SOMATIC EMBRYOGENESIS AND TRANSFORMATION OF CASSAVA FOR
ENHANCED STARCH PRODUCTION.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of
Philosophy in the Graduate School of The Ohio State University

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*****

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Cassava (*Manihot esculenta* Crantz), a member of the family Euphorbiaceae, is one of the most important food crops of sub-Saharan Africa. It is grown throughout the tropics including Asia and Latin America. The five main producers are; Nigeria, Brazil, Thailand, Zaire and Indonesia, which together account for 63% of the total world production of 120-130 million tons of fresh roots per year. The roots are the primary plant part consumed and store abundant amounts of starch. They provide a valuable source of cheap calories for about 500 million people, many of whom are subsistence farmers. In addition, cassava leaves and tender shoots are eaten as a vegetable in many parts of Africa and are an excellent source of vitamins, minerals and protein. Although most cassava is consumed by humans, it is also used in the production of ethanol for fuel, for animal feed, and as a raw material for the starch industry.

Cassava’s high photosynthetic rate, ability to grow on poor soils and its resistance to many pests and herbivores due to the presence of cyanogens make it an ideal crop for subsistence farmers. Furthermore, cassava is largely propagated clonally making it an ideal plant for improvement through genetic engineering.
One of the requirements for the generation of genetically engineered cassava is an efficient and reproducible plant regeneration and transformation system. One of my research objectives was to improve upon current technologies used for cassava transformation. We describe here improved methods for plant regeneration of recalcitrant African cassava cultivars. This technology will allow us to increase the range of cultivars that can potentially be engineered using recombinant DNA technologies.

One of the constraints for cassava starch production is the long growing season. Cassava typically takes 9-12 months to yield a good harvest. This is longer than other major starch-producing crops such as corn and potatoes. The longer growing season of cassava also means that it may need more maintenance in the field than other crops. One objective of my research program was to increase the starch biosynthesis capacity of cassava by enhancing the enzyme activity of ADP-glucose pyrophosphorylase (AGPase), the rate-limiting enzyme in starch biosynthesis. To do this, we transformed cassava with a modified *E. coli* *glgC* gene that encodes AGPase. The *glgC* gene was modified by site-directional mutagenesis (K296E/G336D) to remove the allosteric regulation (enhancement by fructose-1, 6-P and inhibition by adenine monophosphate (AMP) sites and to increase the velocity of the enzyme. Root-specific expression of the *glgC* gene product was achieved using the tuber-specific patatin promoter of potato. We obtained antibiotic-resistant putative transformed plants which have been shown to have integrated
and expressed the transgene by PCR, Southern blot, RT-PCR, and enzyme activity analyses. AGPase enzyme activity in transformed plants was increased by more than 65%. Transgenic plants expressing the bacterial \( glgC \) gene had two-fold greater stem and root biomass than wild-type plants.

Cassava has a high efficiency of photosynthetic conversion of carbon dioxide into assimilates. We also postulated that we could also enhance starch biosynthesis by increasing sucrose biosynthesis in cassava leaves. This was done by transforming cassava with maize \( sps \) gene that encodes sucrose phosphate synthase, the enzyme that catalyzes sucrose synthesis in leaves. Expression of the \( sps \) gene in leaves was driven by the CAB1 promoter which is leaf specific. Southern blotting, RT-PCR and sucrose phosphate synthase enzyme assays were used to demonstrate enhanced expression of the maize \( sps \) gene and increased SPS enzyme activity (58% to 82%). Given the long-term nature of field trials it is estimated that growth analysis studies will extend an additional year from the current time.
To my parents, Simon D. Ihemere and Maggie A. Ihemere
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ABREVIATION.

2,4-D – 2,4-dichlorophenoxyacetic acid; 3D-1, 3D-2, 3D-3 – glgC transformed plants; ACMV – African cassava mosaic virus; ADP- adenine diphosphate; AGPase - ADP-glucose pyrophosphorylase; AMP- adenine monophosphate; ANOVA – analysis of variance; ATP – adenine triphosphate; B5 - Gamborg et al medium; BA - 6-benzyladenine; C3 – carbon photosynthetic cycle; CAB1- chlorophyll a/b promoter; CAMV – cauliflower mosaic virus; CN – cyanide; DNA - deoxyribonucleic acid; CYP D1 – cytochrome P450 D1 gene; FEC – friable embryogenic callus; glgC - gene that encodes ADP-glucose pyrophosphorylase in E. coli; GBSS – granule bound starch synthase; GA3 – gibberellic acid 3; HCN – hydrogen cyanide; HNL - hydroxynitrile lyase; hr – hour; NOS – nopaline synthase; npt II - neomycin phosphotransferase II; MCol 122 – cassava cultivar Manihot columbia 122 ; MCol 2215 – cassava cultivar Manihot columbia 2215; MPer 183 – cassava cultivar Manihot Peru 183; µL – micro liter; µM – micro molar; MS – Murashige and Skoog medium; NAA – naphthalene acetic acid; NADPH – nicotinamide adenine diphosphate, reduced; PCR - polymerase chain reaction; PCS – plasmid containing CAB1 promoter and SPS; RNA – ribonucleic acid; RT-PCR - reverse transcription- polymerase chain reaction; SAG-12 – senescence associated gene 12; SBE – starch branching enzyme; SPS - sucrose phosphate synthase,
SS – starch synthase; SSS – soluble starch synthase; SuSy – sucrose synthase; T-DNA – transfer DNA; TMS 60444 – cassava cultivar Tropical Manihot Selection 60444; TMS 71173 cassava cultivar Tropical Manihot Selection 71173; TP – transit peptide; TP/glgC cassette carrying TP/glgC; WT – wild type; w/v – weight to volume ratio; UDP – uridine diphosphate;
CHAPTER 1

REVIEW OF LITERATURE

1.1 BIOLOGY OF CASSAVA

Cassava (Manihot esculenta Crantz) is a member of dicotyledonous family Euphorbiaceae (Alves, 2002). The genus Manihot has been reported to have about 100 species of which M. esculenta is the only agronomically important species. Cassava is a shrub which grows between 1-4 meters in height (Figure 1.1) and is characterized by sympodial branching. The main stem is typically divided into two – four branches that may have branches of their own. Branching is followed shortly by flowering. This developmental series has been termed reproductive flowering (Alves, 2002). However, in some cases, the flower buds are aborted before maturity, (a testament to its poor flower set), giving the impression that there might be cassava stem branching without the accompaniment of flowering (Alves 2002).

