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Genetic and Genomic Studies of Cassava

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) is native from South America and is one of the most important tropical foods crops for more than 600 million people worldwide. Cassava storage roots are an excellent source of starch but deficient in proteins, vitamins and other micronutrients. To improve yields, starch quality and nutritional value of cassava roots, many studies have been developed aiming to identify molecular markers and increase knowledge about plant genome and gene function. At present the number of publicity available cassava ESTs is estimated at 36,120. The identification of genes with traits of biological, nutritional and agronomic importance and tissue-specific promoters is essential for biotechnological approaches for cassava improvement, which is underway. In this review important advances in the genetics and genomics of cassava are described.

Keywords: cassava storage root, ESTs, functional genomics, plant biotechnology

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INTRODUCTION

Cassava (*Manihot esculenta* Crantz), belongs to Euphorbiaceae family, is native from South America and one of the most important tropical food crops. Around 600 million people in Africa, Asia and Latin America depend on the plant for their survival. In sub-Saharan Africa, average consumption exceeds 300 kg per person/year (FAO 2003). Cassava roots are an excellent source of starch and rate with respect to furnished calories, in fourth place after rice, maize and sugar cane (Puonti-Kaerlas 1998). Worldwide, cassava acreage is more than 16 million hectares and annually produces root yields of more than 170 million tons. The increasing importance of cassava production as a staple to world food supply is evidenced by an improvement in production of more than 75% during the last 30 years.

In developing countries cassava is often cultivated by subsistence farmers, since this crop presents easy propagation systems, high drought tolerance and low demand for nutrients, producing reasonably well under critical conditions of climate and soil (Nweke *et al*. 2002). While cassava is still mainly grown by poor subsistence farmers, its use is becoming more widespread. Cassava can be processed into chips, pellets, flour, alcohol or starch, and used in a variety of industries.

There is no doubt that the root is the most important product of cassava as food since it is a well-known material of high quality starch, however leaves are consumed by many African cultures and are an excellent source of proteins and vitamins (Latham 1979; Ikoigbo 1980). In comparison with roots, the leaves are almost completely neglected in commercial terms, although they are available in abundance and there is considerable potential for its exploitation.

Although adaptability of this crop for adverse environmental conditions, losses in yields have been significant to cassava farmers and usually resulted from biotic and abiotic stresses, including insect and virus diseases, or from physiological deterioration during the post-harvest storage of roots (Ceballos *et al*. 2004). Major drawbacks of the cassava crop are the low protein content of roots and high content of the toxic cyanogenic glycosides linamarin and lotaustralin in all tissues (Jorgensen *et al*. 2005).

To improve yield, starch quality and nutritional value of

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cassava roots, many studies have been developed aiming to identify molecular markers and increase knowledge about plant genome structure and gene function. Molecular markers have contributed significantly in elucidation of genetic origin and in the assessment of genetic diversity of cassava as well as to construct a molecular map of crop. Efforts also have been focused in the identification of genes with traits of nutritional and agronomic importance and tissue-specific promoters that are essential for genetic improvement of cassava by biotechnological approaches. In this review we describe important advances in the genetics and genomics of cassava.

CASSAVA: THE PLANT, ITS USE AND FACTORS AFFECTING ITS YIELD

Cassava has a chromosome number of 2n=36, although some aneuploids have been reported for certain cultivars (Nassar 1978) but this is not common and polyploids are rare. Magoon *et al*. (1966) suggested that cassava was likely an allotetraploid.

This plant is a shrub, 1-3 m height with tuberous and adventitious roots (Nassar and Ortiz 2006). Basically every part of the plant can be utilized, but the starchy roots (**Fig. 1**) are by far the most commonly used product. The starchy roots are a valuable source of energy and can be boiled or processed in different ways for human consumption. Cassava roots are low protein content with an average of about 2-3% (dry weight basis).

Storage roots cannot be used for reproductive purpose, cassava is propagated vegetatively by stem cuttings which when planted produce sprouts and adventitious roots within one week. Seeds are slowing germinating and normally dormant. Dormancy is not break by seed scarification, but by thermal treatment at 18°C for 16 h and 26°C for 8 h (Nassar and Teixeira 1983).

Cassava is a very rustic crop that grows well under marginal conditions where few other crops could survive. Due to its drought tolerance, ability to grow in poor soils and resistance to herbivory cassava is well suited for cultivation and subsistence farmers (Nweke *et al*. 2002).

Cultivars of cassava have been classified according to morphology, e.g. leaf shape and size, plant height, stem color, petiole length and color, inflorescence and flower color, tuber shape and color and content of cyanogenic glycoside in the roots. Traditionally cassava varieties have been classified solely on the level of HCN content in the edible part of plant, which distinguish poison (bitter) with a high level (>100 mg/kg) of the cyanogenc glycoside, versus non-poison (sweet) cassava, which the glycoside is confined mainly to the peel and it at lower level (Nassar and Ortiz 2006).

Factors affecting yield in cassava include biotic and abiotic stresses, such as diseases, drought and soil acids or from deterioration during the post-harvest storage of roots. Many pathogens and pests reduce cassava yields, especially in Africa (Oerke 2006). Cassava mosaic disease (CMD) transmitted by whitefly (*Bemisia tabaci*) vector and spread by infected cuttings, cassava brown streak disease (CBSD), cassava bacterial blight (CBB) and anthracnose (*Colletotrichum gloeosporoides*) are among the most important diseases.

Fig. 1 Cassava (cv. 'IAC 12-829') storage roots.

