotton has been remarkably successful in the USA and Argentina (James, 2003) and is considered to hold significant promise for crops such as cassava. For example, economic *post ante* studies of the potential impact of introducing herbicide resistant cassava in Colombia, estimated cost savings for producers of 15–25% and a total benefit to that country's economy of USD 300 million over a 14-year period (Pachico *et al*., 2003). These figures do not include the positive environmental effects which reduced tillage would bring for increased sustainability of the crop on marginal land. As both Basta (ammonium glufosinate) and Roundup (glyphosate) are effective for controlling weeds in cassava fields (Silvestre, 1989), development of the crop for resistance to these herbicides would fit with current production practices.

To date, property right issues have prevented the development of cassava plants transgenic for the EPSP-synthase (*epsps*) gene, which imparts resistance to glyphosate. However, plants of cv. Per 183, expressing the *bar* gene which encodes phosphinothricin acetyltransferase (PPT) and provides resistance to the herbicide Basta, have been regenerated at the CIAT (Sarria *et al*., 1995, 2000). Transgenic plant lines displayed protection up to 200 mg/l of active ingredient when sprayed as mature plants in the greenhouse. While this remains below the levels of 1500 mg/l of active ppt commonly applied by commercial spraying with Basta or Finale, these early transgenic plants provide proof of principle for this strategy in cassava. Levels of resistance to Basta application have apparently declined within plants vegetatively propagated since 1994 by stem cuttings from the primary transgenic regenerants. Southern blot analysis of RT-PCR products still detect mRNA from the *bar* gene driven by the PTR2 promoter after eight years of vegetative propagation, but the plants no longer tolerate spraying with PPT at 200 mg/l (Echeverry *et al*., 2002). In contrast, transgenic tobacco plants produced the same year with the same genetic constructs (Sarria *et al*., 2000), have remained tolerant to spraying with up to 1500 mg/l PPT over successive sexual cycles. The need for stable transgene expression over extended growth cycles highlights a requisite for vegetatively propagated crops such as cassava and is an important issue for successful field deployment. New transgenic cassava plants for tolerance

to PPT application are presently being generated at CIAT from Colombian cultivar Ica-Negrita (CM3306-4).

Despite proven technical feasibility in other crops and the desirable agronomic trait, development of transgenic herbicide-resistant cassava is not a major priority within the cassava research community at this time. Present funding for cassava genetic transformation programs tends to originate either from international aid organizations or multinational starch companies. The former favors development of transgenic cassava targeted towards smallholder farmers; those who cannot afford to purchase the chemicals required to benefit from herbicide resistance strategies, while commercial investment is presently concentrated on transgenic approaches to modifying the quality of cassava starch. There is good probability, however, that a concerted program to produce transgenic herbicide-resistance cassava could develop successful products within a 4–6 year time span.

Manipulation of starch content

Cassava has developed as a crop of major importance due to its ability to store large quantities of starch (74–85% dry weight) within secondary root structures. This is exploited as a source of human food, feed for livestock and as raw material for the food processing and chemical industries. Many improvement programs, both conventional and transgenic are aimed at increasing starch production though alleviating the constraints which limit dry weight yields. In other cases, efforts are being targeted directly at manipulating the starch biosynthetic pathways to enhance both quality and quantity of the harvestable product.

Plant starch consists of two polymers: amylopectine (70–80%) and amylose (20–30%). Amylose is a linear molecule of (1–4)-linked α-D-glucopyranosyl residues while amylopectin is a highly branched molecule in which (1–4)-linked α-D-glucopyranosyl residues are connected by α(1–6) linkages. Branching occurs at regular intervals in such a way that a cluster of side-chains is formed. The length and the distribution of the side-chains in the molecule are under genetic control and are important parameters for the physical–chemical properties of the starch. The ability to manipulate starch quality *in planta*, by transgenic up- or down-regulation of genes within the biosynthetic pathways offers important opportunities for creating new products and increasing the value of cassava starch. The use of antisense genes to reduce starch accumulation and increase the sugar content of cassava storage roots also holds promise for producing a sugary cassava and improving the crop as a source of industrial alcohol. Such potential is recognized by the major multinational starch companies who have invested in transgenic modification of cassava for enhanced starch content over the past decade.

cDNAs encoding ADP-glucose pyrophosphorylase (AGPase), branching enzyme (BEI, BEII), and granule-bound starch synthase (GBSS), involved in the starch biosynthetic pathway, have been cloned from cassava (Munyikwa *et al*., 1998). AGPase converts glucose-1-phosphate to ADP-glucose, the first step in starch formation, while GBSS converts ADP-glucose to amylose. BEI and BEII are involved in the formation of the branched molecules in amylopectin. Researchers at Wageningen University (Netherlands) cloned the *gbss* gene from cassava in the antisense orientation under control of the CaMV 35S promoter and the *gbss* promoter from potato (Raemakers *et al*., 2003). A total of 50 transgenic plants with the *gbss* antisense gene were recovered, two of which were subsequently shown to produce and store amylose-free starch in their thickened, secondary roots (Raemakers *et al*., 2003). With further development, it is hoped that such amylose-free starch will enable the production of transparent pastes with stable viscosity for industrial applications. Such results are representative of work currently underway to modify the quality of cassava starch. The commercial nature of such research, however, precludes further description of this exciting area of cassava genetic modification.

