Chapter 10
Cassava Biotechnology

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Introduction

Cassava ranks second to sugarcane and is better than both maize and sorghum as an efficient producer of carbohydrate under optimal growing conditions. It is the most efficient producer under suboptimal conditions of uncertain rainfall, infertile soils and limited inputs encountered in the tropics (Loomis and Gerakis, 1975; El-Sharkawy, 1990). This makes cassava an attractive source of food, feed and renewable industrial raw material in under-developed regions of the world. However, biological constraints of a long growth cycle (8–24 months), vegetative propagation and perishability of the bulky roots lessen the crop's potential as an engine of rural development. Securing sufficient clean planting material for the production of a healthy crop can be an ordeal for many small farmers, and the relatively low inputs required for cassava production contrasts sharply with the high inputs/risks involved in processing, transporting and marketing the highly perishable roots (Fresco, 1993).

Biotechnology can contribute to the solution of these problems and realize great benefits for cassava farmers. Since the late 1970s, CIAT has worked and engaged in partnerships with Advanced Research Institutes (ARIs) in Europe and the USA to bring biotechnology to bear on the problems of cassava production, especially those that cannot be dealt with effectively through conventional methods. The Cassava Biotechnology Network (CBN), funded by the Netherlands Development Assistance (NEDA), is an example of an interdisciplinary forum of cassava researchers, farmers, end users and scientists in ARIs, hosted by CIAT with an agenda to find solutions to the problems encountered in the crop (Thro and Fregene, 1999). Cassava biotechnology falls into the following broad areas: molecular genetic markers for germplasm assessment, gene cloning and cassava breeding, genetic engineering for root quality, pest and disease resistance, and tissue culture for rapid multiplication of healthy planting material and cryopreservation. This chapter considers recent advances in molecular genetic markers and genetic engineering.

Molecular markers

Genetic markers of cassava

Genetic markers have become fundamental tools for understanding the inheritance and diversity of natural variation. They provided Gregor Mendel, the father of modern-day genetics, the tools for his ground-breaking experiment on heredity and more recently markers have made possible the construction of genetic maps, the cloning of genes known only by their phenotypes and position on a genetic map, and whole genome

 Cassava: Biology, Production and Utilization
(eds R.J. Hillocks, J.M. Thresh and A.C. Bellotti)

sequencing. The earliest genetic markers in cassava were morphological. Graner (1942) described the inheritance of two markers, leaf shape morphology and root colour, more recently, eight morphological markers, located on the stem, leaves and roots have been described (Hershey and Ocampo, 1989). These markers are governed by alleles with a major phenotypic effect with little or no environmental effects. The second generation of markers were biochemical, such as isozymes, and they provided a useful tool for genetic 'fingerprinting' and the study of genetic diversity in cassava (Hussain *et al.*, 1987; Ramirez *et al.*, 1987; Ocampo *et al.*, 1992; Lefevre and Charrier, 1993a). Isozymes have been applied to characterizing relationships among accessions of African cassava germplasm (Lefevre and Charrier, 1993b; Wanyera *et al.*, 1994) and fingerprinting of the international cassava collection held at CIAT (Ocampo *et al.*, 1992). The alpha beta esterase system was found to be most informative, providing 22 alleles, which have complemented morphological descriptors for the identification of duplicates in the collection at CIAT (Ocampo *et al.*, 1995).

With the discovery of the molecular basis of natural variation, molecular or DNA markers have rapidly gained importance in the study of genes, genomes and genetic diversity. They represent a limitless source of neutral markers for the quantitative assessment of genetic diversity and 'signposts' in gene and genome mapping. Their abundance in any organism facilitates the resolution of genetic relationships and genome/gene mapping unknown until the coming of molecular markers. They represent differences in the nucleotide sequences of either nuclear or organellar genomes and can be uncovered using diverse methods based upon PCR (Mullis, 1990), DNA–DNA hybridizations (Botstein *et al.*, 1980; Fodor *et al.*, 1993), or both. The most prominent molecular marker systems include minisatellites (Jeffreys *et al.*, 1985), restriction fragment length polymorphisms (RFLPs; Botstein *et al.*, 1980) and randomly amplified polymorphic DNAs (RAPDs; Williams *et al.*, 1990). Others are microsatellites, also known as simple sequence repeat markers (SSRs; Litt and Lutty, 1989a,b), amplified fragment length polymorphisms (AFLPs; Vos *et al.*, 1995) and DNA sequencing of the internal transcribed spacer (ITS) of ribosomal DNA (Baldwin, 1992) and of single-copy nuclear genes. More recently DNA chips or oligonucleotide arrays have been added to an ever growing list of molecular markers (Fodor *et al.*, 1993). All these marker systems, other than DNA chips, have been used in cassava to assess genetic diversity, genome mapping and gene tagging.

Molecular marker assessment of genetic diversity

The genetic resources of cassava and its wild relatives represent a critical resource for the future of the crop. It is therefore understandable that germplasm collections and the study of genetic relationships between accessions have been made using virtually every available molecular marker. Minisatellite markers, fingerprints of highly variable tandemly repeated arrays of nuclear DNA (Jeffreys *et al.*, 1985), were the first markers used to study relationships among cassava accessions and their wild relatives (Bertram, 1993; Ocampo *et al.*, 1995). The 'fingerprints' were obtained by Southern hybridization of M13 phage DNA sequences to cassava genomic DNA digested with a panel of restriction enzymes (Rogstad *et al.*, 1988). They were used to derive relationships amongst cassava accessions and *Manihot* spp. from Central America and to identify 29 possible duplicate accessions in a subset of the international cassava collection held at CIAT. RFLP analysis of chloroplast DNA and nuclear ribosomal (rDNA) sequences, using heterologous probes, were also applied to assessing phylogenetic relationships among *Manihot* species from South and Central America and cassava (Bertram, 1993). The evidence from molecular analysis contradicted the widely held view, based on morphological characters, of an origin of cassava in a Meso-American *Manihot* species, *Manihot aesculifolia* and suggested a possible domestication from some close wild relatives from Brazil, including *Manihot tristis* and *Manihot esculenta* sub-spp. *flabellifolia* (Chapter 1). AFLP markers were employed to obtain a quantitative assessment of genetic relationships in a representative sample of the crop's diversity and six wild taxa (Roa *et al.*, 1997). The study again demonstrated that

the Brazilian *Manihot* species *Manihot esculenta* sub-spp. *flabellifolia*, *M. tristis* and *Manihot peruviana*, are more similar to cassava than its Mexican relative *M. aesculifolia*, and that cassava might have its origin in these close relatives (Roa *et al*., 1997). Conclusive evidence on the origins of cassava came from a phylogeographic study based on sequencing the single-copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*; Olsen and Schaal, 1999). They demonstrated that cassava originated from natural populations of *M. esculenta* sub-spp. *flabellifolia* from the southern border of the Amazon basin in Brazil. Markers have also been used to obtain a quantitative assessment of genetic similarity in cassava (Beeching *et al*., 1993; Second *et al*., 1997; Elias *et al*., 2000), and to study the genetic structure of germplasm resistant to disease (Sanchez *et al*., 1999; Fregene *et al*., 2000). Other studies have sought to determine the genetic structure and the basis of genetic differentiation of cassava landraces in Africa (Mkumbira *et al*., 2002; Fregene *et al*., unpublished).

Beeching *et al*. (1993) employed cloned cassava genes coding for enzymes involved in cyanogenesis as RFLP markers to obtain genetic similarity estimates in a small collection of cassava and some wild relatives. They were able to identify possible duplicates, confirm the intermediate position of interspecific hybrids between individuals of the parent material and postulated undocumented interspecific crossing with *Manihot glaziovii*, to explain the distant position of certain cassava accessions in respect of the majority of samples analysed. Evidence of introgression into cassava from *M. glaziovii* was also observed in an AFLP evaluation of genetic diversity in a large collection of cassava from the South American centre of diversity (Second *et al*., 1997). Bonierbale *et al*. (1994) reported a comparison among elite cassava germplasm held at CIAT, adapted to five edaphoclimatic production conditions. They found that germplasm from certain edaphoclimatic zones (ECZ) showed a broader genetic base than others, but the accessions could not generally be assigned to a particular ECZ pool based on molecular patterns, due to considerable overlap of allele frequencies. Other approaches to the assessment of genetic diversity involved analysis of the structure of genotypes resistant to disease. Multiple correspondence analysis (MCA) of AFLP data, using two primer combinations, of cassava genotypes resistant and susceptible to two strains of *Xanthomonas axonopodis*, permitted an elucidation of the genetic structure of cassava germplasm resistant to cassava bacterial blight (CBB; Sanchez *et al*., 1999a,b). Results revealed a random distribution of resistance/susceptibility, suggesting that resistance to CBB has arisen independently many times in cassava germplasm. In contrast, AFLP assessment of 29 landraces and improved varieties from Africa resistant and susceptible to the sometimes devastating cassava mosaic disease (CMD) revealed a non-random distribution of resistant/susceptible varieties (Fregene *et al*., 2000). African landraces resistant to CMD were also found to be genetically differentiated from resistant elite lines and from susceptible landraces, suggesting two different sources of CMD resistance and that the landrace source may have arisen recently as a single mutational event.