Cassava carries out C₃ photosynthesis (Edwards et al., 1990; Angelov et al., 1993; Ueno and Agarie, 1997). The maximum photosynthetic rates varies from 13 to 24 µmol CO₂ m⁻² s⁻¹ under greenhouse or growth chamber conditions (Mahon et al., 1977, Edwards et al., 1990) and from 20 to 35 µmol CO₂ m⁻² s⁻¹ in the field (El-Sharkawy
Figure 1.1: Cassava plants growing in the field (IITA website, www.iita.org)
and Cock 1990). Cassava has a high CO$_2$ compensation point, typical of C3 plants. The optimal temperature for photosynthesis for field grown cassava is 35 °C but the range for optimal photosynthesis is 25 to 45 °C (El-Sharkawy and Cock 1990). Thus, cassava is adapted to the tropical environment.

All cassava organs apart from seeds contain cyanogenic glycosides. Cultivars with less than 100 mg CN equivalents kg$^{-1}$ fresh weight in their roots are called ‘sweet’ while cultivars with 100 – 500 mg CN equivalents kg$^{-1}$ are classified as ‘bitter’ cassava (Wheatley et al., 1993). The most prevalent cyanogenic glycoside is linamarin (95%) with lesser amounts of lotaustralin. The total amount of cyanogenic glycoside is dependent on cultivar, cultural practice, environmental conditions and plant age (McMahon et al., 1995). Linamarin is synthesized in the leaf and transported to the roots (Wheatley and Chuzel, 1993; Siritunga and Sayre, 2003). The hydrolysis of linamarin and breakdown of acetone cyanohydrin by hydroxynitrile lyase or elevated pH (> 5.0) leads to the release of HCN which is poisonous (Cooke and Coursey, 1981). Compartmentalization of linamarase in cell walls and laticifers and linamarin in the vacuole guards against the release of HCN in intact plant tissues (McMahon et al, 1995). Processing of cassava roots brings enzyme and substrate together leading to the hydrolysis of linamarin and breakdown of acetone cyanohydrin to produce cyanide (Mkpong et al., 1989, Wheatley and Chuzel, 1993, McMahon et al., 1995, White et al., 1998).
Post harvest deterioration is a major constraint for marketing fresh cassava roots. Roots are very perishable and deteriorate within 24-72 h after harvest. This deterioration is manifested by the production of phenolic compounds whose polymerization leads to the discoloration of the roots (Wheatley and Chuzel, 1993). This process is oxygen dependent and can be inhibited by placing the roots in an anaerobic environment (Wheatley and Chuzel, 1993). Tissue dehydration at the sites of mechanical damage enhances the rapid onset of deterioration (Ghosh et al., 1988). Secondary deterioration is caused by microbial infection of damaged tissues (Wheatley and Chuzel, 1993).

The main carbohydrate storage organ in cassava is the root (Fig. I.2). Cassava is propagated by stem cuttings. Plants propagated in this way don’t have the typical dicot taproot system. Instead adventitious roots arise from the base of the cut surface or from subterranean buds. Some of the adventitious roots transform to storage roots while others remain fibrous and serve to supply water and nutrients to the plant. Cassava storage root is not anatomically a tuber or stem due to the organization of the vascular tissue (Wheatley and Chuzel, 1993). The fully developed cassava storage root comprises three sections: the periderm (bark), cortex (peel) and the parenchyma. The parenchyma is the edible part which consists mainly of starch and makes up about 85% of the total root mass. The parenchyma is interspersed with xylary tissues (Wheatley and Chuzel, 1993). The cortex is made up of sclerenchyma, cortical parenchyma and phloem and accounts for 11-20% of the root weight (Barrios and Bressani, 1967). The periderm comprises about 3% of the total root mass and is a thin layer that can be readily removed from the
Figure 1.2: Cassava root freshly harvested from the field (IITA website, www.iita.org)
exterior of the root. The size and shape of roots is contingent on the cultivar and environmental factors (Wheatley and Chuzel, 1993).

Cassava leaves are lobed with palmate veins. The number of leaf lobes range from three to nine. Leaves produced at the time of flowering are reduced in size and lobe number. The leaves closest to the inflorescence are commonly simple and have no lobes. The leaves have an alternate arrangement and a phyllotaxy of 2/5. The mature leaves are glaborous and leaves are surrounded by two stipules. The adaxial leaf surface is characterized by a waxy epidermis that gives it a shiny appearance. The stomata are located mainly on the lower leaf surface (Cerqueira, 1989). Only 2% of the 1500 cultivars studied had stomata on their adaxial surface (El-Sharkaway and Cock, 1987). Stomata are functional on both sides of the leaf, but those on the upper surface of the leaf are larger (Cerqueira, 1989).

Cassava is monoecious producing both male (pistillate) and female (staminate) flowers on one plant. The inflorescence is located in the juncture between branches. The staminate flower is located on the lower part of the branches opposed to the pistillate flower on the upper part of the branch. The pistillate flowers are more numerous than the staminate flowers. The female flowers open 1-2 weeks before the male flowers on the same inflorescence. The plants are therefore prone to cross-pollination which might explain the high level of heterozygosity in cassava (Alves, 2002). The flowers lack corolla or calyx but have a perianth that has five tepals varying in color (yellow, red,
purple) depending on cultivar. The male flower is about half the size of the female. The male flower has ten stamens (Alves, 2002).