GENETIC BREEDING OF CASSAVA

The utilization of inter-specific crosses in cassava breeding program was initiated by Storey and Nichols (1938) in Amani (Tanzania), where they obtained plants with resistance to CMD using cassava cultivars and accessions of wild relative of cassava, *Manihot glaziovii*. Each cross was followed by three backcrosses to cassava to recover desirable root characteristics. Hybrid clones were not only resistant to CMD but also productive and tolerant to drought (Otim-Nape 1993). These clones are still maintained at Amani and have been used by breeders of the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria) as a source of CMD resistance. In 1980, Hahn and coworkers also used inter-specific crosses between *M. esculenta* and *M. glaziovii* to develop plant with resistance to CMD and CBB.

In the 1950s, cassava improvement programs were initiated in Brazil by breeders of the Instituto Agronômico de Campinas (IAC, Campinas, Brazil) that identified the parental source of new cultivars which were selected on the basis of their productivity and resistance to diseases and insects. In this work were selected clones raised from natural inter-specific hybridization of wild species of *Manihot* normally growing in close proximity to cultivated cassava. Progeny seedlings of these natural crosses grow simultaneously and some of them were selected by farmers, reproduced vegetatively, giving rise to new clones that were grow in commercial plantations and subject to auto-pollination due to the monoclonal systems of plantations. Emerging homozygous plants have genes of wild species introgressed into their genomes (Nassar and Ortiz 2006).

In the 1970s, The Centro Internacional de Agricultura Tropical (CIAT, Cali, Colombia) initiated a cassava breeding program; since then, many cassava cultivars have been released in many countries, mainly in Asia (Kawano 2003). However, cassava breeding in Latin America seems to have achieved only limited success, e.g. cassava yield in Colombia in 2003 was just above 10t/ha – the same as in the early 1980s (Nassar and Ortiz 2006). Yield reduced in the whole continent from 13.8 t/ha in 1960s to 12.8 t/ha in the 1990s (FAO 2003). In contrast to Latin America, significant advances were achieved in cassava breeding programs of Asia, where cassava has changed from subsistence crop to an industrial cash crop. At the Central Tuber Crops Research Institute (CTCRI, India) cassava breeders produced the highest world yields, with productivity increased from 7.2 t/ha in 1961 to 26.9 t/ha in 2000 (FAO 2003).

Breeding programs in cassava have been conducted by different research centers in Brazil (Embrapa Mandioca e Fruticultura, Cruz das Almas, BA), Colombia (CIAT, Cali), Nigeria (IITA, Ibadan) and India (CTCRI). Breeding objectives depend on the ultimate use of the cassava; however the main breeding goal is high yield per unit area, particularly in marginal or pest-prone environments. Productivity plays a major role in the industrial use of cassava (i.e. starch production and dried roots for animal feed), whereas stability of production will be fundamental in the many regions where cassava is the main subsistence crop. Industrial uses of cassava require high dry matter content as the main quality trait for the roots, whereas human consumption will frequently emphasize cooking quality or starch characteristics over productivity (Ceballos *et al*. 2004). Cassava varieties with enhanced dry matter content and improved processing qualities have been

developed (Gonçalves Fukuda *et al*. 2000; de Vries and Toenniessen 2001; Gonçalves Fukuda and Saad 2001; Nweke *et al*. 2002; Jennings and Iglesias 2002). Other root quality traits relevant to different cassava breeding programs of the world are the cyanogenic potential in the roots (Dixon *et al*. 1994), higher protein content and reduced post-harvest physiological deterioration. Stability of cassava production is associated with resistance or tolerance to major biotic stresses, such as CMD and CBSD diseases. Manipulation of genes from wild species has led to new cultivars that resist prevailing diseases and pests, allowing the avoidance of large-scale famine in sub-Saharan Africa (Nassar and Ortiz 2006).

During the past 30-50 years, significant progress has been achieved in genetic improvement of cassava, however it is recognized that traditional breeding presents limitations in improving the crop to suit the varying needs of small farmers and commercial production in tropics (Jennings and Iglesias 2002; Kawano 2003). The development of new technologies in the area of molecular markers, genetic mapping and genomics have increased the knowledge about genetic diversity and genome of cassava as well as allowed the identification of new genes which could be integrated into farmer's preferred cultivars and landraces cassava through biotechnological approaches.

STUDYING THE ORIGIN AND GENETIC DIVERSITY OF CASSAVA BY MOLECULAR MARKERS

The genus *Manihot* contains about 100 species distributed throughout the New World tropics, from Mexico to Argentina. Most species occur in northern South America (~80 species), and there is a secondary center of species diversity in Central America and Mexico (~17 species).

For many years the genetic origin of cassava has been an intriguing theme for researchers; however only within the last decade has the relationship between cassava and its wild relatives begun to be clarified. Comparative studies of reproductive characteristics, botanic origin and phylogenetic relatedness of cassava with other *Manihot* species had been used as parameters to determine the origin of cassava. In the last taxonomic revision of *Manihot* described by Rogers and Appan (1973) it was proposed the cassava probably had multiple origins in Central and South America, with Central American species likely playing a key role in the crop's evolution; however studies reported by Allem (1987, 1994) indicated the *M. esculenta* ssp. *flabellifolia* as a potential direct wild progenitor of cassava.

The development of molecular markers has contributed significantly to the study of plant genetics, since they provide more useful information than morphological markers for studies on the domestication and evolution of plants. Morphological markers generally correspond to the qualitative traits that can be scored visually, while a molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be monitored.