In other studies of cassava landraces, the genetic variability of 31 varieties of cassava traditionally grown by Makushi Amerindians from Guyana, and a representative sample of 38 varieties from an *ex situ* world collection held at CIAT, Cali, Colombia, was assessed by AFLP markers (Elias *et al*., 2000). Twenty-one varieties presented intravarietal polymorphism, suggesting a variety could be made up of more than one genotype. The amount of diversity found in the cassava cultivars from a single site in Guyana was equal to that in the group representative of the CIAT collection and non-correspondence was found between the structure of molecular diversity and variation observed for agronomic traits that are targets for selection by cultivators. In contrast, genetic diversity was found to be structured according to taste, bitter as against sweet varieties in a study of cassava varieties from Northern Malawi (Mkumbira *et al*., 2002). In another study of genetic relationships in 96 landraces collected from ten villages in southern Tanzania 68 SSR markers were employed in a principal component analysis to reveal genetic differentiation amongst the landraces not based on taste or location (Fregene *et al*., unpublished). Although the basis of clustering is unclear, it is thought to represent different introduction events. Like maize, cassava appears to have highly differentiated gene pools and a large

percentage of dominant/recessive gene action loci, which are two key characteristics required for heterosis. Once the wealth of data has been analysed and crosses between clusters tested, hopefully molecular markers can be used to predict heterosis. SSR markers have also been used to identify duplicates in the CIAT core collection (Chavarriaga-Aguirre *et al.*, 1999) and to analyse germplasm from the littoral and Amazonian regions of Brazil (Mueller *et al.*, unpublished).

Genome mapping in cassava

Markers have also been used to generate a molecular genetic map of cassava. The map was constructed from the segregation of RFLP, SSR, RAPD and isozyme markers in an intraspecific cross between TMS 30572, an improved line from IITA, Ibadan, Nigeria, and CM2177–2, an elite line from CIAT, Cali, Colombia (Fregene *et al.*, 1997). The F_1 mapping progeny consists of 150 individuals. The RFLP markers were derived from several genomic and cDNA libraries and cloned genes of known functions, while the SSR markers were generated from one small-fragment genomic library (Chavarriaga-Aguirre *et al.*, 1998). A total of 150 RFLP, 30 RAPD, five microsatellite and three isoenzyme loci segregating as single-dose restriction fragments (SDFs; Wu *et al.*, 1992) in the gametes of the female parent, define 20 linkage groups and span 950 cM with an average marker density of one per 6 cM. From another 120 RFLP, 50 RAPD, four microsatellite and one isoenzyme single-dose markers in the gametes of the male parent, 24 linkage groups were drawn with a total distance of 1220 cM and average marker density of one marker every 8 cM. Thirty RFLP and two SSR markers detected a unique segregating fragment in each parent and a common allele in both parents and were mapped to similar positions on the male/female-derived linkage group. Such allelic bridges (Ritter *et al.*, 1991) are crucial for identifying the analogous linkage groups in the male- and female-derived maps, as they detect the same locus on both parental chromosomes, except when they represent dupicated sequences. Comparison of intervals in the male- and female-derived maps, bounded by markers heterozygous in the allelic bridges of both parents (Ritter *et al.*, 1991), revealed significantly less meiotic recombination in the gametes of the female compared to the male parent.

Cassava is thought to be an allopolyploid (Magoon *et al.*, 1969) and a segmental allopolyploid (Umanah and Hartmann, 1973). Genetic mapping of its genome is expected to provide conclusive evidence of this. In well-known allopolyploids such as maize, wheat and cotton, blocks of duplicated loci have been clearly identified by RFLP mapping (Helentjaris *et al.*, 1988; Devos *et al.*, 1993; Reinisch *et al.*, 1994). Results from the genetic mapping of the cassava genome revealed only a few randomly distributed duplicated loci, less than 5% of the total number of markers, a number corresponding roughly to that reported in many diploids (Causse *et al.*, 1994; Tanksley *et al.*, 1995). Moreover, more than 90% of the markers detected only one locus compared to more than 50% in maize and cotton (Helentjaris *et al.*, 1988; Reinisch *et al.*, 1994). A similar low percentage of duplicated loci was found during RFLP mapping of *Hevea*, a member of the family Euphorbiaceae which has the same number of chromosomes as cassava ($2n = 36$; Lespinasse *et al.*, 2000). Evidence of the allopolyploid origin of cassava ($2n = 36$) is cytogenetic and relies heavily on the identification of two sets of dissimilar nucleolar-organizing regions, on the repetition of chromosome types (Magoon *et al.*, 1969; Umanah and Hartman, 1973), and on the basic chromosome numbers of other genera in the Euphorbiaceae, which range from six to 11 (Perry, 1943). No evidence of tetrasomic inheritance or of wild *Manihot* relatives with chromosome numbers of $2n = 18$ has been found to support the allopolyploid theory in cassava. The karyology of the 18 haploid chromosomes of cassava reveals six identical pairs and three different pairs of homologous chromosomes (Magoon *et al.*, 1969). Assuming random assortment between homologous chromosomes of the six pairs of identical chromosomes, a reduction of 67% would be expected for all markers linked in repulsion (17% as against 50%). The percentage of markers linked in the repulsion phase in the genetic map of cassava reported here, 30%, is significantly higher than this although lower than the 50% expected for full diploids. This suggests that a small amount of random pairing may occur. Consequently, a purely disomic mode of inheritance has been

concluded for cassava, although it is not currently clear if this represents the vestiges of an ancient allopolyploid or a true diploid.

Together the female- and male-derived maps have more than 300 markers and are estimated to cover 80% of the cassava genome. The cassava map therefore requires completion. However, the majority of markers on the map are RFLP markers and do not lend themselves easily to large scale, high-throughput marker-assisted analysis of plant populations, the principal application of the map. In an attempt to make marker technology more widely applicable in cassava, work began on a second generation map made up of highly polymorphic PCR-based markers, such as SSRs and sequence-tagged sites (STSs). Two SSR-enriched genomic DNA libraries were constructed and about 6000 clones were screened for the presence of the following SSR motifs: TC, GT, CAA, CAG, ACG, AAT, and CAGA and GATA (Mba *et al.*, 2000). A cDNA library constructed from leaf and root mRNA isolated from the elite cassava clone TMS 30572 was also screened for the SSR motifs mentioned above. More than 87,000 clones were screened. A total of 322 SSR markers were obtained from the enriched libraries, of which 92 have currently been mapped to the existing map of cassava (Mba *et al.*, 2000; Fregene *et al.*, unpublished data). Another 200 SSR markers were obtained from the cDNA libraries of which 10 have been mapped (R.E.C. Mba, unpublished data). The level of polymorphism of the SSR markers derived from the cDNA library in the parents of the cassava map population was 40%, considerably less than that found in the enriched genomic DNA libraries (60%).

The genetic map of cassava comprises of 250 RFLP markers on the male- and female-derived maps. It is an important resource that can be converted into PCR-based co-dominant markers known as STSs. The RFLP probes used in generating the markers have therefore been sequenced and primers designed to amplify them. The primers will be used in PCR reaction and gel electrophoresis without or with restriction enzyme digestion (cleaved amplified polymorphisms; CAPs) to discover polymorphisms which can serve as markers. This will ensure the rapid completion of a PCR-based map of cassava.

Another initiative toward completing the cassava map is the generation of expressed sequence tags (ESTs). Isolation of genes that are differentially expressed in the parents of a mapping population, has been proposed as a way of developing ESTs around specific traits for the candidate locus approach to increase the accuracy of mapping quantitative traits (Boventius and Weller, 1994; Suarez *et al.*, 2000). The cDNA/AFLP technique (Bachem *et al.*, 1996) was applied to mRNA from the parents of the cassava genetic map population and more than 500 transcript-derived fragments (TDFs) were obtained that were unique in either parent (Suarez *et al.*, 2000). A subset of 50 TDFs were cloned and sequenced. Sequence alignment of the expressed sequence tags (ESTs) revealed mostly genes of unknown function. Six of the TDFs have been mapped as RFLP markers to the existing molecular genetic map of cassava; the TDFs as RFLP markers were more polymorphic than random cDNAs. A number of cloned genes of known function have also been included on the molecular genetic map of cassava. They include two cytochrome P-450 genes that convert the amino acids L-valine and L-isoleucine during the biosynthesis of cyanogenic glucosides linamarin and lotaustralin in cassava (Andersen *et al.*, 2000), the AGPase phosphorylase, and the granule-bound starch synthase (GBSSII) gene involved in the biosynthesis of starch (Munyikwa *et al.*, 1997).