The pollen is yellow or orange and the pollen grains range in size from $122 – 148 \mu m$ (Ghosh et al., 1988). The female flower has a ten-lobed basal disk. The ovary is tricarpellary with six ridges. The three locules each contain one ovule which matures into carunculate seeds. The seed coat is smooth and dark brown mottled with grey.

Not much is known about flowering of cassava plants (Alves, 2002). Some clones are known not to flower. Flowering can start six weeks after planting but the time it takes to flower is a function of cultivar and environment. Moderate temperatures (approximately 24 °C) are known to be conducive for flower initiation. Photoperiod also is known to affect flowering. Flower initiation occurs best at photoperiods $> 13.5$ hours (Keating et al., 1982).

The distribution of dry matter in cassava is dependent on the age of the plant. Up to 60 – 75 days after planting dry matter accumulation is mainly in the leaves. However, after the fourth month more dry matter is accumulated in the storage roots than in the rest of the plant (Howeler and Cadavid, 1983; Tavora et al., 1995). At 12 months of growth, the dry matter is present mainly in the storage roots followed by stem and leaves. The time for the maximal accumulation of dry matter depends on the cultivar and the environment. The growth phase in high latitudes is 4 – 6 months (Lorenzi 1978) and 7 months for high
altitudes (Oelslglke 1975). In the tropics, where growth is faster, the maximum rate of dry matter accumulation is at 3 – 5 months age (Howeler and Cadavid 1983).

1.2 HOW CASSAVA CAME TO AFRICA

Botanical, genetic and archeological evidence supports the South American origin of cassava, pointing to the Amazon region as the center of cassava domestication (Olsen and Schaal, 1999, Hillocks, 2002). Scientists have attempted to determine the geographical and evolutionary origin of cassava by taking advantage of the high DNA sequence variation in the non-coding regions (introns) of the glyceraldehyde 3-phosphate dehydrogenase ($G3pdh$) gene. Olsen and Schaal (1999) used this technique to demonstrate that cassava was domesticated from wild $M. esculenta$ populations along the southern border of the Amazon basin.

Portuguese navigators took cassava with them from Brazil to West Africa in the 16th century (Jones, 1959) then later to East Africa (Jennings, 1976). Even though cassava was grown in Fernando Po in the Gulf of Benin and around the Congo River in the 16th century, cassava’s dispersal into West Africa did not take place until the 20th century (Hillocks, 2002). Cassava was initially grown mainly in the coastal areas of Africa. The inland spread of cassava cultivation was by African traders who were drawn to cassava for its fabled characteristic of providing security against famine. Cassava is now grown in most African countries especially south of the Sahara desert. Realizing the importance of cassava, the International Institute of Tropical Agriculture (IITA) was established with its headquarters in Ibadan, Nigeria in 1972 under the guidance of the Consultative Group on International Agricultural Research (CGIAR) to oversee the development of the crop
in Africa. Together with Centro Internacional de Agricultura Tropica (CIAT) in Colombia, IITA has the global mandate for cassava improvement (Hillocks, 2002).

1.3 CASSAVA PRODUCTION

Although cassava is native to the Amazon region, Africa now produces more cassava than the rest of the world combined (Table 1.1). Africa’s largest producers of cassava are Nigeria (35%), Democratic Republic of Congo (19%), Ghana (8%), Tanzania (7%) and Mozambique (6%). The top producers in Africa have increased their production greatly in the past two decades especially Nigeria (22% - 35%) and Ghana (4% - 8%) (IITA, 1997). Other countries are on the decline.

The increases in production of cassava has come as a result of an increase in the area of land cultivated as opposed to an increase in yield per hectare. Total yields have increased by 33% in the past two decades while the cultivated land for cassava increased by 70% during the same time period (IITA, 1997). In Africa, cassava yield per hectare declined 1.2% to 0.6% in the past decade. Only Ghana has increased yield from 1990 – 1995. According to the survey conducted by Collaborative Study of Cassava in Africa (COSCA), funded by Rockefeller Foundation, the main reason for the increase in cultivation is response to famine, hunger and drought. This confirms the value of cassava as a security crop (Hillocks, 2002).
Table 1.1: Yield for cassava producing countries in Africa from 1996–1998 (FAO 1998).
<table>
<thead>
<tr>
<th>Country</th>
<th>Yield (kg ha(^{-1}))</th>
<th>Production (1000 million tonnes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angola</td>
<td>5,573</td>
<td>2,500</td>
</tr>
<tr>
<td>Benin</td>
<td>8,747</td>
<td>1,452</td>
</tr>
<tr>
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1.4 CASSAVA AND FOOD

Cassava serves as the main staple food for more than 500 million people in the tropical and subtropical regions of the world (Balagopalan, 2002). It also contributes significantly to the livelihood of these people. After removing the skin, cassava is eaten raw in Africa and other parts of the world. However, the cultivars that are eaten raw are the ‘sweet cultivars’ which have low cyanogenic glycoside content. The cultivars that have high cyanogenic glycoside content are processed and cooked before consumption (Balagopalan, 2002).

Cassava is used as food in various ways across the world. In South America, cassava tubers are grated and pounded into pulp. The dewatered pulp is often shaped into pies and cakes, wrapped in leaves and baked on fire. In West Africa cassava roots are fermented in a pot for 4-7 days, boiled and pounded into fufu and eaten with vegetable soup supplemented with fish and meat (Lancaster et al., 1982).

In Liberia, cassava is made into dumby which is prepared by placing boiled cassava roots in mortar followed by pounding. Following pounding, the dumby is cut into pieces and put in soup supplemented with vegetables or meat. It is then swallowed whole. Cassava is also made into farina in South America and West Indies. Farina is prepared by removing the skin of the cassava root and grating. The mash is then depulped, sieved and

12
roasted in a slow fire. It could then be stored for several months and can be eaten as a cereal and in combination with other foods (Omole, 1977).