Analysis of RFLPs (restriction fragment length polymorphism) and AFLPs (amplified fragment length polymorphism) indicated that South America and Central America *Manihot* species form two distinct lineages, and that cassava is more closely related to the South America lineage (Bertram 1993; Fregene *et al*. 1994; Roa *et al*. 1997). Studies reported by Olsen and Schaal (1999, 2001) revealed that cassava probably originated in wild *M. esculenta* populations growing along the southern rim of the Amazon Basin in Brazil. Cassava haplotypes of the gene glyceraldehyde-3-phosphate dehydrogenase (G3pdh) are present in these natural populations of wild *M. esculenta* subspecies, but absent in populations from others regions of Brazil (Olsen and Schaal 1999). Colombo *et al*. (2000) used RAPD (randomly amplified polymorphic DNA) and AFLP molecular markers to investigate the genetic relatedness between cultivated cassava and two naturally occurring species, *Manihot flabelifolia* and *Manihot peruviana*. The results obtained by Colombo and coworkers were consistent with the hypothesis that *M. flabellifolia* and *M. peruviana* gave rise to the cultivated species. SNPs (single nucleotide polymorphism) and SSR (single sequence repeat) analysis reported by Olsen (2004) confirmed that cassava originated in the Southern Amazon basin and it was domesticated from a single wild *Manihot* species, *M. esculenta* ssp. *flabellifolia*, rather than from multiple hybridizing species, as traditionally believed.

Biochemical and molecular markers have also been used to evaluate the genetic diversity within cassava plants including cassava landraces (Beeching *et al*. 1993; Bertram 1993; Chavarriaga-Aguirre *et al*. 1998; Fregene *et al*. 2000; Mühlen *et al*. 2000; Carvalho and Shall 2001; Fregene *et al*. 2003; Balyejusa *et al*. 2003). The term landraces, folk variety and primitive variety have been defined as distinct populations ecological or geographically originated from the local selection carried out by farmers (Brown 1978). There are several germplasm collections with hundreds to thousands of cassava accessions in national programs of Brazil, Thailand, Nigeria, Mozambique and Tanzania. In addition, the CIAT (in Colombia) and the IITA (in Nigeria) hold large germplasm collections. SSR markers has been identified in cassava and used with successful to evaluate the genetic diversity of the cassava core collection at the CIAT, in Colombia (Chavarriaga-Aguirre *et al*. 1998). Mühlen *et al*. (2000) have distinguished bitter from sweet cassava varieties studying the genetic diversity of cassava folk varieties from four Brazilian regions by AFLP, RAPD and SSR markers. SSR markers have also been used to study the genetic diversity in cassava germplasm from collection in Brazil (Carvalho and Schaal 2001) and in cassava landraces from Africa and Neotropics (Fregene *et al*. 2000, 2003).

Molecular markers associated with traits of agronomic interest have made a significant contribution in marker-assisted breeding programs of cassava, such as SSR and AFLP markers linked to the CMD-resistance gene identified in cassava landraces with highest resistant to this disease (Akano *et al*. 2002) and RAPD markers linked to resistance to anthracnose identified in cassava landraces by Akinbo *et al*. (2007).

Markers have also been used to generate a molecular genetic map of cassava. A first genetic map of cassava, estimated to cover 80% of the genome of the crop, was constructed with a total of 300 RFLP, RAPD, SSR and isozyme markers; more than 70% are RFLP markers (Fregene *et al*. 1997). Recently a new genetic map of cassava based on SSRs was created by Okogbein *et al*. (2006); while FRLPs are expensive and require facilities for radioactive procedures, PCR-based markers systems such as SSR are easy to implement in most laboratories and they produce a result within a day.

The genetic resource of cassava and its wild relatives represent a critical resource for the future of the crop. Studying the organization of genetic diversity is expected to benefit cassava germplasm conservation and it's exploiting as source of agronomic traits useful to genetic improvement of cassava. While wild *Manihot* species had been used in inter-specific crosses with cassava cultivars to introgress useful alleles, the exploiting of cassava landraces as a new source of agronomic traits is recent. Studies have indicated that cassava landraces have acquired thorough their domestication a large diversity in relation to many economic traits, such as a high content of carotenoids (Carvalho *et al*. 2002a, 2004a; Nassar *et al*. 2007) and accumulation of unusual carbohydrates (Carvalho *et al*. 2004b).

CASSAVA GENE DISCOVERY

The gene cloning procedure was created in early 1970s with the development of recombinant DNA technology, since then, rapid progress have been achieved in developing tools for the manipulation of genetic information in plants; many genes coding for agriculturally and socio-economically important traits have been identified and transferred to crop varieties by biotechnological approaches.

The recent development of high-throughput analysis such as transcriptome and proteome has allowed functional genomic studies where genes and proteins expressed in different tissues, developmental stages and/or environmental conditions are identified in a large scale. The technology of sequencing expressed sequence tags (ESTs) offers a relatively cheap alternative to whole genome sequencing and has become a valuable resource for gene discovery. ESTs sequences from many plant species have been isolated and used for development of functional molecular markers, preparation of transcript maps and construction of cDNA microarrays.

Despite application of genomic approaches in cassava have initiated few years ago significant advances have been achieved; at present the number of publicity available cassava ESTs is estimate in 36,120. Progress in gene discovery of cassava is described below.

PROSPECTING GENES RELATED TO BIOTIC AND ABIOTIC STRESS RESISTANCE

Losses in yields to cassava farmers are usually attributed to biotic and abiotic stresses such as diseases, drought and acidic soils. Virus and bacterial diseases are major contributors to cassava yield reductions in Africa and South America. Cassava Mosaic Disease (CMD), caused by a complex of geminiviruses, represents a major constraint on cassava production in Africa, while Cassava Bacterial Blight (CBB), caused by the bacterium *Xanthomonas axonopodis* pv. *manihots* (*Xam*), is an important disease, endemic to Latin America and Africa. 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) and is caused by a virus of the genus *Ipomovirus* (Monger *et al*. 2001).

The development of gene cloning procedures and genomics technologies has allowed the characterization of many genes from different plant species that provide resistance to a variety of pathogens and pest diseases. The identification of genes related to disease resistance is essential for understanding plant-pathogen interactions and known molecular mechanisms involved in resistance process; furthermore, disease resistance genes have been employed successfully in the production of transgenic plants of different crops (Latha *et al*. 2005; Zuo *et al*. 2007).