Research at CIAT to produce waxy cassava has been initiated recently with a project funded by the Colombian Ministry of Agriculture to genetically engineer industrial cassava varieties, using an antisense and sense construct of a full-length GBSSI gene. Constructs have been integrated into FEC of the model cv. 60444, via *A. tumefaciens*, as demonstrated by GUS stable expression that revealed a successful incorporation of the gene. Regeneration of the transformed plants is nearing completion and will be tested for waxy phenotype (CIAT, unpublished results).

Workers at Ohio State University have been successful in up-regulating overall starch biosynthesis by expressing a modified version of the *E. coli glgC* gene under control of the tuber-specific patatin gene from potato (Lloyd *et al.*, 1999; Ihemere *et al.*, 2003). *glgC* encodes AGPase which is the rate-limiting step in cassava starch biosynthesis. Seven transgenic cassava plants expressing the transgene were recovered and shown to have a greater than 65% increase in AGPase activity compared to controls. Most importantly, when grown under greenhouse conditions, these plants were shown to generate significantly increased shoot and root biomass and to accumulate almost twice the dry weight starch content as the non-transgenic mother plants. Efforts are now being directed at field trailing these plants to determine if such increased yields can be sustained under field conditions with associated biotic and abiotic stresses.

Elevated protein content for nutritional enhancement

Cassava roots are an excellent source of starch and dietary energy but are very poor in protein (only 1–2% of dry weight). Increasing nutritional protein levels in cassava storage roots could beneficially impact diet and health in many regions, especially Central and West Africa. As a first investigation to determine whether this may be feasible through transgenic technologies, researchers at ETH, Switzerland, genetically transformed plants of cv. 60444 with an artificial storage protein *ASP1* gene, designed to be rich in essential amino acids (Kim *et al.*, 1992). Analysis of regenerated tissues confirmed expression of the transgene at both the RNA and protein levels. Total protein content of *in vitro* leaves were found not to differ from the non-transgenic plants, but levels of the amino acids proline and serine were elevated and asparagine, alanine and methionine depressed compared to controls (Zhang *et al.*, 2003c). Lack of vigor in these transgenic plants, most probably due to culture-induced somaclonal variation, prevented production and analysis of storage roots. However, these data demonstrate that it is possible to manipulate protein content in cassava through a transgenic approach. More recently, new ASP1 transgenic cassava plants have been produced and phenotypically normal plants recovered. Strong expression of the ASP1 protein has been detected in the leaves from several of these lines growing in the greenhouse (ETH, unpublished results).

It should be recognized that developing and deploying transgenic cassava with traits such as enhanced protein or other nutritional qualities presents significant technical and regulatory challenges. The new product should be preferentially accumulated in the storage roots, requiring that the transgene be under control of efficient tissue-specific control elements. It must be non-allergenic, and not washed out or degraded during post-harvest processing. In order to be accepted by consumers, the modified product should not adversely affect root yields, cooking, palatability or storage qualities of the final foodstuffs. Combining good pest and disease resistance with the new nutritional quality trait will also be important to ensure that farmers choose to plant such enhanced germplasm over their traditional varieties.

Future direction and applications of transgenic programs

Future applications

Additional applications for the transgenic improvement of cassava have been suggested and in some cases preliminary investigations initiated. These can be separated into short-, medium-and long-term prospects with respect to possible deployment. Extended leaf retention and resistance to cassava bacterial blight (CBB) disease fall within the first category. Leaf longevity in cassava varies significantly between cultivars and has been correlated with increased yields and improved root quality. Transgenic plants expressing the cytokinin regulation *ipt* gene (Gan and Amasino, 1995) under control of the senescence-controlling promoter have been produced and are currently being analyzed at ETH, Switzerland. If successful this technology would have application for increasing yields for both smallholder and commercial-scale farmers. Significant progress has also been made in mapping and cloning genes imparting resistance to CBB, a sporadic but serious disease, capable of causing complete crop loss and rendering the stems unusable as planting material for subsequent cropping cycles (Verdier *et al.*, this issue). It is predicted that genes from CBB-resistant cultivars will soon be available for transfer to susceptible farmer-preferred germplasm. Similar efforts with

the *Xa21* gene, which imparts resistance to leaf bacterial blight disease in rice, have proved successful (Datta *et al*., 2000).