Cassava macaroni is prepared by blending cassava flour, groundnut flour and wheat semolina in the ratio 60:12:15. It is enriched with 12% protein. The food is used to feed children because of its high protein content (Balagopalan, 2002).

In Ghana, Nigeria, Guinea, Togo and Benin, *gari* is one of the most important cassava foods. Cassava roots are skinned, grated and dewatered in sacks made from jute fibers and allowed to ferment for 2-4 days. This is followed by sieving to remove fibers from the roots. It is then fried in shallow iron pans and stirred continuously until it becomes dry and crisp. Palm oil is added sometimes during frying to prevent burning and also as a source of carotene in the food. Gari is prepared with boiling water to make a thick paste. Cassava is also used in preparing fast food. It is used in preparing wafers, and made into fried chips, cakes and doughnuts (Balagopalan, 2002).

Cassava is also important in animal feeds. Various parts of the plant such as leaves, stem and roots are used to feed animals. The high energy value of cassava makes it a good source of carbohydrate in animal diets (Omole, 1977). However, roots have low protein content. This short-coming is often overcome by supplementing it with soya which is rich in protein.
Fresh roots are used in feeding farm animals such as cattle, goats and sheep in the developing countries. Sometimes, the roots are boiled before feeding animals. Feeding livestock with fresh roots might cause cyanide toxicity depending on the level of cyanogenic glucosides in the roots. It has been reported that replacement of cereals with cassava up to 50 – 100% did not affect the milk quantity and quality of dairy animals (Mathur et al., 1969). In some cases higher milk yield has been reported up to 19.5% as a result of increased energy from cassava (Balagopalan, 2002).

Cassava leaves are used as forage in many developing countries especially in the dry season when other feeds are scarce. There is some opposition to the use of cassava leaves as forage, however, for fear of cyanogenic poisoning of the livestock (Balagopalan, 2002).

1.5 CASSAVA AND INDUSTRY

Cassava is an important commodity in industry mainly because of its starch which is used in the production of various items. Cassava starch is used in the production of adhesives (Balagopalan, 2002). Gums are made from cassava starch by heat treatment. Other varieties of adhesives are made from cassava starch by the addition of chemicals (Balagopalan, 2002, Cock, 1985).

Cassava gum, made without additives, is produced by cooking cassava starch with water. Preservatives may be added later if needed. This type of gum is used in bill pasting, making bags, and in the tobacco industry. Copper sulphate may be added to the paste to prevent microbial damage. Cassava starch is used for paste production because of its
cohesiveness and clarity. Cassava starch is preferred for food packaging over other types of starch because of its bland taste (Balagopalan, 2002; Cock, 1985).

The cassava gums containing chemicals are made by the addition of calcium chloride, magnesium chloride, borax, urea and carboxymethyl cellulose during the process of gelatinization. The chemicals increase viscosity, flowability and are used for controlling hydration. Such gums are used in the lamination of papers, production of wall-papers and pasting labels (Balagopalan, 2002; Abraham, 1996).

Cassava starch is also used in production of dextrins. An aqueous solution of dextrin is used for bonding similar and dissimilar surfaces. Dextrins are not as strong adhesives as starch films but are preferred to starch because they can be used at higher concentrations than starch and accelerate drying. Dextrins are used as envelope gums, bottle-labeling adhesives, postage stamps adhesives, in making cardboard boxes and photographic mounting materials (Balagopalan, 2002; Cock, 1985; Trim et al., 1996).

Glucose and dextrose, products of starch hydrolysis, are liquefied into sugar-syrups. The syrup can be used in making confection and in the pharmaceutical industry. Cassava starch is also used in making fructose syrup and fructose crystals which are used in the substitution of sucrose, glucose and synthetic sweeteners. Fructose is 1.7 times sweeter than sucrose and four times sweeter than glucose (Abraham, 1996). Cassava starch is also used in the manufacture of maltodextrin which substitutes for glucose as a sweetener. It is also used as a thickening agent (Balagopalan, 2002; Balagopalan, et al., 1988). Cassava starch oxidized with hypochlorite or chlorine is useful in the paper industry because of its low viscosity, film strength and clarity in making glossy papers.
These starches are also used in the textile industry for sizing warps of cotton and spun rayons and for laundry finishes. Cationic starches made by treating starch with amino, imino, ammonium, sulphonium or phosphonium groups are used in the paper industry to provide glaze and strength to paper (Abraham, 1996; Cock, 1985; Trim et al., 1996).

Cassava starch is used in blending synthetic polymers to give it biodegradable characteristics. Cassava starch, because of its low swelling and gelatinization temperature, is easily saccharified to simple sugars. These simple sugars are used in the production of sugar alcohols such as sorbitol, mannitol and maltol. Sorbitol can be used to replace glycerin in the production of tooth paste, cosmetics and oil-based paints. It is also used as a raw material in the production of ascorbic acid (Ren, 1996). Mannitol has a wide range of applications in medicine as a dehydrating agent in blood vessel diastolic preparations. Mannitol is also used in the treatment of cerebral thrombosis and other circulating disorders and in the production of polyester, polyethylene and solid foam plastics. Maltol, a food flavoring agent, is used in the confectionery industry (Ren, 1996).

1.6 CASSAVA TISSUE CULTURE.

Cassava, a very important crop in the tropics, however, it is very difficult to breed by classical genetics. It takes ten years to generate a new cultivar by conventional breeding (Alves, 2002). This is in large part due to inconsistent flowering coupled with poor seed set. Cassava is also heterozygous and clonally propagated (Jennings and Iglesias, 2002). In this circumstance, genetic engineering becomes attractive alternative for plant
breeding for cassava improvement. One of the requirements for the generation of transgenic cassava is an efficient and reproducible plant regeneration system. Nearly all parts of cassava plant have been used to establish in vitro cultures (Roca, 1984).