Genetic mapping of genes controlling agronomic traits

Molecular genetic maps provide a set of 'landmarks' for the complete genome and consequently a high probability of detecting linkage with any gene(s) of interest in genetics or breeding. Practical applications have been seen for many crops in 'tagging' genes of agronomic interest, usually as a more efficient selection parameter in breeding schemes, and for dissecting quantitative genetic variation into simpler Mendelian components (Tanksley *et al.*, 1989; Lee, 1995; Mohan *et al.*, 1997). One of the primary objectives of genetic mapping and gene tagging efforts in cassava is to provide tools that can increase the cost-effectiveness and efficiency of cassava breeding. Desirable characters that

are difficult to evaluate using traditional methods are logical targets for 'molecular breeding' of cassava. These include resistance to pests and pathogens (especially those that are subject to quarantine exclusion), traits expressed only at the end of the crop's growing cycle, and traits for which the phenotype is difficult to measure. Prerequisites for the molecular mapping of agronomic traits generally include importance of the trait in question, the difficulty of screening by direct methods and a large variable population with a simple pedigree. Other requirements include a source of genome-wide markers and a reliable phenotypic screening method. The population selected for the construction of a genetic map of cassava was designed to segregate for traits regarded as priorities for the development of molecular markers and marker-assisted selection (MAS; Fregene *et al.*, 1997). They include resistance to CMD, bacterial blight, early bulking and root quality characters such as cyanogenesis, postharvest deterioration, culinary quality and starch content.

Cassava mosaic disease (CMD)

CMD is the most important disease of cassava in Africa (Chapter 12), and a potential threat to the crop in Latin America where the disease is still not known, although the whitefly vector has recently been found on cassava. Host plant resistance is the principal method of control, and was first identified in third backcross derivatives of an interspecific cross between cassava and *M. glaziovii*. Resistance is thought to be polygenic with a recessive component. The female parent TMS 30572 of the cassava map population has this source of resistance. Recently, several Nigerian cassava landraces have been identified that show strong resistance to CMD. Several mapping populations were developed that segregate for the old and new landrace sources of resistance. They include a half-sib backcross population, derived from crossing five F_1 progeny of the mapping population to the CMD-resistant parent and F_1 of the resistant landraces to susceptible varieties. The crosses were evaluated at two sites with high disease pressure over 2 years in Nigeria. Classical genetic analysis confirmed the polygenic nature of the *M. glaziovii* source of resistance and a major dominant gene control for the new resistance source (Akano *et al.*, 2002).

A bulk segregant analysis (BSA) approach was used to quickly identify markers linked to both sources of resistance. An SSR marker, SSRY40, on linkage group D of the genetic map of cassava, was found to be associated with CMD resistance and explains 48% of the phenotypic variance of CMD resistance ($P < 0.001$). This gene(s) has been designated *CMD1*. An SSR marker, SSRY28, and a RFLP marker, GY1, located on linkage group R explain 68% and 70%, respectively, of phenotypic variance of the new source of resistance (Akano *et al.*, 2002). The dominant CMD resistance gene has been designated *CMD2* and it is flanked by SSRY28 and GY1 at 9 and 8 cM, respectively. *CMD2* and markers associated to it are particularly valuable tools for breeding resistance to CMD in Latin America where the absence of the disease makes it impossible to select for resistance to CMD. In Africa, where a rapid deployment of strong resistance into cassava gene pools is required to protect cassava from CMD, selecting for the high level of resistance with a marker maybe more efficient compared to conventional breeding. The advantage of MAS is that it enables the breeder to eliminate at an early stage CMD-susceptible genotypes, which in the case of the heterozygous CMD-resistant landraces, is 50%. This halves the cost of disease evaluation and increases selection efficiency. The elimination of inferior genotypes at an early stage increases the efficiency of selection by allowing the breeder to concentrate on fewer genotypes at the seedling and crucial single-row trial stages, where progenies are reduced by up to 95%.

Cassava bacterial blight (CBB)

CBB, caused by *X. axonopodis* pv. *manihotis* (*Xam*), is a major disease of cassava in Africa and South America. Resistance to CBB was evaluated in individuals of the F_1 cross by controlled greenhouse inoculations and symptoms were assessed visually at 7, 15 and 30 days after inoculation, using a scale where 0 = no disease and 5 = maximum susceptibility (Jorge *et al.*, 2000). Five *Xam* strains, CIO-84, CIO-1, CIO-136, CIO-295 and ORST X-27 were used. Area under the disease progress curve (AUDPC) was used as a quantitative measure of resistance in quantitative trait loci (QTL) analysis by single-marker regression. Based on the AUDPC values, 12

QTLs, located on linkage groups B, C, D, G, L, N and X of the female-derived framework map, were found to explain 9–27% of the phenotypic variance of response to the five *Xam* strains. A scheme to confirm the usefulness of these markers in evaluating segregating populations for resistance to CBB has also been proposed (Jorge *et al.*, 2000).

Early bulking

Early bulking is another trait evaluated in the F_1 mapping progeny. A preliminary assessment of early bulking was conducted in 1998 by harvesting the F_1 mapping population at 7 months after planting (MAP) in the CIAT Palmira site (Fregene *et al.*, 2001). Dry matter yield was determined on three plants per genotype. Based on results from this evaluation, 40 early-bulking genotypes and 40 late-bulking groups were selected. Carefully picked healthy cuttings of the 80 selected genotypes were planted in a new experiment in December 1998 at Palmira. An early-bulking cassava landrace (*Mandioca de tres meses*) introduced from Brazil was used as control. Nine harvests were done within a 7-month period after which the experiment was terminated (July 1999). At each harvest, four plants in a row within a plot, per genotype, were evaluated for root yield and other traits assumed related to bulking. The traits evaluated were plant height, plant vigour, leaf area index (LAI), fresh root yield, fresh foliage, number of roots per plant, root diameter of the biggest five storage roots. Others were harvest index (HI), measured as the ratio of root yield to total harvested biomass, root dry matter and dry foliage. Multiple regression analyses of the evaluated traits (independent variable) and dry matter root yield (dependent variable) revealed that early bulking is affected mostly by HI and dry foliage. QTL analysis was done for traits significantly linked to early bulking using QGENE (Nelson, 1997) and markers linked to the traits associated with early bulking were significant at $P < 0.005$. Three QTLs each were found for dry foliage weight that explain 25–33% of phenotypic variance while five QTLs were found for harvest index that explain 18–27% of phenotypic variance (Okogbenin, unpublished). Kawano *et al.* (1998) showed that selection of HI in breeding scheme is an efficient indirect selection parameter for root yield. Based on the marker analysis of the early-bulking study, it is apparent that early bulking (and by extension yield), can be increased more effectively by using a selection criteria based on markers for foliage (or total plant biomass) and HI.

Other traits

Several other gene-tagging projects ongoing at CIAT include resistance to the cassava whitefly (*Aleurotrachelus socialis*), CBB, using different crosses, the cassava root rot (*Phytophthora* spp.) and root qualities such as postharvest deterioration (PHD), starch quality and culinary quality.

Cloning of genes of agronomic interest

The heterozygous nature of cassava implies that attempts to introduce any trait, even when it is controlled by a single gene, may lead to the loss of a favoured variety. A more efficient way to introduce traits controlled by a single gene, such as CMD- resistance, is through genetic engineering. However, it is necessary first to clone the genes controlling the trait of interest. There are several approaches to cloning known only by its phenotype, or by its biochemical role in a biosynthetic pathway. The first is that of positional cloning (Martin *et al.*, 1993; Tanksley *et al.*, 1995) and cloning of genes via heterologous genes (Bothwell *et al.*, 1990). Three important criteria for positional cloning are a fine map based on a large mapping population of the appropriate genome region, a bacterial artificial chromosome (BAC) library and an efficient transformation protocol for complementation analysis.

A BAC library has been constructed for cassava, for positional cloning of genes identified during genetic mapping of traits of agronomic interest (Fregene *et al.*, 2000, 2001). DNA was isolated from the cassava variety TMS 30001 and embedded in agarose plugs as described by Zhang *et al.* (1995). TMS 30001, developed at the International Institute for Tropical Agriculture (IITA), shows strong resistance to CMD and resistance to some strains of CBB. Large genomic DNA, in one-third of an agarose plug, was partially digested with *Hin*d III, 1.5 units (U) for 20 min at 37°C, and DNA fragments of

100–300 kb, size-selected by pulse field gel electrophoresis (CHEF MAPPER, Bio-Rad Corp.). Size-selected DNA was ligated into the Hind III cloning site of pBeloBAC11 in a vector:insert ratio of 10 : 1, using 14 U of ligase, in a final volume of 100 µl. Twenty microlitres of DH10B competent cells (GIBCO BRL) were transformed, with 2 µl of the ligation reaction, by electroporation, and white colonies picked for DNA insert sizing. Colonies were grown for 14 h in LB+ 30 mg ml^{-1} Chloramphenicol and plasmid DNA isolated by the Autogen automatic plasmid isolation robot (Kurabo Inc.). Plasmid DNA was digested with 10 U of *Not* I to liberate inserts and separated on a 1% agarose gel by pulse field gel electrophoresis. The rest of the ligation was transformed, plated out and 55,000 clones with average size of 80,000 base pairs picked with the Q-bot robot (Genetix PLC). The library has a 5× coverage of the cassava genome.