Medium-term applications include the genetic modification of cassava for improved post-harvest qualities and nutritional enhancement. Cassava roots take 9–18 months to reach maturity depending on the cultivar and can be harvested at any time after this period. This highly flexible harvest window is an attractive feature, allowing farmers to 'store' the crop in the ground until they require the food or until local market prices are favorable. However, after harvesting, cassava roots must be consumed or processed within 24–48 h, or the tissues quickly degenerate becoming unpalatable and unmarketable. Such a short shelf-life restricts the development of cassava as commercial crop, especially in areas with poor transportation infrastructure, making it difficult for large-scale processors to obtain reliable supplies of raw material and for farmers to obtain reliable prices for their product. Extending the storage time of harvested roots would have a major impact on the economics of cassava production, processing and utilization. Conventional breeding has not been successful in generating germplasm with significantly improved storage qualities. However, biochemical dissection of post-harvest deterioration in storage roots and cloning of genes involved in these pathways is providing important new information as to the underlying processes (Cortez *et al*., 2002; Reilly *et al*., this issue). It is predicted that knowledge generated from these studies will form the basis of transgenic strategies to counteract the rapid degeneration of cassava roots, which presently restricts full development of the crop.

Efforts to enhance the storage protein content of cassava roots are described above. In addition to elevated protein levels, there is interest in improving the levels of iron, folate, vitamin A and other micronutrients such as zinc in staple food crops like cassava (Dellapenna, 1999; Fregene and Pounti-Karleas, 2002). While technical capacity to achieve these aims is already available or being developed, the challenges faced for deployment of germplasm modified in this manner would be similar as those for enhanced protein content.

Longer-term goals for transgenic improvement of cassava include apomixis and bioplastic production, modified plant architecture and more rapid bulking of the storage roots. Introduction of traits for apomixis would revolutionize the crop, allowing disease-free planting materials to be produced and facilitating the rapid bulking and dispersal of elite clones. Exploiting the large capacity inherent to cassava for producing and storing polymers makes the crop an attractive target for molecular farming technologies. For example, if starch accumulation can be eliminated by the use of antisense sequences or gene silencing technologies, it may be possible to genetically engineer cassava to synthesis and accumulate high-value bioplastic polymers, such as poly-hydroxybutyrate, within its storage roots.

Future technical developments

In order to achieve many of the above goals, genetic transformation technologies for cassava must continue to be improved. It is recognized that progress in the era of post-genomics is resulting in identification and cloning of genes of agronomic interest from model species such as *A. thaliana* and from cassava itself. Progress towards the latter at CIAT, Colombia, will most likely yield sequences of interest from *M. esculenta* for resistance to CBB (Lopez *et al*., 2003; Verdier, this issue), CMD (Akano *et al*., 2002) and in the longer term to whitefly (Bellotti and Arias, 2001) and post-harvest deterioration (Reilly *et al*., this issue). By screening for homology, it is also hoped to access genes coding for beneficial traits from cassava's wild relatives. The challenge will be to integrate these into the most relevant cassava farmer-preferred germplasm and elite breeding lines. In many cases the desire will be to transfer large segments of DNA such as BAC clones and cosmids or, in the case of metabolic engineering, to simultaneously integrate several genes into the cassava genome and express these in a coordinated manner. While such capacities are established in the other major crops, further investment is required to develop transgenic systems in cassava to facilitate these aims.

Production of genetically transformed crop plants without marker genes is desirable but remains technically demanding. Possibilities of segregating out selectable markers exist if the initial transformation events will pass through sexual cycles. However, in vegetatively propagated crops such as cassava this is not always available, especially if the desire is to preserve the genetic background of the target germplasm, as in the

case of transgenic improvement of existing farmer-preferred cultivars. Recent advances for removal of marker transgenes such as the Cre-lox system are promising (Hare and Chua, 2002), as are recent reports describing recovery of transgenic plants by mating high-throughput PCR to screen for regeneration of transgenic plantlets from systems without chemical selection (Vetten *et al.*, 2003). In all cases, however, highly efficient *in vitro* plant regeneration systems are required to exploit the above technologies. At this time, morphogenic capacity of the four transformation systems for cassava illustrated in Figure 1 remain below that which would allow recovery of practical numbers of transgenic plants, if they were to be coupled with the available marker-free systems. This is especially true for cultivars outside the established model genotypes, 60444, Col. 2215 and Col. 22, most often used in cassava transformation studies. Progress towards genetically transformed, marker free cassava plants will be made as transgenic technologies for the crop are improved. However, it is considered that use of selectable markers will remain a requirement both for experimental purposes and field deployment for the near future.