1.6.1 Somatic embryogenesis.

Somatic embryogenesis is the method of choice in regeneration of cassava. Explants have been mostly restricted to young leaves and shoot meristems (Schopke et al., 1993). Somatic embryos have been induced from cassava young leaf lobes and cotyledons. Plants have also been regenerated from these tissues (Stamp and Henshaw, 1982, 1986, 1987a, b; Stamp, 1987; Szabados et al., 1987; Taylor and Henshaw, 1993; Mathews et al., 1993; Raemakers et al., 1993; Konan et al., 1994). Even though it is now possible to regenerate cassava plants from a limited number of cultivars, the efficiency of regeneration achieved so far is low (Schopke et al., 1993). In their best experience, Szabados et al., (1987) obtained an average of 1.15 plantlets per primary explant. Stamp and Henshaw (1987a) studied the regeneration of somatic embryos derived from cotyledon explants and found that 14% of the embryos with which they started eventually regenerated into plants.

1.6.2 Organogenesis

Cassava organogenesis is independent of callus formation and auxin treatment. Unlike most plants, there is no intermediary of callus required for organogenesis. Instead shoot primordia are induced directly from cotyledons of somatic embryos and young leaf lobes
on MS (Murashige and Skoog, 1962) medium supplemented with 6-benzyladenine (BA). (Li et al., 1996; Mussio et al., 1998).

1.6.3 Meristem culture.

Meristem culture and regeneration of cassava have been reported (Kartha et al., 1974; Bajaj, 1983). This could provide an alternative source of explants for transformation studies, but it is difficult to obtain the number of meristems adequate for transformation experiments (Schopke et al., 1993). Apart from serving as explants for cassava transformation, meristem culture serves a more important role in providing disease-free plants, especially for plants infected with viruses as meristems are generally devoid of viruses (Schopke et al., 1993). Meristems therefore serve as a means for viral decontamination as well as in multiplication of cassava.

1.6.4 Protoplast culture

The isolation and culture of cassava protoplasts has been performed (Mabanza and Jonard, 1983; Mabanza, 1984; Szabados et al., 1987; Villegas et al., 1988; Nzoghe, 1989). Prior to the report of Sofiari (1996), only one successful regeneration from protoplasts has been reported (Shahin and Shepard, 1980) but this has not been reproduced.

Currently, the most reliable method for cassava regeneration is through the young leaf lobes (and perhaps, through suspension cultures which is more time consuming and requires more dexterity). Somatic embryos have been obtained from a number of cultivars, but there is still the need for optimization of the regeneration procedures.
Results from the field tests of cassava plants derived from embryogenesis showed that somaclonal variation is not a problem in cassava (Schopke et al., 1993). This makes plant regeneration via somatic embryogenesis a reliable tool for genetic improvement of cassava.

1.7 CASSAVA TRANSFORMATION

Cassava transformation has been reported via Agrobacterium-mediated transformation (Li et al., 1996; Arias-Garzon et al., 1997; Sarria et al.; 2000, Zhang et al., 2000a; Siritunga and Sayre, 2003; Siritunga et al., 2003) and particle bombardment (Raemakers et al., 1996; Schopke et al., 1996; Zhang et al., 2000b). Agrobacterium-mediated transformation is favored over particle bombardment because of the greater stability of transformants. Agro-mediated transformation usually results in the transfer of 1-3 copies of the transgene to plants compared to the particle gun which has been known to deliver up to 12 copies of a transgene. Importantly, the introduction of multiple transgene copies can lead to gene silencing (Alien et al., 1993; Matzke and Matzke, 1995; Meyer and Saedler, 1996).

The first published attempt to transform cassava cells was that of Calderon (1988). He inoculated leaf pieces, stem pieces and embryogenic callus with Agrobacterium containing plasmids with the coding sequences for neomycin phosphotransferase II (npt II), phosphinotricin acetyltransferase (bar) or β-glucoronidase (uidA). He isolated callus lines expressing the phenotype expected from the transformation tissue.
Subsequent reports of transient gene expression followed (Franche et al., 1991; Arias-Garzon and Sayre, 1993; Luong et al., 1995). Arias-Garzon and Sayre (1993), observed a tissue-specific inhibition of transient gene expression in cassava associated with elevated DNase activity. Using a cauliflower mosaic virus 35S promoter, they observed several hundred localized regions which expressed β-glucuronidase activity in cassava leaves but virtually none in the roots. This shows that different parts of cassava plant respond in different ways to transient transformation treatments. Luong et al., (1995), reported transient expression of the uidA gene in cassava tissues by electroporation. However, in 1996 two reports of stable cassava transformation came out simultaneously; one using Agrobacterium mediated transformation (Li et al., 1996) and the other using microparticle bombardment (Schopke et al., 1996).

The first transformation of cassava with a gene of agronomic importance was that of Arias-Garzon (1997) and White et al., (1998) who successfully transformed cassava with the gene encoding hydroxynitrile lyase (HNL). Siritunga et al., (2003) also transformed cassava with the gene encoding HNL driven by the 35S promoter. HNL catalyzes the breakdown of acetone cyanohydrin, a product of linamarin hydrolysis, to acetone and cyanide (Siritunga et al., 2003). This was an important development in cassava processing because cassava roots contain little HNL (0-6%) relative to that in leaves. The rate of acetone cyanohydrin breakdown in the transformed plants was 41-75 % faster than wild-type cassava (Siritunga et al., 2003). These plants are advantageous for
detoxification and processing of cassava food products especially for the poor people of the African continent who are perennially dependent on cassava for food.

Additional reports of the genetic transformation of cassava have followed (Zhang et al., 2000a; Zhang et al.; 2000b; Siritunga and Sayre, 2003). But in each case only marker genes of little agronomic importance were introduced into cassava. Sarria et al., (2000) reported the transformation of cassava with bar gene which confers resistance to the herbicide basta. Transformed plants tolerated 200 mg/L basta which proved toxic to the untransformed cassava plants.