Discovery of genetic markers linked to the gene controlling the new landrace source of resistance to CMD and construction of a BAC library, facilitates positional cloning of the CMD resistance gene. The nearest marker to the dominant CMD resistance gene is 8 cM and it is not appropriate for the construction of an array of overlapping BAC clones (contigs) across the genome intervals; contigs across > 1 cM is not feasible. Therefore, fine or dense maps of the genomic regions identified carrying the resistance gene are needed. The most efficient way of fine mapping in cassava is by employing a variant of the bulk segregant analysis and AFLP markers (Giovannoni *et al.*, 1991). The method utilizes the unique ability of the RAPD and AFLP technique to sample many loci throughout the genome, using many primer combinations, and the ability to find additional linked markers in any region, by screening bulks of genotypic classes of markers adjoining that region. This method is currently being used with a large mapping population of 700 genotypes to identify more markers in the region of the cassava genome carrying the *CMD2* resistance gene. Once fine maps have been obtained, a relationship between genetic distances and physical distances in the relevant regions will be estimated. Based upon the estimated physical distance required to transverse the region bearing resistance genes, a BAC contig will be constructed by BAC clone digestion and fingerprinting. Finally candidate BAC clones will be introduced into cassava genotypes susceptible to CMD via genetic transformation using the BIBAC system.

Other genes of agronomic interest have also been cloned by the use of heterologous probes. One of the most important is the biosythesis gene for the generation of cyanogenic glucosides. Cassava produces the cyanogenic glucosides linamarin and lotaustralin in the roots, that can be toxic to human and animal health if not removed by processing. To block the production of these glucosides it is necessary to identify the key genes in the biosynthesis of linamarin and lotaustralin. The first steps in the biosynthesis of the two cyanogenic glucosides are the conversion of L-valine and L-isoleucine, respectively, to the corresponding oximes. Two full-length cDNA clones that encode cytochromes P-450 catalysing these reactions have been isolated using a heterologous probe (Anderson *et al.*, 2000). The two cassava cytochromes P-450 are 85% identical and share 54% sequence identity to CYP79A1 from sorghum. They have been designated CYP79D1 and CYP79D2. Functional expression in the methylotrophic yeast, *Pichia pastoris*, reveal that each cytochrome P-450 metabolizes L-valine as well as L-isoleucine, consistent with the co-occurrence of linamarin and lotaustralin in cassava. Both CYP79D1 and CYP79D2 are actively transcribed in the cassava genome and production of acyanogenic cassava plants would therefore require down-regulation of both genes. Cassava trangenics with CYP79 anti-sense constructs have been generated and are currently being tested for the production of linamarin and lotaustralin (Moller, 2000, personal communication).

Genes involved in the *in situ* breakdown of the cyanogenic glucosides of cassava following tissue damage leading to the production of hydrocyanic acid, have also been cloned. A linamarase cDNA clone (pCAS5) was isolated from a cotyledon cDNA library using a white clover beta-glucosidase heterologous probe (Hughes *et al.*, 1992). Concanavalin A affinity chromatography and endoglycosidase H digestion demonstrate that linamarase from cassava is glycosylated, having high-mannose-type *N*-asparagine-linked oligosaccharides. Consistent with this structure and the extracellular location of the active enzyme is the identification of an *N*-terminal signal peptide on the deduced amino

acid sequence of pCAS5. Several genes controlling starch biosynthesis in cassava are also included in the list of cloned cassava genes (Munyikwa *et al.*, 1997). They include the ADP glucose pyrophosphorylase (AGPase) B and S gene that catalyses the synthesis of ADP glucose and the granule-bound synthetase (GBSSII) gene, the predominant starch synthase gene that catalyses the conversion of ADP-glucose to amylose. They were cloned using homologous genes from potato. Other starch biosynthesis genes cloned include the starch branching enzyme that gives rise to amylopectin, the branched starch polymer. Potato transgenics expressing the cassava AGPase B gene in anti-sense orientation had reduced levels of AGPase B mRNA and 1.5–3 times less starch and more than five times more soluble sugars than the controls (Munyikwa *et al.*, 1997). Similarly, cassava transgenics expressing GBSSII in the anti-sense orientation, produced little or no GBSSII transcripts and almost 100% amylopectin starch (T.R.I. Munyikwa, personal communication). These starch biosynthesis genes open up an opportunity to produce a range of cassava roots with different quantities of soluble sugars and starches with different amylose/ amylopectin ratios.

Another trait of economic interest for which cloned genes are needed to block its biosynthetic pathway, is PHD. In cassava, this is thought to be a physiological wound response mechanism that undermines the nutritional integrity of tuberous roots (Wheatley, 1982; Wheatley and Schwabe, 1985). Several genes known to be involved in wound healing in plants have been cloned and characterized for their expression during PHD. They include the ACC oxidase that catalyses the last reaction of ethylene biosynthesis in plants, phenylalanine ammonia-lyase (PAL), a key enzyme of the phenyl propanoid metabolism pathway, and catalase, involved in the breakdown of hydrogen peroxide (Li *et al.*, 2002a,b; Reilly *et al.*, 2002). A cDNA, mSOD1, encoding cytosolic copper/zinc superoxide dismutase (CuZnSOD) has also been cloned and characterized from cell cultures of cassava which produce a high yield of SOD (Lee *et al.*, 1999). The mSOD1 gene is highly expressed in cultured cells, as well as in intact stems and tuberous roots. Levels of mSOD1 transcript increased dramatically a few hours after heat stress at 37°C and showed a synergistic effect with wounding stress.

In conclusion, genes, molecular tags and the knowledge accumulated can be used by plant breeders and genetic engineers to address the constraints cassava faces worldwide that have no ready solution using conventional tools. This will require sustained support from both public and private institutions and has the potential to produce a highly productive and profitable crop.

Genetic Engineering

Genetic engineering is a powerful tool that complements traditional breeding and can extend the genetic pool of useful gene sources beyond the species. Transgene technology also offers the advantage of transferring single or even quantitative traits, without the problems of linkage encountered in traditional breeding. The areas where genetic engineering can have an impact in cassava include yield and root quality improvement, pest and disease resistance, and the production of novel compounds for value-added products from cassava. Other important unsolved problems are the low protein content of the roots, the poor storability of freshly harvested roots and the cyanogenic nature of cassava.

Production of stably transformed plants requires an efficient *in vitro* culture system that allows regeneration of plants. Plant cells are generally considered to be totipotent, thus being able to regenerate whole plants from single cells *in vitro*. The ability to regenerate *in vitro* is, however, often limited to certain tissues and developmental stages, and the requirements for transformation and regeneration competence may not always be compatible. Furthermore, a method for efficient transfer and stable integration of the transgenes into the plant genomic DNA is essential for transformation, as well as a means for identifying and selecting transformed cells. The main constraint is usually not the delivery of foreign DNA to the regenerable cells, but the recovery of the transformed cells. Finally, the introduced genes must be correctly expressed in the primary transgenic plants and transmitted stably to their progeny. As cassava is vegetatively propagated, the transgenes can be fixed already at the level of the primary transgenic plants, and stable inheritance is of concern only when the

transgenic plants are to be incorporated in breeding programmes.

Cassava transformation and regeneration methods

Of the several methods for delivering foreign DNA into plant cells (Potrykus and Spangenberg, 1995), the most commonly used are *Agrobacterium*-mediated gene transfer and particle bombardment. As stable transformation frequencies are low, the use of different marker genes is necessary to allow the identification and to be susceptible to *Agrobacterium* (Calderon-Urrea, 1988), but the pathogenicity of different strains is highly variable and genotype-dependent (Chavarriaga-Aguirre *et al.*, 1993; Sarria *et al.*, 1993; Li *et al.*, 1996; Puonti-Kaerlas *et al.*, 1997b). Until recently, mainly due to selection problems, cassava was considered recalcitrant to genetic engineering. The first reports on successful regeneration of transgenic cassava plants have been published only in the second half of the 1990s (Li *et al.*, 1996; Raemakers *et al.*, 1996; Schöpke *et al.*, 1996). Table 10.1 shows the current status of cassava transformation.

The most commonly used visual markers are GUS-encoded by the *uidA* gene (Jefferson *et al.*, 1986; Jefferson 1987), the luciferase genes from the firefly *Photinus pyralis* (Ow *et al.*, 1986) and soft coral *Renilla reniformis* (Mayerhofer *et al.*, 1995), and the green fluorescent protein (GFP; Chalfie *et al.*, 1994). Selectable marker genes can render transformed plant cells resistant to an antibiotic, a metabolic analogue, or a herbicide, thus allowing cells containing a transgene to survive and proliferate, while the wild-type cells are either arrested in their growth or killed. The most commonly used selectable marker genes encode resistance to aminoglycoside antibiotics (*npt*II) (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983), hygromycin (*hpt*; van den Elzen *et al.*, 1985; Waldron *et al.*, 1985), and phosphinotricin, the active ingredient in many herbicides including Basta (*pat* and *bar*; Murakami *et al.*, 1986; De Block *et al.*, 1987; Thompson *et al.*, 1987; Wohllenben *et al.*, 1988). As public concern regarding the use of antibiotic-resistance genes in transgenic plants has become an important factor, development is moving towards other selection systems. An example is the use of antibiotic-resistance genes whose expression can be inhibited by the introduction of introns into the coding region (Wang *et al.*, 1997). A selectable marker system based on the *ipt* gene from the T-DNA of *Agrobacterium tumefaciens*, either linked to the *Ac* transposable element, to allow its removal from regenerating shoots (Ebinuma *et al.*, 1997), or under the control of an inducible promoter (Kunkel *et al.*, 1999) is another example with potential. New non-antibiotic methods have been developed based on positive selection, which favours the regeneration and growth of transgenic cells while suppressing the growth and proliferation of non-transgenic ones. The use of a glucuronide derivative of the cytokinin benzyladenine (Joersbo and Okkels, 1996), xylose (Haldrup *et al.*, 1998a,b) and mannose (Joersbo *et al.*, 1998), have been shown to improve significantly the frequencies of transgenic plant regeneration in tobacco, potato and sugarbeet when compared to antibiotic selection schemes.