Plants employ a variety of defense mechanisms during a resistance response to pathogens, including the use of mechanical barriers, defense proteins and defensive enzymes. This change in the biochemistry activity of the plant includes, among others, the production of proteases, pathogen-related proteins and several enzymes, such as peroxidase (Develey-Rivière and Galiana 2007; He *et al*. 2007). The presence of conserved domains in the protein sequence of resistance gene (*R*-gene) products allows their classification in different groups (Deng 2006). The most common group of *R*-genes is characterized by a presence of a Leucine Rich Repeat (LRR) domain in the corresponding protein products (Hammond-Kosack and Jones 1997).

Studies aiming to isolate and characterize disease resistance genes in cassava have been initiated recently (Lopez *et al*. 2003; Pereira *et al*. 2003; Anderson *et al*. 2004; Fregene *et al*. 2004; Lopez *et al*. 2004, 2005). Resistance Genes Analogues (RGAs) have been identified in cassava using PCR assays with degenerate primers targeting the Nucleotide Binding Site (NBS) and Toll/Interleukin-1 Receptor (TIR) domains of *R*-genes by Lopez *et al*. (2003). About 60% of *R*-genes are clustered in genomes, as shown by genetic and molecular maps (Hulbert *et al*. 2001) and *R*-genes present in a given cluster can confer resistance to different strains of the same pathogen or to diverse pathogens (van der Vossen *et al*. 2000). RGAs identified in cassava could be related to CMD and CBB resistance and they may provide markers tightly linked to *R*-gene loci, being valuable in marker-assisted selection of breeding for resistance. The cloning of the peroxidase gene offers a potential marker for selecting cassava clones for resistance to bacterial blight (Pereira *et al*. 2003).

As a first investigation using genomic approaches in cassava, Lopez *et al*. (2004) reported the identification of a unigene set of 5700 sequences obtained from different cDNAs libraries of plant tissues challenged by the pathogen *Xam* and root tissues of varieties with high and low starch contents. Preliminary analyses indicated genes putatively involved in the plant defense response to pathogen infection detected only in infected libraries, such as, genes homolog to R-proteins, chitinase and receptor-like protein kinase. In additional studies, these 5700 sequences were used in a cDNA microarray to analyze the incompatible interaction between cassava and *Xam* strain CIO151 (Lopez *et al*. 2005). Expression profiling and cluster analysis indicate that, in response to inoculation with *Xam*, cassava induces dozens of genes, including principally those involved in oxidative burst, protein degradation and pathogenesis-related genes.

ESTs homologous to genes expressed during systemic acquired resistance (SAR) in plants and other genes involved in cell-to-cell and cytoplasm-to-nucleus virus trafficking were isolated from cassava resistant CMD genotypes by Serial analysis of gene expression (SAGE) (Fregene *et al*. 2004). In addition, Anderson *et al*. (2004) isolated about 23,000 ESTs from various cassava tissues and genotypes that will be a valuable resource in study of genetic diversity, stress resistance and growth and development not only cassava, but also other members of the Euphorbiaceae family.

Drought is one of the major abiotic stresses limiting cassava productivity in worldwide. In plants, drought triggers a wide variety of response including changes in gene expression, the synthesis of specific proteins (e.g., chaperones proteins and late-embryogenesis-abundant proteins), and the accumulation of metabolites or osmotically active compounds. Physiologically, tolerance to drought is a complex phenomenon involving drought escape, dehydration avoidance, dehydration tolerance and desiccation tolerance mechanisms (Blum 1998). Genetically, tolerance to dehydration stress is a multifactorial trait, which makes breeding for drought tolerance arduous. In the past decade, significant progress has been made in elucidation of abiotic stress pathways at the molecular level in plants, and this knowledge has been useful to the production of transgenic plants tolerant to drought, cold and salt (Dai *et al*. 2007; Peng *et al*. 2007).

Studies aiming to increase knowledge about cassava drought tolerance at the molecular level were reported by Lokko *et al*. (2007) who characterized 18,166 ESTs resulting in a set of 8577 sequences. cDNA libraries were prepared with dehydration-stressed and control well-watered tissues from a cassava landrace (genotype TME117) grown in humid and sub-humid conditions. The dehydration-stressed library uncovered numerous ESTs with recognized roles in drought response, including those that encode late-embryogenesis-abundant proteins, transcription factors, heat-shock proteins as well as proteins related to signal transduction and oxidative stress.

IDENTIFICATION OF GENES AND PROTEINS RELATED TO STORAGE ROOT FORMATION IN CASSAVA

The storage root is the most important product of cassava as food. It is a rich source for carbohydrates but is low in proteins and micronutrients (Cock 1985). Depending on the cassava cultivar, the protein content ranges from less than 1% to 5% (dry weight); furthermore, this very low protein amount presents a reduced nutritional value due to its low level of essential amino acids (Gomez and Noma 1986; Ngudi *et al*. 2002).

Cassava storage roots result from swelling of fibrous roots by secondary growth. The physiological process by