1.7.1 Transformed cassava plants

There are more than 1500 cultivars of cassava worldwide (Alves, 2002). The high level of heterozygozity could be a function of cross-pollination in cassava resulting from the differences in timing of male and female flowers. Not all cassava cultivars are transformable to date. Only a few cultivars have been successfully transformed viz:

- TMS 60444 (Zhang et al., 2000a, b; Gonzalez et al., 1998; Schopke et al., 1996)
- TMS 71173 (Ihemere et al., this thesis)
- MCol 2215 (Arias-Garzon, 1997; White et al., 1998; Siritunga and Sayre, 2003)
- MCol 122 (Zhang et al., 2000 a, b)
- MPer 183 (Sarria et al., 2000)
Regardless of the paucity of transformation success among cultivars, not all transformable cassava cultivars respond to the available methods of transformation. Only TMS 60444 has been successfully transformed using particle bombardment of friable embryogenic callus (Raemakers et al., 1996; Gonzalez et al., 1998; Zhang et al., 2000b). MCol 2215 (White et al., 1998; Siritunga and Sayre, 2003) and TMS 71173 (Ihemere et al., this thesis) are the most responsive to Agrobacterium-mediated transformation of cassava somatic embryo explants. Success with other cultivars is limited (Fregene and Puonti-Kaerlas, 2002).

1.7.2 Methods of cassava transformation

Cassava has been transformed using different types of tissues and explants as starting material including; somatic embryo cotyledons (White et al., 1998; Siritunga and Sayre, 2003; Zhang et al., 2000a), young leaf lobes (White et al., 1998), and friable embryogenic callus (Raemakers et al., 1996; Gonzalez et al., 1998; Zhang et al., 2000b) (Table 1.2). Embryogenesis has been reported from friable embryogenic calli, somatic embryo cotyledons and young leaf lobes. Direct organogenesis has been reported with somatic embryo cotyledons and young leaf lobes (Zhang et al., 2000a). The efficiency of transformation of the different explants and methods was scored by scientists in the Advanced Cassava Transformation Group who met at CIAT in Palmira, Colombia (June 9-10 2003).
The somatic embryogenesis method using embryo cotyledons as explants was chosen to be the best method (Table 1.3) based on efficiency of regeneration, incidence of chimeras, speed of regeneration of transformed plants, and simplicity of transformation technique. The different methods were scored 1-5, with 5 being the best score.
Table 1.2: Methods used in cassava transformation (modified from Fregene and Puonti-Karlas, 2002)
<table>
<thead>
<tr>
<th>Target tissue</th>
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<th>Gene transfer system</th>
<th>Selection</th>
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Table 1.3: Relative ranking (1(low) – 5 (high) of three transformation systems.

$^\Phi$ Genotypes used: FEC (friable embryogenic callus) – TMS 60444, Adera4, MCol 1505, B. Rouge, Mtai5, R60 (Mtai 8), CM3306-4, Organogenesis – MCol22, TMS60444; GSE (germinated somatic embryo – MCol2215, TMS 71173, Mper183, MCol22. Plant regeneration: FEC – 50% of the genotypes tested can be transformed. Organogenesis – lower success on transformation of new varieties. GSE – 50% of the genotypes tested can be transformed.
1.7.3 Promoters used in cassava transformation

Gene promoters are very important in the transformation process because they drive the tissue- and temporal-specific expression of genes. Without different promoters controlled gene expression is not possible. Many promoters have been used in cassava transformation and many more are still in use in the cassava transformation process. Some of the promoters used to date are listed in Table 1.4 with some of their characteristics.

Only a few of the promoters have been identified or shown to be root/tuber-specific, namely, P15-1.5, P54-1.0, patatin and potato GBSS.
<table>
<thead>
<tr>
<th>Promoter</th>
<th>Source plant/organism</th>
<th>Size (kb)</th>
<th>Tissue specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTR-1, PTR-2 (putative transcription regulator)</td>
<td>Methanococcus jannaschi</td>
<td>2.5 kb</td>
<td>constitutive</td>
<td>Ouhammouch and Geiduschek, 2001</td>
</tr>
<tr>
<td>P15-1.5</td>
<td>cassava</td>
<td>1.46 kb</td>
<td>root</td>
<td>Ihemere and Sayre, in prep</td>
</tr>
<tr>
<td>P54-1.0</td>
<td>cassava</td>
<td>1.08 kb</td>
<td>root</td>
<td>Zhang et al., 2003</td>
</tr>
<tr>
<td>SAG-12 (Senescence-Associated Gene -12)</td>
<td>tobacco</td>
<td>1.5 kb</td>
<td>leaf</td>
<td>Chang et al., 2003</td>
</tr>
<tr>
<td>CAB1 (chlorophyll a,b)</td>
<td>Arabidopsis</td>
<td>0.4 kb</td>
<td>leaf</td>
<td>Siritunga and Sayre, 2003; Ihemere and Sayre, in prep.</td>
</tr>
<tr>
<td>PATATIN</td>
<td>Potato (Solanum)</td>
<td>1.3 kb</td>
<td>tuber/root</td>
<td>Ihemere and Sayre, in prep</td>
</tr>
<tr>
<td>Granule Bound Starch Synthase 1</td>
<td>Potato (Solanum)</td>
<td>1 kb</td>
<td>tuber/root</td>
<td>Salehuzzaman et al., 1994</td>
</tr>
<tr>
<td>African Cassava Mosaic Virus (ACMV)-DNA1</td>
<td>ACMV</td>
<td>2.7 kb</td>
<td>constitutive</td>
<td>Frey et al., 2001</td>
</tr>
</tbody>
</table>

Table 1.4: Promoters that have being used in cassava transformation.
1.7.4 Selectable markers in cassava transformation

The need for selectable markers in transformation arises from the need to distinguish between transformed and non-transformed tissues and plants. In cassava transformation, the selection of transformed tissues has been done using; kanamycin, geneticin and paromomycin (enabled by the nptII gene), hygromycin (enabled by hpt gene), phosphinotricin and basta (enabled by bar gene), and mannose (enabled by phosphomannose isomerase) (See Table 1.2).