Of the different explants used for regeneration, meristems are the tissue of choice as they represent 'growth centres' of plants. In *Arabidopsis thaliana* and *Medicago truncatula* (Trieu *et al.*, 2000), developing flower meristems can be transformed efficiently using *Agrobacterium*. In cassava, meristems can be induced to form multiple shoots on cytokinin-containing medium. Most of the shoots are derived from pre-existing axillary meristems, but also *de novo* formation of new meristems and shoots occurs (Konan *et al.*, 1994a, 1995, 1997). Transient and stable expression of both GUS and luciferase have been demonstrated in meristems and meristem-derived somatic embryos and multiple shoot clusters after particle bombardment (Puonti-Kaerlas *et al.*, 1997a) or co-cultivation with *Agrobacterium* (Konan *et al.*, 1995; Puonti-Kaerlas *et al.*, 1997a). Transgenic sectors were detected in the developing shoots, but no fully transgenic plants have been regenerated. Somatic embryogenesis is now the most commonly used regeneration method for cassava. Somatic embryogenesis in cassava is restricted to meristematic and embryonic tissues. Somatic embryos can only be induced on a limited number of explants such as cotyledons or embryonic axes from zygotic embryos (Stamp and Henshaw,

Table 10.1. Methods used for the genetic transformation of cassava.

Target tissue	Regeneration mode	Gene transfer system	Selection	Transgenic tissues	Analysis	Reference
Somatic embryos	Somatic embryogenesis	Electroporation	–	Chimeric embryos	Transient GUS expression	Luong *et al.* (1995)
Somatic cotyledons	Shoot organogenesis	*Agrobacterium*	Hygromycin geneticin	Transgenic plants	Southern, Northern	Li *et al.* (1996)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Paromomycin	Transgenic plants	Southern	Schöpke *et al.* (1996)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Luciferase	Transgenic plants	Southern	Raemakers *et al.* (1996) Schöpke *et al.* (1997a)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	–	Chimeric suspensions	Transient gene expression	Munyikwa *et al.* (1998a)
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Luciferase and phosphinotricin	Transgenic plants	Southern, Northern	González *et al.* (1998)
Embryogenic suspension	Somatic embryogenesis	*Agrobacterium*	Paromomycin	Transgenic plants	Southern	Sarria *et al.* (2000)
Somatic cotyledons	Somatic embryogenesis	*Agrobacterium*	Basta	Transgenic plants	Southern	Zhang *et al.* (2000)
Somatic cotyledons	Shoot organogenesis	Particle bombardment	Hygromycin	Transgenic plants	Southern, RT-PCR	Zhang and Puonti-Kaerlas (2002)
Somatic cotyledons, Embryogenic suspension	Shoot organogenesis, Somatic embryogenesis	Particle bombardment	Mannose, hygromycin	Transgenic plants	Southern, Northern, RT-PCR	Zhang *et al.* (2001)
Embryogenic suspension	Shoot organogenesis, Somatic embryogenesis	*Agrobacterium*	Mannose, hygromycin	Transgenic plants	Southern, Northern, RT-PCR	

1982; Konan *et al*., 1994a,b) and immature leaf lobes (Stamp and Henshaw, 1987a; Szabados *et al*., 1987; Matthews *et al*., 1993; Raemakers, 1993; Raemakers *et al*., 1993a; Li *et al*., 1995, 1996, 1998a; Puonti-Kaerlas *et al*., 1997a,b). Other tissues include meristems and shoot tips (Szabados *et al*., 1987; Narayanaswami *et al*., 1995; Frey, 1996; Puonti-Kaerlas *et al*., 1998), anthers (Mukherjee, 1995) and immature inflorescences on auxin-containing media (Woodward and Puonti-Kaerlas, 1998). Primary somatic embryos can be induced to produce secondary somatic embryos by further subculturing on auxin-containing medium (Stamp and Henshaw, 1987b). By constant subculturing of somatic embryos, a cyclic embryogenesis system can be established either in liquid or solid medium, where the embryos rarely pass the 'torpedo' stage, until transferred to germination medium.

In cassava, both primary and secondary somatic embryos develop from groups of cells, usually located at or near the vascular tissue (Stamp, 1987; Raemakers *et al*., 1995b). The multicellular origin of cassava somatic embryos makes them poorly suited for genetic engineering. Moreover, their location under the plant epidermis also limits their accessibility to *Agrobacterium*. Transgenic sectors were detected in somatic embryos after electroporation, but no transgenic plants were regenerated from these embryos (Luong *et al*., 1995, 1997). Particle bombardment of embryogenic clusters led to high transient expression of visible marker genes in several laboratories, but only sectorial transgenic embryos could be regenerated from bombarded embryos. Transgenic callus can be obtained readily from explants from cycling somatic embryos, but the competence for embryogenesis is lost when the cultures are treated with antibiotics to select for transformed cells (Chavarriaga-Aguirre *et al*., 1993; Schöpke *et al*. 1993; Puonti-Kaerlas, unpublished). There is only one report on successful regeneration of transgenic cassava plants by secondary somatic embryogenesis, supported by molecular data to show the presence of transgenes in one of the 15 regenerated Basta-resistant plant lines (Sarria *et al*., 1995, 2000). In this case, secondary somatic embryos were induced on cotyledon explants from primary somatic embryos of the cassava landrace MPeru183, after co-cultivation with the wild-type *Agrobacterium* strain CIAT1182, carrying the genes encoding GUS and phosphinotricin resistance. Selection on 8–32 mg l^{-1} Basta allowed the regeneration of putative transgenic secondary embryos that could be regenerated to mature plants. MPeru183 and the *Agrobacterium* strain CIAT1182 were selected after a screen for the most efficient combination to ensure high transformation frequency (Sarria *et al*., 1993). The reproducibility of this method has not been assessed so far, and the CIAT1182 strain is oncogenic, thus its use for production of transgenic plants on routine basis is still limited. Disarming the vector should offer great potential for further improvement of *Agrobacterium*-mediated transformation methods. The use of immature cassava leaves to regenerate transgenic plants after co-cultivation with *Agrobacterium* was reported (Arias-Garzon and Sayre, 1998) but needs verification at the molecular level.

As transformation of somatic embryos can lead to the formation of chimerics (Sarria *et al*., 1995), a more promising option is the use of friable embryogenic callus (FEC). A fraction of the cycling somatic embryos maintained on a MS medium (Murashige and Skoog, 1962), or even more efficiently, on a GD medium (Gresshoff and Doy, 1974) supplemented with picloram, produces a highly FEC (Taylor *et al*., 1996). Pure FEC can be transferred easily to liquid culture to establish a rapidly proliferating embryogenic suspension in SH (Schenk and Hildebrandt, 1972) medium supplemented with 6% sucrose and 10–12 mg l^{-1} picloram. Maturing embryos develop on transfer to hormone-free medium. In contrast to the primary or secondary somatic embryos, the new embryogenic units in FECs develop from the surface cells of the globular embryo clusters, and appear to be of single cell origin, which makes them good targets for transformation (Taylor *et al*., 1996). Particle bombardment of embryogenic suspensions of 'TMS 60444' allowed regeneration of transgenic cassava plants using three different approaches. The first is based on antibiotic selection using paromomycin (Schöpke *et al*., 1996; González *et al*., 1998) or hygromycin (Zhang and Puonti-Kaerlas, 2002). The second method employs visual selection using firefly luciferase as a screenable marker gene (Raemakers *et al*., 1996), or on a combination of antibiotic

selection and luciferase screening (Munyikwa *et al.*, 1998a,b) and the third on positive selection (Zhang and Puonti-Kaerlas, 2002). With paromomycin selection the bombarded tissues were first grown for 2 weeks after transformation in liquid medium containing 12 mg l^{-1} picloram, after which, paromomycin was added to the liquid culture at 15 mg l^{-1}. After 4–5 weeks of liquid selection, the developing embryogenic units were transferred to solid culture medium under the same selective conditions and cultured for another 4 weeks. Regeneration of plants was only possible when no selection was applied.