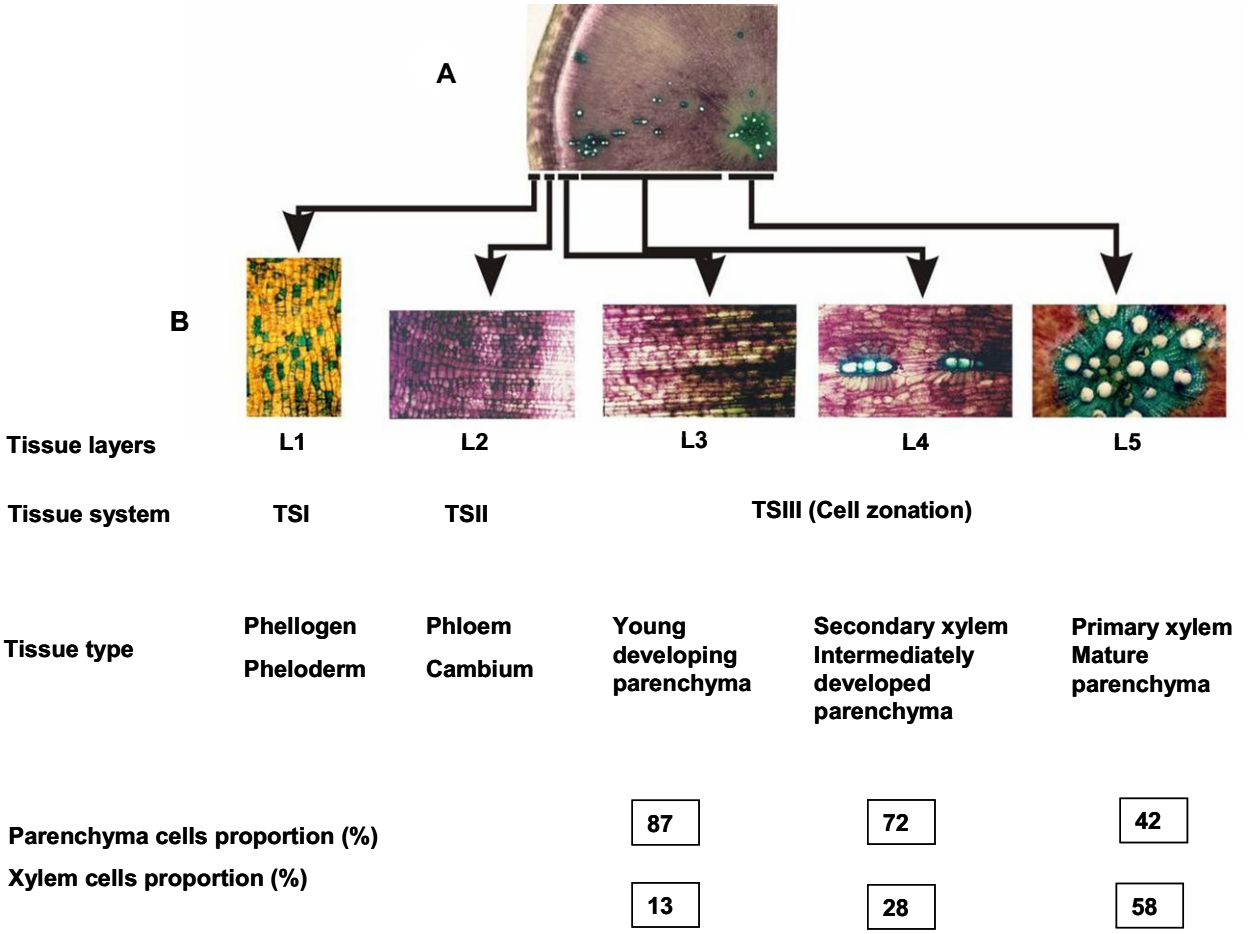

Fig. 2 Identification of tissue systems and layers in a cassava storage root. (**A**) Cross section with *O*-toluidine blue staining. (**B**) Tissue layers (L1 to L5), tissue systems (TSI to TSIII) and tissue type (phellogen, phelloderm, phloem, cambium, young developing parenchyma, intermediately developed parenchyma, mature parenchyma, primary xylem and secondary xylem). Note that layers L3, L4 and L5 belong to the same tissue system. Adapted from de Souza *et al*. (2006).

which a stem section or a root undergoes morphological changes to become a special storage organ is termed tuberization (Melis and van Staden 1984). The tuberous root of cassava is a vegetative structure, and has none of the reproductive properties associated with other storage organs such as potato tuber. Cassava storage root formation occurs through massive cell division and differentiation of parenchyma cells of the secondary xylem which is originated from vascular cambium (Ramanujam and Indira 1984).

Progress in cassava *in vitro* tuberization was recently reported by Medina *et al*. (2007). Anatomical analysis of storage roots formed *in vitro* showed radial expansion as a consequence of massive proliferation and enlargement of parenchyma cells occurring in the middle cortex, but not from cambial activity as in roots formed *in vivo*; however this cortical expansion could be related to dilatation growth induced by hormone treatments.

The identification of genes and proteins expressed in cassava storage roots might help to understand how the storage root is formed as well as to elucidate mechanisms involved in low protein content. Furthermore, specific promoter or regulatory elements of these genes might be used to drive efficient storage root expression.

Several proteins related to storage root formation in cassava have been identified using genomics and proteomics approaches (Cabral and Carvalho 2001; de Souza 2001; de Souza *et al*. 2002, 2003; Zhang *et al*. 2003a; de Souza *et al*. 2004, 2006; Sheffield *et al*. 2006). An anatomical model with three tissue compartmentalization systems has been used in gene expression studies of cassava storage root (Cabral *et al*. 2000; de Souza 2001, de Souza *et al*. 2002, 2003; Carvalho *et al*. 2004b; de Souza *et al*. 2006). According to this model: tissue system I, is composed of phellogen and phelloderm, tissue system II of phloem and vascular cambium, and tissue system III of secondary xylem with its highly specialized starch-rich parenchyma cells (**Fig. 2**).

The Pt2L4 is a glutamic acid-rich protein codified by the *Mec1* gene predominately expressed in the tissue system III (de Souza *et al*. 2002, 2006) and related to secondary growth in storage roots of cassava (Carvalho *et al*. 2002b). Zhang *et al*. (2003a) isolated two endogenous cassava promoters (C15 and C54) with strong activity in vascular tissues and in parenchyma cells of storage roots; therefore these promoters are important candidates for regulating transgene expression in cassava storage roots. The cassava C15 protein has high similarity to cytochrome P450 proteins from other species while C54 is very similar to the Pt2L4. The Pt2L4 and C54 cassava proteins are 60% identical with 18.0 and 16.7 kDa molecular weights and 3.70 and 3.97 isoelectric points, respectively. There are two or more homologue genes coding for glutamic acid-rich proteins in cassava genome according to Southern blot analysis (Zhang *et al*. 2003a; de Souza *et al*. 2006). Recently we isolated a promoter sequence of *Mec1* gene that contains some conserved *cis*-acting regulatory elements, such as those related to expression in vascular tissues of roots and light response, according to *in silico* analysis (unpublished data). Further experiments will focus in the functional studies of this promoter.