1.7.5 Problems with cassava transformation

The successes recorded in cassava transformation not withstanding there are still many problems associated with cassava transformation. First and foremost cassava has a low efficiency of transformation compared to other crops. The best recorded transformation efficiency for cassava is in the range of 3-5%. Most other crops have transformation efficiencies greater than 10% (Raemekkers et al., 1997). This makes it difficult to work with cassava. Added to the low efficiency of transformation is the difficulty to characterize transformed cassava plants molecularly. Southern blot analysis of cassava is very difficult. Part of the problem is that the cassava genome is large. Siritunga, (2002) reported that gene insertions from independently transformed cassava plants may have similar restriction patterns making it difficult to identify independently transformed lines.

Scientists working on cassava transformation have tried a variety of tissues for cassava transformation. The most prolific being the friable embryogenic callus (FEC) which has been reported to generate more transformed cassava (Raemekkers et al., 1996; Gonzalez et al., 1998). However, this has its own problems. Production of FEC requires that the
cassava embryogenic tissues stay on high auxin (50 µg/L) media for up to six months compared to the one month requirement for somatic embryogenesis. This long exposure to high auxin concentrations leads to somaclonal variation in cassava. The severity of the somaclonal variation has led to production of stunted cassava plants and those with variegated leaves.

It is also speculated that cassava transgenes are prone to deletion. Sarria et al., (2000) imparted herbicide resistance to cassava through genetic engineering. Proof of transformation events included positive GUS stains, RT-PCR and Southern blotting. Some of their positive transformants lost the transgenes with time, however.

1.8 STARCH

Starch is the most important form of carbon reserve in plants with respect to the amount produced, its distribution among different plant species, and its commercial importance (Martin and Smith, 1995). Starch comprises different polymers of glucose arranged in a three-dimensional, semi-crystalline structure called the starch granule.

Starch is composed of two types of glucan polymers: amylose and amylopectin (Martin and Smith, 1995). Amylose is made up of linear chains of α (1, 4)-linked glucose residues which are typically about 1,000 residues long. It may have one α (1, 6)-linkage per 1,000 residues and makes up about 30% of total starch (Martin and Smith, 1995; Okita, 1992; Smith et al., 1995). On the other hand, amylopectin consists of highly branched glucan chains and accounts for about 70% of total starch in most plants. Chains
of about 20 α (1, 4)-linked glucose moieties are joined by α (1, 6)-linkages to other branches. Some branches of amylopectin are not substituted on the number six position and are called A chains. These are α (1, 6)-linked to inner branches called B chains which may be branched at one or more points. A single chain per amylopectin molecule has a free reducing end, the C chain (Kainuma, 1988; Smith and Martin 1993; Martin and Smith, 1995).

1.8.1 Phosphorylated starch

The phosphorylation of starch is almost universal in plants (Blennow et al., 2002). Glycogen, which is the analogue of starch in animals, also contains phosphate groups an indication that phosphorylation is a requirement for storage α-glucan metabolism (Vikso-Nielsen et al., 2001). However, the level of starch phosphorylation varies among plants. Phosphorylation in cereal starch is low (less than 1 nmole Glc-6-P/mg starch). Cassava starch has 2.5 nmole Glc-6-P/mg starch, a low level of phosphorylation. In contrast, potato starch is highly phosphorylated (8-33 nmole Glc-6-P/mg starch) (Blennow et al., 2000; Blennow et al., 2002). The phosphate groups are bound mainly to the amylopectin moiety of starch as monoesters at the C-6 and C-3 positions of glucose units (Bay-Smidt et al., 1994; Blennow et al., 2002).

The phosphorylation of starch serves to increase the hydration capacity following gelatinization. Phosphorylation of starch prevents its crystallization and affects the viscosity of the final product. Engineering crop starch for higher phosphate content is
important because it reduces expensive and environmentally hazardous chemical processing by industry (Blennow et al., 2002).

It has been reported that starch granules having long amylopectin chains, as the result of inhibition of starch synthase III, had severe granule fissuring (Fulton et al., 2002). Potato starch with reduced starch phosphorylation is also prone to granule fissuring (Blennow et al., 2002). This is thought to be caused by the steric interaction between amylose and amylopectin and that starch phosphorylation serves to prevent such interactions.

The phosphorylation of starch is catalyzed by a 160 kDa R1 protein, an α-glucan water dikinase (GWD) (Ritte et al., 2002). The enzyme phosphorylates starch by transferring the β-phosphate of ATP to the C-3 or C-6 positions of the glucose moiety and the γ-phosphate to water (Ritte et al., 2002).

1.8.2 Cassava starch

Cassava starch is stored in the amyloplasts of thickened roots. The starch content of cassava roots ranges from 74 – 85 % of the dry weight (Rickard et al., 1991; Munyikwa et al., 1997). Cassava starch granules are round, flat on one side and contain a conical pit which extends to a well-defined eccentric helium, ranging in size from 5 – 40 µm (Moorthy, 1994; Munyikwa et al., 1997). An 18 month study of the growth of cassava starch granules indicates growth continues up to six months. No further growth was observed after six months (Moorthy and Ramanujan, 1986).
Cassava starch comprises 14 – 24% amylose (Ketiku and Oyenuga, 1972; Kawabata, et al., 1984). Starch is classified into three types based on different X-ray diffraction patterns, viz: A, B and C. Cassava starch comprises mainly the A-type pattern that is characteristic of cereal starches (Guilbot and Mercier, 1985). This A-type pattern is characterized by closely packed double helices compared to the more open B-type arrangement. Cassava starch also consists of 0.08% - 1.54% crude fat, 0.03% - 0.6% crude protein, and 0.75% - 4% phosphorus (Soni et al., 1985; Munyikwa et al., 1997).