After the transgenic units were multiplied as FECs, shoot regeneration was initiated by sequential transfer and culture on a series of media to induce the differentiation of globular and torpedo-stage embryos (1.2 mg l^{-1} picloram), development of cotyledons (0.93 mg l^{-1} NAA) and maturation of embryos (0.5% activated charcoal, no growth regulators). Before attempting to root the regenerants, a multiplication step to induce multiple shoot formation from the apical meristem of the germinating embryos was applied (1 mg l^{-1} BA). The capacity for regeneration differed greatly between the selected lines, and sometimes no plants regenerated. Southern data were published to prove the presence of the *uidA* gene in one regenerated shoot. When hygromycin was used, selection was started 3 days after bombardment. The cultures were grown in a liquid medium with 12 mg l^{-1} picloram and 50 mg l^{-1} hygromycin for 2–4 weeks after which they were transferred to a solid medium supplemented with 1 mg l^{-1} NAA and 25 mg l^{-1} hygromycin for 2–8 weeks to allow FEC formation and embryo emergence. The hygromycin-resistant embryos developed into shoots on transfer to an elongation medium containing 0.4 mg l^{-1} BA and no hygromycin. GUS assays, PCR, reverse transcribed (RT)-PCR as well as Southern and Northern analyses confirmed the transgenic nature and stable expression of the transferred genes in two regenerated plant lines. This protocol allows transgenic plants to be regenerated in 15 weeks, thus reducing considerably the time required for *in vitro* culture. Selection systems based on paromomycin (Schöpke *et al.*, 1997a; González *et al.*, 1998) and hygromycin have also been adapted to *Agrobacterium*-mediated transformation of embryogenic suspension cultures.

After a 2-day co-cultivation period of the suspension cells with *Agrobacterium* strain ABI containing a plasmid carrying the *nptII* and intron-interrupted *uidA* genes, the cultures were grown for 8–10 days without selection, followed by culture in liquid SH medium containing paromomycin for 5–6 weeks and on solidified selective medium for 4 weeks. They were then transferred to regeneration medium without selection for embryo differentiation (4 weeks), cotyledon development (4 weeks), maturation (2 weeks), shoot development (4 weeks) and rooting (4–8 weeks) using the media described for bombarded suspensions. Southern blot analysis demonstrated stable integration of the *uidA* gene into the cassava genome in two plant lines. After 3–4 days co-cultivation with *Agrobacterium* strain LBA4404 containing a plasmid carrying intron-interrupted *hpt* (Wang *et al.*, 1997) and *uidA* genes, the tissue was cultured for 3 days without selection and then for 15 days in liquid medium containing 12 mg l^{-1} picloram and 50 mg l^{-1} hygromycin. After another 15 days' liquid culture using 25 mg l^{-1} hygromycin, the suspensions were transferred to solid medium with1 mg l^{-1} NAA and 25 mg l^{-1} hygromycin. Embryos developed on this medium in 4–8 weeks, and shoots were regenerated from the cotyledonary stage embryos on a medium with 0.4 mg l^{-1} BA without hygromycin in 4 weeks. Molecular analyses confirmed the transgenic nature of 12 regenerated lines. Raemakers *et al.* (2000) have further modified the protocols based on antibiotic selection.

When firefly luciferase was used to select for transgenic tissues, the embryogenic suspensions were cultured for 1 day after bombardment on solid medium containing 6% sucrose. They were then transferred either to liquid medium containing 6% sucrose, or, to solid medium with reduced sucrose concentration of 6% to 2% in two 3-day subculture steps. Two weeks after bombardment, the cultures were monitored for luciferase activity, and clusters of embryogenic units at and around the luciferase positive spots, were isolated and cultured further. The luciferase screen and tissue selection was repeated at 2-week intervals, until 2 months after bombardment the friable embryogenic calli clusters containing at least 1% luciferase-positive units were transferred to maturation medium containing 1 mg l^{-1} picloram for development of somatic

embryos. The maturing luciferase-positive embryos were further multiplied by secondary somatic embryogenesis on media containing either 10 mg l^{-1} NAA or 8 mg l^{-1} dinitrophenol (2,4-D), and in subsequent steps of cyclic somatic embryogenesis 10 mg l^{-1} NAA. After desiccation, the somatic embryos were induced to grow on a medium containing 1 mg l^{-1} BA, and then multiplied as shoot cultures by nodal cuttings. The efficiency of the secondary embryo formation from selected embryos was 83%, but only 1–15% of the embryos grow into transplantable shoots. Southern hybridization data were presented to support the transgenic nature of three plants.

A method combining antibiotic selection, using phosphinotricin, 20 mg l^{-1}, and luciferase, allowed the development of both transformed and non-transformed maturing embryos from bombarded FECs, but the luciferase screening could be used to exclude escapes (Snepvangers *et al.*, 1997). The inclusion of 20 mg l^{-1} phosphinotricin in the maturation medium could not block the maturation of non-transgenic embryos completely. Later studies demonstrated that strict visual selection using luciferase was more efficient and led to the production of transgenic lines faster than antibiotic selection. Southern analysis confirmed the transgenic nature of 18 plant lines produced by combining antibiotic and visual selection (Munyikwa *et al.*, 1998a). The selection protocol using luciferase has been further refined by Raemakers *et al.* (2000). A positive selection system was developed for embryogenic suspensions (Zhang and Puonti-Kaerlas, 2002; Zhang *et al.*, 2002). After transformation, the cells were grown for 4 weeks in liquid culture using a modified SH medium containing 12 mg l^{-1} picloram, 4% mannose and 1% sucrose, before transfer to solid medium containing 2% sucrose, 2% mannose and 1 mg l^{-1} NAA for 2 weeks. After another 4 weeks culture on the same medium without mannose, shoots were regenerated in 4 weeks from embryos cultured on a medium containing 2% sucrose and 0.1 mg l^{-1} BA. GUS assays and molecular analyses confirmed the transgenic nature of 14 regenerated plant lines.

The use of the protocols described above depends on the availability of embryogenic suspensions, the establishment of which is still genotype-dependent and labour-intensive. The low regeneration rates and the regeneration of possibly abnormal plants reduce the transformation efficiencies obtained after transformation of embryogenic suspensions (Taylor *et al.*, 1996; Raemakers *et al.*, 1997b, 2000; Snepvangers *et al.* 1997; Schöpke *et al.*, 1997b). The use of paromomycin selection reduces the regenerative potential of the transgenic material and relatively complicated, time- consuming and labour-intensive regeneration schemes must be followed. This has led to somaclonal variation and reduced growth rates in up to 50% of the regenerants, and to lower survival rate (40% versus 90% in controls) of the plantlets upon transplanting to the greenhouse (Raemakers *et al.*, 1997b; Munyikwa *et al.*, 1998a). Luciferase, though a non-invasive detection method, requires access to costly equipment including a coupled device camera for detection and localization of the bioluminescence. In contrast, combining GUS assays with hygromycin or mannose selection, should allow rapid and easy selection of cultures for regeneration, resulting in 100% selection efficiency (Zhang and Puonti-Kaerlas, 2002) and possibly reducing the length of time required for tissue culture before plant regeneration.

An efficient alternative regeneration system using organogenesis was developed in order to circumvent the problems encountered when regenerating plants by germinating somatic embryos (Li *et al.*, 1995, 1996, 1998a). Shoot primordia were induced directly on cotyledon explants from germinating cycling somatic embryos on a medium containing cytokinins. A cycling system where the secondary somatic embryos were induced on cotyledon explants from maturing somatic embryos was established. To induce shoot organogenesis, cycling somatic embryos were transferred to maturation medium containing 1.0 mg l^{-1} BA and 0.5 mg l^{-1} IBA. After a passage on elongation medium containing 0.4 mg l^{-1} BA, the regenerating shoots were easily rooted on hormone-free medium and transplanted into soil in the greenhouse. Shoot induction frequency of different cassava cultivars was 42–67% and shoot primordia could be induced on cotyledons from cycling embryos maintained either on 2,4-D or picloram. Cotyledon explants derived from cycling somatic embryos showed the highest competence for organogenesis, while those from primary

somatic embryos responded very poorly. The organogenesis frequencies have improved further by using silver nitrate in the medium (Puonti-Kaerlas, unpublished). The transferability of this protocol has been demonstrated with ten different Latin American, African and Asian cultivars, and the system is currently being implemented at IITA, Ibadan.

Compared to regeneration via germination of embryos derived from suspensions, shoot regeneration via organogenesis is faster, and requires less time in tissue culture. Using organogenesis, transplantable shoots can be regenerated from cotyledon explants within 60–65 days. In addition, the germination/maturation steps in the protocol ensure the selection for highly regeneration competent embryos, hence minimizing the risk of producing embryogenic cultures that will be arrested in their development. The shoots regenerated via organogenesis develop from cells at or close to the cut edges of the cotyledon explants, which makes them good targets for *Agrobacterium*-mediated gene transfer. In contrast to the protocols based on somatic embryo production, both callus and shoot development were inhibited by similar amounts of antibiotics, and both geneticin, hygromycin and phosphinotricin could be used as selective agents (Li *et al.*, 1996, 1998b; Puonti-Kaerlas *et al.*, 1997a,b). The choice of an appropriate *Agrobacterium* strain and pre-induction of the bacteria with acetosyringone were essential for high transformation frequency. Extending the co-cultivation time to 4 days resulted in the highest transient transformation rates, without excessive bacterial contamination. The developmental state of the explants was also found to be a critical factor in the transformation procedure. Cotyledons from newly germinated embryos were very sensitive to *Agrobacterium*, and survived the co-cultivation procedure poorly, which resulted in low transformation rates. Cotyledon explants from older embryos survived better, but explants from germinating embryos older than 20 days regenerated less efficiently. The highest regeneration and transformation frequencies could be obtained by using cotyledon explants from somatic embryos of 'M Col 22' cultured for 15 days on maturation medium.