Differential gene expression analysis in fibrous and storage roots has increased our understanding about the molecular mechanisms underlying cassava tuberization. Protein polymorphism of adventitious and storage roots of cassava have been studied in a 2-D gel system and the results showed over 260 proteins unique to the storage root and

possibly related to secondary growth (Cabral and Carvalho 2001). Comparative gene expression study using northern blot analysis have revealed five genes up-regulated in storage roots, among them, the *Mec1* gene and cassava genes coding to putative RING Zinc Finger, calmodulin and TCTP proteins (de Souza *et al*. 2004). Recently, Sheffield *et al*. (2006) reported a large-scale protein identification study in fibrous and storage roots of cassava using high-resolution 2-DE. They identified 237 proteins span various functional categories from energy, primary and secondary metabolism, disease and defense, destination and storage, signal transduction, protein synthesis, cell structure and transcription to cell growth and division. Gel image analysis has shown unique, as well as up and down-regulated proteins, present in the storage and the fibrous tissues.

IDENTIFICATION OF GENES EXPRESSED DURING POST-HARVEST PHYSIOLOGICAL DETERIORATION

Post-harvest physiological deterioration (PPD) is an endogenous root disorder affecting the storage roots of cassava. After 24-72 h of harvest, roots exhibit a blue-black discoloration of the xylem vessels known "vascular streaking" and display other organoleptic properties such as undesirable flavor and odor. Vascular streaking initiates at wound sites caused by mechanical damages during harvesting (Booth 1976) and it is resulted from peroxidase mediated oxidation of scolpoletin (Tanaka *et al*. 1983). Other biochemical changes during PPD include increases in respiration (Hirose 1986), changes in lipid composition (Lalaguna and Agudo 1989), secondary metabolite accumulation (Rickard 1981; Uritani *et al*. 1983), the synthesis of the phytohormone ethylene (Hirose 1984) and the occurrence of a wound-induced oxidative burst (Reilly *et al*. 2003). Increases in activity of a range of enzymes including phenylalanine ammonia-lyase (PAL), acid invertase, catalase, dehydrogenase, peroxidases and polyphenol oxidase (Rickard 1981; Tanaka *et al*. 1983) are detected during PPD. The secondary metabolites that accumulate during PPD include diterpenic and phenolic compounds (Sakai and Nakagawa 1988). Peaks of reactive oxygen species (ROS) and increased activity of enzymes that modulate ROS are detected during deterioration (Reilly *et al*. 2001).

Several genes coding to proteins involved in post-harvest physiological deterioration have been isolated and used in gene expression analysis (Han *et al*. 2001; Huang *et al*. 2001; Reilly *et al*. 2001, 2003) that demonstrated altered regulation of proteins and enzymes involved in signal transduction, ROS modulation, phytohormone synthesis, senescence and programmed cell death (PCD) responses, synthesis of anti-microbial, anti-oxidant and other defensive compounds. Recently, Reilly *et al*. (2007) employed a cDNA-microarray constructed from cassava roots submitted to deterioration in order to identify PPD-responsive genes. In this work were identified 72 non-redundant expressed sequence tags which showed altered regulation during the post-harvest period. Of these 63 were induced while 9 were down-regulated. Many of the up-regulated and PPD specific expressed sequence tags were predicted to play a role in cellular process including programmed cell death, reactive oxygen species turnover, cell wall repair, signal transduction, stress response and activation of protein synthesis.

ADVANCES IN GENETIC ENGINEERING OF CASSAVA

Genetic engineering is a powerful tool that complements traditional breeding and can extend the genetic pool of useful gene source beyond the species. Transgenic technology also offers the advantage of transferring single or even quantitative traits, without the problems of linkage encountered in traditional breeding such as outcrossing, and inbreeding depression, inherent to this vegetatively propagated crop.

Biotechnological approaches require necessity to dominate transformation and regeneration of the host crops. Another important requirement is the availability of suitable transgenes coding for agriculturally and socio-economically important traits and tissue and/or development-specific promoters that guarantee the transgene's correct expression.

In the last five years significant advances were achieved in the establishment of methodologies to genetic transformation of cassava.

GENE TRANSFER TECHNOLOGIES IN CASSAVA

According to a report by Taylor *et al*. (2004) four distinct transformation systems have been employed to produce genetically transformed cassava plants. All transformation systems require as an initial step, the production of embryogenic tissues from in vitro leaf-lobe explants. Embryogenic tissues are used to produce four target tissues (friable embryogenic callus (FEC), cotyledons fragments, embryogenic structures and immature leaf explants) that can be transformed by microparticle bombardment or *Agrobacterium tumefaciens* and plants can be regenerated by somatic embryogenesis or shoot regeneration (Taylor *et al*. 2004). Due problems of somaclonal variation within plant regenerated from embryogenic suspensions, studies have been addressed to the utilization of FEC cultures as a target for transgene insertion (Raemarkers *et al*. 2001; Taylor *et al*. 2001; Ibrahim *et al*. 2007) (**Fig. 3**). Cassava transformation procedures using *A. tumefaciens* have been employed with more frequency due high recovery of transgenic plants con-