Control of transgene expression

Description of the major traits being addressed by transgenic modification illustrate some of the technical challenges facing cassava biotechnologists. Transgenically derived products must be expressed and directed to the relevant tissues at levels that impart agronomically useful traits. As we have seen, this may be to stem tissues for *Bt* protection against stem borers, to leaf tissues for herbicide tolerance and resistance to CMD and to the storage roots for starch modification and improved post-harvest characteristics. As a heterozygous, vegetatively propagated crop, cassava brings additional considerations. Levels of transgene expression must be maintained over many successive vegetative cycles under varying conditions of biotic and abiotic stress encountered in farmers' fields in the tropics. Achieving homozygosity for the transgene is problematic due to inbreeding depression, and in the case of established farmer-preferred cultivars, a desire to maintain the existing genetic background by avoiding sexual crossing. Proposals by the Rockefeller Foundation and CIAT to develop technologies for the production of double haploids in cassava could have an important impact in this area. If successful, such systems will circumvent problems associated with sexual recombination and facilitate the production of cassava homozygous for desired transgenic (and non-transgenic) traits.

Access to tissue-specific and inducible promoters, free from restrictive property right issues, is required for the successful development and deployment of transgenic cassava. To date, a variety of promoter sequences have been employed to drive transgene expression in the crop. Constitutive promoters including CVMV 35S and the CsVMV have proven effective, as have tissue specific promoters derived from other plant species such as the *Arabidopsis cab1* promoter (Siritunga and Sayre, 2003), and patatin from potato (Siritunga *et al.*, 2004). The CsVMV promoter was cloned from the cassava vein mosaic virus and developed as an alternative to 35S for use in biotechnology programs for developing countries (Verdaguer *et al.*, 1996). As a full-length (526 bp) version, it drives strong constitutive expression (Taylor *et al.*, 2001), while root and phloem specific versions have also been generated by deletion of control elements within its sequence (Verdaguer *et al.*, 1998).

Progress has also been made in the isolating and characterization of endogenous promoter sequences from cassava itself. In a recent report, two cDNAs (c15 and c54) were identified from a storage root cDNA library, the transcripts of which were detected in roots but not leaves. The deduced amino acid sequences indicated homology to cytochrome P450 and a glutamate-rich protein respectively (Zhang *et al.*, 2003). Promoter sequences were isolated from the corresponding clones within a cassava genomic library, and 1465 bp (for c15) and 1081 bp (for c54) fragments fused to the *uidA* visual reporter gene. Production and analysis of transgenic cassava plants expressing the GUS protein under control of these promoters showed both to drive transgene expression most strongly in phloem, cambium and xylem vessels of vascular tissues from leaves, stems and root systems, including the starch-storing xylem parenchyma cells within secondary roots (Figure 2E and F) (Zhang *et al.*, 2003a). In a similar study, a cassava

PAL promoter was identified and isolated after screening a library generated from deteriorating storage roots. GUS expression driven by a 840 bp fragment in transgenic cassava plants revealed localized expression within the xylem parenchyma of mature stem and root tissues and in the phellogen (Taylor *et al*., 2003). Availability of these, and addition control elements developed through similar research programs, are providing important new tools for improving transgene expression and producing cassava with beneficial traits.

Progress and challenges for deployment of transgenic cassava

The ultimate aim of cassava transgenic programs is to generate improved germplasm for deployment to farmers in the tropics. While integration and expression of transgenes in cassava is now routine and is generating important new knowledge, it is the agronomic potential of traits such as increased resistance to pests and disease and enhanced starch which motivates commercial and public funding for these projects. Researchers are being forced, therefore, to address not only the required plant science and biotechnologies, but also matters of intellectual property and the regulatory processes involved in environmental and food safety. Description of the on-going research programs above illustrates that while some projects are still within the phase of proof of principle (for example, insect resistance and protein enhancement) others at a stage where the challenges of field trails and development for commercial deployment have become a reality. Important milestones will be reached in 2004 with the first field trails of transgenic cassava plants in Africa and South America. If successful, the relevant strategy will have be to transferred to farmer-preferred and/or elite breeding lines and taken through the regulations required for release. The latter is especially problematic, as few countries in the tropical regions have the required legislation in place or the capacity to perform the necessary testing. With deregulation presently costing several million dollars per transgenic integration event, the issues of how this will be financed are not trivial. A journal dedicated to plant molecular biology is not the place to examine these issues in depth. However, it is hoped that the reader appreciates that while good laboratory science underpins agricultural biotechnology, it will not alone improve the well-being of farmers in developing countries. Indeed, it represents only the first, and perhaps most straightforward, step towards this goal.

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