Following co-cultivation, callus and small resistant shoot primordia developed on selection medium containing 15 mg l^{-1} hygromycin or 20 mg l^{-1} geneticin from the cotyledon explants co-cultured with LBA4404 (pBin9GusInt) or LBA4404 (pTOK233), respectively. In GUS assays, three of 27 regenerated geneticin-resistant shoot primordia and six of 30 hygromycin-resistant shoots stained blue. After rooting, the putative transgenic shoots were transferred to soil in the greenhouse (Li *et al.*, 1996). Cloned plant material was stained for GUS activity in order to assess the expression of the 35S promoter in different cassava tissues. In contrast to earlier reports based on transient assays after particle bombardment (Arias-Garzón and Sayre, 1993), the 35S promoter was shown to be highly expressed in all cassava tissues, including all parts of the roots. The highest expression levels, as determined by the intensity of the blue colour, were in the youngest tissues, including apical and axillary meristems and root tips (Puonti-Kaerlas *et al.*, 1997a,b). The stable integration of the transgenes into cassava nuclear DNA was demonstrated in five transgenic plants and Northern data to prove the transcriptional activity of the transgenes were presented. The selection system needs further improvement, as many escapes occurred. Southern analysis of 18 lines selected on geneticin verified their transgenic nature (Li *et al.*, 1998b, Puonti-Kaerlas, unpublished). Recently, the organogenesis protocol was adapted to use with the Particle Inflow Gun to allow the widest possible range of cultivars to be transformed (Zhang and Puonti-Kaerlas, 2002; Zhang *et al.*, 2000). The bombardment parameters were partially optimized and the selection procedure was modified. Using 7.5 mg l^{-1} hygromycin for the first 10 days after bombardment, and then 15 mg l^{-1} hygromycin for 2 weeks, followed by culture of the developing shoot primordia on 10 mg l^{-1} hygromycin, increased the stringency of the selection. Molecular analyses confirmed the transgenic nature of nine regenerated plant lines.

The positive selection system using mannose was shown to be compatible also with the organogenesis-based regeneration mode of cassava (Zhang and Puonti-Kaerlas, 2002). Bombarded cotyledon pieces were first cultured for 3–4 weeks on a medium containing 1.0 mg l^{-1} BA, 0.5 mg l^{-1} IBA, 1% mannose and 0.5% sucrose, then the developing shoot primordia were transferred for 3 weeks to

elongation medium containing 0.4 mg l^{-1} BA, 2% sucrose and 1% mannose. The regenerated shoots were maintained on a medium supplemented with 1% mannose. Molecular analyses confirmed the transgenic nature of two plant lines. In order to improve the selection efficiency of both antibiotic and positive selection, rooting assays were developed. The rooting of axillary shoots of non-transgenic cassava was inhibited by 5.5 mg l^{-1} hygromycin and 8 mg l^{-1} geneticin (Zhang *et al.*, 2000) and 1% mannose (Zhang and Puonti-Kaerlas, 2002), while the transgenic plants could root normally. The rooting tests allow an efficient secondary screening for elimination of escapes from primary selection.

Prospects for genetic engineering

Disease resistance

The use of agrochemicals to protect plants against most fungal and bacterial diseases is in principle possible, even if not economically viable or environmentally sustainable, but in the case of viral diseases this option does not exist, as suitable 'viricides' are not available for routine use. Resistance strategies are therefore of the highest priority. *Cassava common mosaic virus* (CsCMV), which causes up to 20% yield losses (CIAT, 1991), is currently the most important viral disease of cassava in Latin America. No vector is known for this virus, and it is spread mainly by mechanical transmission and the use of infected cuttings. The coat protein gene of CcSMV has been transferred to cassava as a potential source of resistance (Schöpke *et al.*, 2000). CMD causes losses of up to 20–80% of total yields throughout the African continent, and can locally result in complete crop failure (Lozano and Booth 1974; Thresh *et al.*, 1994a,b; Otim-Nape, 1995). It has been ranked as the most important vector-borne disease of all African food crops (Geddes, 1990). CMD is caused by a number of viruses transmitted by the whitefly (*Bemisia tabaci*), and disseminated in vegetative planting material, but not via true seed (Swanson and Harrison, 1994). The most recent pandemic caused by a new recombinant strain has spread in eastern Africa from north to south through Uganda and western Kenya at 15–20 km $year^{-1}$ (Otim-Nape *et al.*, 1994a,b, 1997a,b; Otim-Nape, 1995; Gibson *et al.*, 1996). In Uganda the use of new cultivars and disease-free planting material have met with some success in managing the disease (Otim-Nape *et al.*, 1997a,b). The heterozygous nature of cassava implies that breeding of new CMD-resistance cultivars using traditional methods can lead to the loss of favoured local landraces and improved lines, and genetic transformation technology may be necessary to transfer the desired traits to preferred varieties. So far CMD has not reached Latin America, but a new biotype of the vector that feeds on cassava has been found in the neotropics (CIAT, 1990; Franca *et al.*, 1996). This now makes CMD a serious threat to cassava production in Latin America, as the germplasm in America is highly susceptible to at least one of the causal viruses referred to as the cassava mosaic geminiviruses (CMGs).

African cassava mosaic virus (ACMV) is one of at least four CMGs having a genome consisting of two covalently closed circular single-stranded DNA (ssDNA) molecules known as DNA A and DNA B (Stanley, 1983). DNA A is responsible for the replication of both DNA components (Townsend *et al.*, 1986; Etessami *et al.*, 1991) and virus proliferation rates (Haley *et al.*, 1992; Hong and Stanley, 1995) and, together with DNA B, for vector transmission and virus spread (von Arnim *et al.*, 1993; Haley *et al.*, 1995; Liu *et al.*, 1997; Briddon *et al.*, 1998). Both genomic components are also necessary for the systemic infection of susceptible host plants (Stanley, 1983). DNA B is involved in cell-to-cell and long-distance virus spread and production of disease symptoms (von Arnim *et al.*, 1993; Haley *et al.*, 1995). The promoter activity of ACMV is regulated by its own gene products (Haley *et al.*, 1992; Hong and Stanley, 1995). Understanding the regulation of ACMV replication and gene expression will allow the development of new resistance strategies using transgene technology. Until recently all studies were conducted with herbaceous model species, but now studies on the simultaneous regulation of both sides of the bi-directional promoter of ACMV using dual luciferase assays, have shown differences between cassava and model plants (Frey *et al.*, 2002). A virus replication assay has been developed for cassava, which should allow more

detailed experiments using cassava instead of model systems (Puonti-Kaerlas, unpublished).

There are indications from heterologous species that resistance against ACMV could also be transferred to cassava. Expression of the defective interfering DNA (Stanley *et al.*, 1990; Frischmuth and Stanley, 1991) and the movement protein of *Tomato golden mosaic virus* (von Arnim and Stanley, 1992) have been shown to increase their resistance to the virus in variable degrees. So too has the expression of the AC1 gene of ACMV (Hong and Stanley, 1996), dianthin (Hong *et al.*, 1996, 1997) and a mutated replicase (Sangare *et al.*, 1999) in transgenic model plants. Different constructs carrying the AC1 gene have been used to transform cassava (Schöpke *et al.*, 2000). In addition to these, novel strategies are being designed for engineering sustainable resistance to ACMV (Berrie *et al.*, 1998; Schärer-Hernandez *et al.*, 1998). Preliminary results using a virus-induced cell death system show that it is possible to reduce ACMV replication by 37–99% in transgenic model plants (Frey, 2000), and transgenic cassava plants containing this construct are being developed.

Resistance to insect pests

Due to its long growth period, cassava is subject to prolonged and repeated attacks from numerous insect pests (Bellotti, 1979; Bellotti *et al.*, 1994, 1999; Chapter 11). In Latin America, lepidopteran insects are currently the main cassava pests. These include stem borer (*Chilomina clarkei*) and hornworm (*Erinnyis ello*). Hornworm causes variable yield losses along the northern coast of South America and in the Caribbean. Stem borer causes considerable damage to cassava in Colombia (Bellotti *et al.*, 1999). It not only causes considerable yield loss, but as the larvae live and feed inside cassava stems, they are protected from external applications of insecticides, and a severe attack can lead to reduced quality or even complete loss of planting material. The soil bacterium *Bacillus thuringiensis* (Bt) carries a set of *cry* genes encoding insect-specific endotoxins (Bt toxins), which are efficient in combating a variety of insects. Spraying Bt was shown to be efficient in biological control of cassava hornworm (Bellotti and Arias, 1979). Expression of the *cry* genes in transgenic cassava would complement the available methods for pest control in an environmentally and economically sustainable way. Several groups are currently working on this topic.