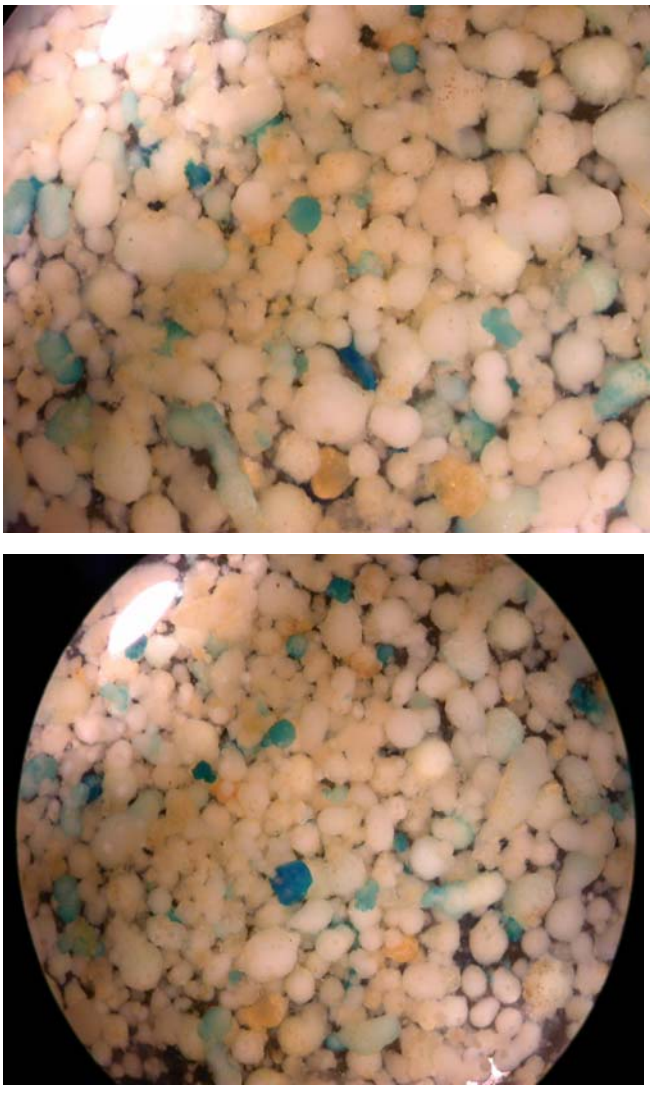

Fig. 3 Transient expression of GUS gene in cassava friable embryogenic *callus* (Note: Figure kindly provided by Ibrahim *et al*. 2007).

taining single-copy insertions of transgene when compared to microparticle bombardment (Taylor *et al*. 2004). Molecular confirmation of transgene integration in plant regenerated has been developed by southern analysis and PCR amplifications. A range of selectable and visual markers genes have been used to monitor genetic transformation procedure and investigate transgene expression pattern, such as the *npt*II gene, GUS, GFP and luciferase.

With the establishment of methodologies to cassava transformation many plants expressing traits of agronomic interest have been generated. Examples of applications of transgenic technologies in cassava are described below.

PRODUCING AN ACYANOGENIC CASSAVA

The leaves and roots of cassava contain potentially toxic levels of cyanogenic glycosides (linamarin (95%) and lotaustralin (5%) (Conn 1979, 1994). These cyanogens have been demonstrated to protect the plant from herbivory by animals and generalized insect feeders (Bellotti and Arias 1993). Significantly, cyanogenic levels in leaves (200-1300 mg CN equivalents/kg dry weight) and roots (100-500 mg CN equivalents/kg dry weight) are higher than the maximum levels (10 mg CN equivalents/kg dry weight) recommended for foods by the FAO. Therefore, cassava foods must be processed to remove cyanogenic *priori* to consumption. In Africa, a number of cyanide-associated health disorders have been attributed to ingesting poorly processed cassava (Cliff *et al*. 1985; Delange *et al*. 1994). The severity of these disorders depends on the level and frequency of cyanogenic exposure and the state of nutrition of the consumer (Osuntokun 1981).

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. In the last years some studies have been addressed for production of acyanogenic cassava by biotechnological approaches (Siritunga and Sayre 2003, 2004; Jorgensen *et al*. 2005). Transgenic cassava plants with a 95% reduction in cyanogenic glycoside content in roots and acyanogenic (<1% of wild type) in leaves were obtained by RNA interference to block expression of the CYP79D1 and CYPD79D2 genes encode two highly similar cytochrome P450s that catalyze the first-dedicated step in linamarin and lotaustralin synthesis (Jorgensen *et al*. 2005) (**Fig. 4**).

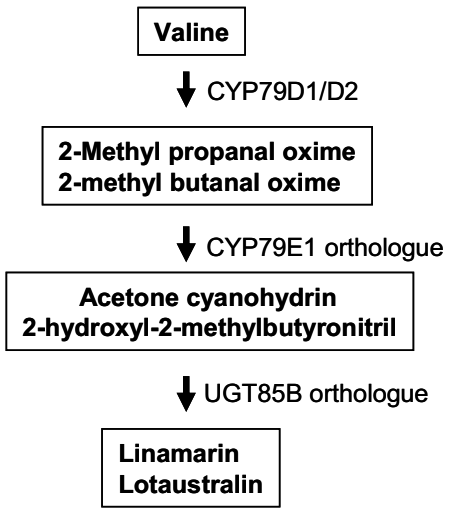

Fig. 4 Linamarin and lotaustralin biosynthesis in cassava. Adapted from Jorgensen *et al.* (2005).

IMPROVEMENT OF NUTRITIONAL VALUE OF ROOTS

Storage root of cassava provides a rich source for carbohydrates but is low in vitamins and proteins. Furthermore, this very low protein amount presents a reduced nutritional value due to its low level of essential amino acids (Gomez and Noma 1986; Ngudi *et al*. 2002). Consequently, individuals consuming exclusively or predominantly cassava usually suffer from protein-deficiency symptoms. Therefore, elevation of cassava's protein content among those people could play a crucial role in the reduction of protein energy malnutrition.