Root quality and yield

CYANOGENESIS. Cassava is cyanogenic, i.e. hydrogen cyanide (HCN) is produced in all parts of the plant when the tissues are damaged. To prevent cyanide poisoning, linamarin and lotaustralin have to be removed by labour-intensive processing, and shortcuts in processing can lead to fatal consequences (Akintonwa and Tunwashe, 1992; Mlingi *et al.*, 1992; Akintonwa *et al.*, 1994). All known cassava cultivars contain cyanogenic glucosides, and despite considerable efforts, no acyanogenic cultivar has been found or produced (Jennings, 1976; Bokanga, 1994; Dixon *et al.*, 1994). High cyanide cultivars are favoured in many areas as they are considered tolerant to environmental stress and also relatively safe from theft by humans and mammals (Rosling *et al.*, 1993; Kapinga *et al.*, 1997). The occurrence of severe neurological disorders is closely linked to prolonged exposure to cyanide (Osuntokun and Monekosso, 1969; Tylleskär *et al.*, 1991, 1992, 1995; Tylleskär, 1994). Recent studies also seem to indicate a neurotoxic effect of linamarin itself (Banea-Mayambu *et al.*, 1997). In addition to the health risks of cassava-based food, the effluent from cassava processing plants often contains toxic amounts of cyanide, and consequently can be a serious pollutant if not properly managed (Manilal *et al.*, 1983).

By manipulating the key enzymes in linamarin synthesis, the cytochrome P-450 oxidases, through down-regulation using anti-sense technology, the levels of cyanogenic glucosides in cassava roots could be reduced. Acyanogenic cassava could considerably increase household income by eliminating the fear of buying fresh cassava roots in some regions and reduce the emission of pollutants from starch factories. Of the genes in the biosynthesis pathway, the key enzymes, cytochrome P-450 oxidases (Koch *et al.*, 1994) were recently isolated and characterized (Andersen *et al.*, 2000), and anti-sense constructs to down-regulate the expression of these genes are currently being transformed into cassava (B.L. Møller, personal communication). Alternatively, increased expression of

linamarase and α-hydroxynitrilase, the enzymes breaking down linamarin, would offer a way to enhance the rate of HCN release during cassava processing. The residual cyanohydrins in processed cassava are the main source of dietary cyanide (Tylleskär *et al.*, 1992), and thus ways of maintaining a high activity of the α-hydroxynitrilase during cassava processing would be of great interest. To enhance the breakdown of cyanohydrins during cassava root processing, production of transgenic cassava over-expressing the α-hydroxynitrilase gene is in progress (Arias-Garzon and Sayre, 1998).

PROTEIN CONTENT. One of the disadvantages of cassava is the low protein content of the roots, which can lead to qualitative protein malnutrition in areas where the diet is based predominantly on cassava. Also, protein deficiency has been shown to aggravate symptoms related to cassava toxicity (Rosling, 1988). Synthetic storage proteins designed to improve the protein quality and quantity have been introduced to potato (Yang *et al.*, 1989) and to sweetpotato (Prakash and Egnin, 1997; Demgen, 2000). A novel synthetic storage protein gene (Kim *et al.*, 1992) has been transferred to cassava to improve the nutrient balance of cassava, so as to allow its use as a cheap protein source. The stable expression of the storage protein gene has been verified on transcriptional and translational levels and accumulation of the protein in cassava tissues has been shown in both primary roots and leaves of the regenerated transgenic plants (Puonti-Kaerlas, unpublished). Cassava leaves contain valuable high quality protein and provide a reliable, low cost source of vitamins, minerals and proteins (Balagopalan *et al.*, 1988; Bokanga, 1994; Dahniya, 1994). Excessive leaf harvesting, however, reduces storage root production, and therefore leaves can only be harvested every 2 months in order to minimize losses in root yields (Bokanga, 1994).

Leaf retention

Leaf longevity has been shown to be one of the main traits associated with high yields, together with a leaf area index of 3.0–3.5 (Hunt *et al.*, 1977; Cock, 1979). Furthermore, high drought tolerance and productivity were associated with leaf retention during drought (El-Sharkawy *et al.*, 1992; Osiru *et al.*, 1994). Leaf retention capacity during periodic drought was also positively correlated with root quality. Prolonging the life of individual leaves could help to produce cultivars with improved yield and root quality and to permit more frequent harvesting of leaves while maintaining a satisfactory photosynthetic area to ensure storage root production. As the market value of leaves in the areas where they are consumed is often higher than that of the roots (Lutaladio and Ezumah, 1981), this could also contribute to household economies. Prolongation of photosynthetically active leaf life has already been achieved in transgenic tobacco carrying the *ipt* gene encoding cytokinin production under the control of a senescence-regulated promoter from *Arabidopsis thaliana* (Gan and Amasino, 1995). Transgenic cassava plants containing the same construct have been produced and are currently being analysed (Li *et al.*, 1998b).

Future prospects for genetic engineering

The development of new techniques allowing more efficient improvement of cassava has proceeded rapidly in the past few years. Isolation of promoters and genes as cDNAs and genomic clones from cassava will contribute to the increased knowledge of metabolic pathways, their regulation, and eventually, their genetic engineering, allowing the production of new cassava cultivars resistant to pests and diseases with improved nutritional quality or altered starch composition. Vitamin A is vital to the normal development in humans, and the consequences of vitamin A deficiency range from night blindness through total blindness, to reduced resistance to various terminal diseases (Sommer, 1988; West *et al.*, 1989). According to UNICEF, *c.* 124 million children in the world suffer from vitamin A deficiency (Sommer, 1988; Humphrey *et al.*, 1992). Expressing the corresponding genes from plants producing β-carotene, the precursor for the synthesis of vitamin A, in cassava roots would help to alleviate this problem. Cassava roots have a basic capacity for β-carotene synthesis, as shown by

the identification of cassava cultivars with yellow roots containing carotenoids (Moorthy *et al.*, 1990; Adewusi and Bradbury, 1993a,b). The carotenoid content of cassava roots can be as high as 2 mg per 10 g fresh weight (Iglesias *et al.*, 1997), but a large part of this may exist in non-provitamin A like forms, e.g. as luteolin (Adewusi and Bradbury, 1993a,b). Breeding for high carotenoid content was reported to have produced cassava lines with up to sevenfold higher carotene contents in the roots (Nair and Pillai, 1996). Thus the expression of the first enzyme in the pathway, phytoene synthase, might suffice to produce cassava roots with high β-carotene content in lines favoured by farmers, without changing their other characteristics. Should this prove insufficient, it is now also possible to transfer the whole pathway required for β-carotene production, as recently demonstrated with transgenic rice (Burkhardt *et al.*, 1997).

Similarly, iron deficiency anaemia, one of the most serious deficiencies in the developing countries affecting over 1 billion people, could be combated by increasing iron content and improving its availability in cassava roots using transgene technology (Anon., 1999; Gura, 1999; Lucca, 1999).

The development of cassava cultivars with different starch composition would increase the value of cassava as an industrial crop. Amylose-free cassava starch and cassava roots with increased levels of sugars would create special market niches. The expression of ADP glucose pyrophosphorylase B gene in anti-sense in transgenic cassava has already shown to result in plants with little or no transcript and increased soluble sugars in the storage roots (Munyikwa *et al.*, 1998a,b). Means to control physiological postharvest deterioration of cassava roots may be developed once the process is better understood, possibly by manipulating the regulation of the key enzymes involved in the wound response reaction in roots.

Future possibilities of adding value to cassava as an income-generating crop include also the production of biodegradable plastics. As a first step towards production of biodegradable plastics in plants, accumulation of polyhydroxyalkanoates has been demonstrated in transgenic *Arabidopsis* plants expressing bacterial PHA genes (Poirier *et al.*, 1995), and an *ex ante* study indicated that production of biodegradable plastic in cassava may well be a viable option (Stoeckli, 1998).

Other possible strategies for value-added cassava could include the improvement of iodine content and availability, production of cyclodextrins, improved baking quality of cassava flour, nematode resistance and herbicide resistance.

It is now possible to regenerate transgenic cassava plants, which is the prerequisite for genetic improvement of cassava, but so far there is little information on the transferability of the current protocols to other cultivars beyond the model cultivars, or to other laboratories. The genetic improvement of cassava via biotechnology is constrained by the lack of routine, inexpensive, efficient and genotype-independent transformation methods. Despite the recent breakthroughs, the absence of efficient and reliable technology will be one of the greatest constraints in applying genetic engineering to cassava improvement. Different transformation systems may be needed for different cultivars, and thus both direct gene transfer methods, as well as those based on *Agrobacterium*, should be developed further. The high proliferation rate of embryogenic suspensions will make the multiplication of transgenic tissues efficient. In contrast, organogenesis may be a less genotype-dependent regeneration mode for cassava than germination of somatic embryos, and may also allow more flexibility in the choice of selectable marker genes.

The main limitation to the rapid development of new techniques for cassava remains the lack of funding for research. It is to be hoped that the progress achieved with limited resources will help to increase the interest in this important crop. Cassava biotechnology has potential to alleviate poverty, increase food security and promote the efficient use and conservation of genetic resources, provided that its results are available to cassava-growing countries. Access to this technology should be equitable, and it should not create new dependencies, nor lead to raw material substitution in industrial countries. As cassava remains irreplaceable in marginal environments, the people living in these areas should be the first to benefit from the new technology.

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