Plant biotechnology can make important contributions to food security and malnutrition improvement. "Golden Rice" is a variety of rice (*Oryza sativa*) produced through genetic engineering to biosynthesize the precursors of β-carotene (pro-vitamin A) in the edible parts of rice (Ye *et al*. 2000; Beyer *et al*. 2002). The albumin AmA1 from *Amaranthus hypochondriacus* seeds was successfully expressed in the starch tissue of potato tubers (Chakraborty *et al*. 2000).

Investigations aiming to increase protein content in cassava roots by technological approaches were initiated by Zhang *et al*. (2003b) who produced plants genetically transformed 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 *ASP1* gene at both the RNA and protein levels. Total protein content of *in vitro* leaves were similar from non-transgenic plants, however levels of the amino acids proline and serine were elevated and asparagine, alanine and methionine were reduced compared to controls (Zhang *et al*. 2003b), indicating that the expression of *ASP1* can indirectly affect amino acid metabolism because of its high essential amino acids content (Stupak *et al*. 2006).

Recent studies reported by Nassar and Sousa (2007) revealed a interspecific hybrid originated from crosses between cassava × *Manihot oligantha* containing 10 times more lysine and 3 times more methionine than the common cassava cultivar. Besides wild *Manihot*, exploiting indigenous landraces as a source of nutritional traits, such as high carotene content, could also be used in the improvement of cassava roots. Studies in the areas of genomics, proteomics and metabolomics could increase our understanding about amino acid metabolism and carotenoid synthesis in different cassavas, allowing the development of strategies to manipulate the content of these compounds in cassava farmer-preferred cultivars.

CASSAVA RESISTANT TO INSECTS

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 hornworm (*Erinnyis ello*), whitefly (*Aleurotrachelus socialis*) and stem borers (*Chilomina clarkei*) (Bellotti 2002).

The cassava stem borer is endemic in the Colombian North Coast and can cause losses of 50-100% of cassava stakes and result in a severe shortage of planting material. 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.

Resistance to stem borer has been introduced in important Colombian cassava cultivars expressing the *cry1Ab* gene under control of the CaMV 35S promoter (Ladino *et al*. 2002). The CIAT CORPOIOCA in Colombia released recently a new cassava variety, Nataima-31, with resistance to whitefly (Vargas Bonilla *et al*. 2002).

MANIPULATION OF STARCH CONTENT

Cassava storage roots contain large quantities of starch (74-85% dry weight). Plant starch consists of two polymers:

amylopectin (70-80%) and amylose (20-30%) that have the same basic structure, but differ in their length and degree of branching, which ultimately affects the physicochemical properties of these polymers. Amylose is an essentially linear polymer of glucosyl residues linked via α-1,4 glucosidic linkages, whereas amylopectin exists as a branched α-1,4:α-1,6 D-glucan polymer (Ball *et al*. 1996; Ball and Morell 2003). The relative amounts of amylose and amylopectin are what give starches their unique physical and chemical properties, which convey specific functionality and could be of biotechnological importance (Myers *et al*. 2000; Charles *et al*. 2005). High amylose starches are widely used as thickeners, are strong gelling agents used in the production of jellies and gum candies (confectionery) while starches with high levels of amylopectin are broadly used by the food industry to improve uniformity, stability and texture.

The synthesis of starch in plant cells begins with the enzyme ADP-glucose pyrophosphorylase (AGPase), which catalyses the reaction of glucose-1-phosphate with ATP to form ADP-glucose (liberating pyrophosphate). The ADP-glucose is then used a substrate by granule-bound starch synthase (GBSS), which add glucose units to the end of a growing polymer chain to build up a starch molecule. Branches in the chain are introduced by branching enzymes (BEs), which hydrolyze 1,4-glycosidic bonds, and in their place, create 1,6 bonds with other glucose units. Further alterations in starch structure might occur because of the action of enzymes generally relegated to a degradative role in starch metabolism, the starch debranching enzymes (DBEs), phosphorylases and glucanotransferases. 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 quality. More detailed reviews are available from Ball *et al*. (1996) and Smith (1999).

Approaches altering starch quality and quantity by manipulation of starch biosynthetic enzymes have been conducted in different crops (Stark *et al*. 1992; Regierer *et al*. 2002; Ihemere *et al*. 2006). Transgenic cassava plants producing an amylose-free starch by expression of the *gbss* gene encodes a granule-bound starch synthase from cassava in the antisense orientation under control of the CaMV 35S promoter and the gbss promoter from potato has been reported by Raemakers *et al*. (2003). Recently Ihemere *et al*. (2006) reported the transformation of cassava plants using an *E. coli glgC* gene with modification which reduces allosteric feedback regulation by fructose-1,6-bisphosphate. Interestingly transgenic plants have nearly two-fold higher AGPase activities and two-fold greater root and top (stem and leaves) biomass than wild-types plants.

PERSPECTIVES WITH CASSAVA GENOME SEQUENCING

Functional genomic studies in cassava were initiated generating about 36,000 ESTs currently available in GenBank database. Besides ESTs, cassava germplasm collections, including its wild relatives and indigenous landraces, and molecular genetic maps are also available to support cassava improvement programs.

The sequencing of cassava was recently proposed by The Global Cassava Partnership (GCP-21), an alliance of the world's leading cassava researchers and developers acting under the support of the Food and Agriculture Organization (FAO). Information generated with genome sequencing will complement preliminary data of functional genomic studies, identifying cassava genes targets for biotechnology approaches and providing tools to explore the large biodiversity within cultivated and wild species. A long time the benefits of cassava genome sequencing could generate new higher-yielding or more pest- and disease-resistant cultivars as well as cassava with improved nutritional value to poor people from developing countries.

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