1.9 STARCH SYNTHESIS

Starch synthesis takes place in the leaves during the day from sugars produced through photosynthesis. It is also produced in the other organs namely, meristems and root cells. Starch also accumulates in storage organs such as seeds, fruits, tubers and storage roots. (Okita, 1992; Martin and Smith, 1995; Smith et al., 1995).

Synthesis of starch takes place in plastids which are called amyloplasts located in storage organs whose main function is starch production. However, starch may also be produced in plastids with other specialized functions including, chloroplasts (which function in photosynthetic carbon fixation), plastids of oil seeds (fatty acid biosynthesis) and chromoplasts of roots such as carrots (carotenoid biosynthesis) (Martin and Smith 1995; Okita, 1992; Smith et al., 1995).
1.9.1 Biochemistry of starch biosynthesis

Three classes of enzymes that are directly involved in starch synthesis are: ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.23), starch synthase (SS; EC 2.4.1.21) and starch branching enzyme (SBE; EC 2.4.1.28) (Martin and Smith, 1995; Smith et al, 1995). Amylose and amylopectin are synthesized from ADP-glucose which is produced from glucose-1-phosphate and ATP via AGPase catalysis. The next step involves the $\alpha(1,4)$ linkage of the non-reducing end of a preexisting glucan moiety and the glucosyl residue of ADP-glucose in a reaction catalyzed by starch synthase. The final step in starch synthesis is catalyzed by starch branching enzyme which hydrolyzes an $\alpha(1,4)$ chain within a chain and then ligates the reducing end of the glucan fragment to another glucose residue in an $\alpha(1,6)$-linkage (Martin and Smith, 1995; Okita, 1992; Smith et al., 1995) (Figure 1.3). Branches are generally 20 glucan residues apart.
Figure 1.3: The biochemistry of starch biosynthesis. Glucose-1-phosphate and ATP serve as the substrate for AGPase. The resulting ADP-glucose is used by starch synthase to elongate a pre-existing amylose moiety through $\alpha$-linkage at its non-reducing end. Starch branching enzyme next rearranges the amylose chain through $\alpha_{1,6}$ linkage. PPI = pyrophosphate (Slattery et al., 2000).
1.9.2 Starch synthases

\[(\text{Glucosyl})_n + \text{ADP-Glucose} \rightarrow (\text{Glucosyl})_{n+1} + \text{ADP}\]

Starch synthases produce amylose. Starch synthases are classified into two main groups based on their location in the starch granule: soluble starch synthase (SSS) and granule bound starch synthase (GBSS). Soluble starch synthase is located in the stroma of the amyloplast or chloroplast as a soluble protein. Soluble starch synthase has an extra N-terminal domain compared to GBSS and has a molecular weight of 77 kDa (Kossmann and Lloyd, 2000).

GBSS is a 60 kDa protein, known as the waxy protein because its loss results in synthesis of waxy starch (high amylopectin). It is bound inside the starch granule. Both SSS and GBSS have multiple isoforms. Cassava GBSSI and GBSSII have only 30% homology (Munyikwa et al., 1997). GBSSII is expressed mainly in the leaf whereas GBSSI is expressed mainly in the root (Munyikwa et al., 1997).

1.9.3 Starch branching

The starch branching enzymes have several isoforms in some plants including maize, rice and pea (Fisher et al., 1993; Burton et al., 1995). The isoforms are divided into two families viz.: Family A comprises maize SBE II, pea SBEI and rice SBEIII. This family contains an extra N-terminal domain that is not found in family B. Family B is made up of maize SBE I, Potato SBE, rice SBE, I cassava SBE, and pea SBEII (Fisher et al., 1993; Burton et al., 1995; Martin and Smith, 1995).
The catalytic properties of the two families are different (Guan and Preiss, 1993). Members of family A isoforms have lower affinity for amylose than family B. Family A uses shorter glucan chains in α- (1, 6)-linkages hence they make amylopectins with shorter glucan chains compared to family B. The reason for this catalytic difference is a conserved structural difference between the two isoforms (Martin and Smith, 1995).

Pea SBE mutants having reduced expression of starch branching enzyme have been isolated which have seven-fold lower starch branching enzyme activity than wild-type plants (Smith et al., 1990). The SBE isoform I was found to be absent in the mutant which accounts for this low activity. Starch synthesis also dropped by 40% in the mutant, as a result, photosynthesis was also inhibited in the mutant. These results indicate that SBE activity is required for normal rates of starch synthesis.

A drop in the activity of the branching enzyme decreases the number of non-reducing chain ends on the starch grain. Consequently, the concentration of one of the substrates of starch synthase is lowered. Accordingly, the level of ADP-glucose (the other substrate) increases. The increased concentration of ADP-glucose could affect the pathway in two ways: by acting as a feedback-inhibitor of AGPase and by sequestering adenine nucleotides. Biochemical changes induced by high light intensity in the mutant lead to a decrease in ATP levels which in turn reduces glycerate-3-phosphate reduction, the reaction in Calvin cycle most sensitive to decreasing ATP levels (Smith et al., 1990; Edwards and Walker, 1983). ATP acts as a donor of phosphoryl groups for energy metabolism while other nucleotide pools are used to form the activated nucleotide-
diphosphate precursors for the synthesis of biopolymers. In the plastid, ATP simultaneously acts as: (1) an energy source for Calvin cycle, and (2) a source of ADP-glucose for starch synthesis. The accumulation of glycerate-3-phosphate is enhanced by the falling inorganic phosphate (Pi) supply. This leads to activation of AGPase, thus, stimulating starch synthesis and in turn evoking Pi recycling in the chloroplast (Martin and Smith, 1995).

1.9.4 Starch debranching enzyme

Two types of debranching enzymes have been identified in plants (Doehlert and Knutson; 1991, Nakamura et al., 1996). The pullulanases (also referred to as limit-dextranases and R-enzymes) hydrolyze the α-1, 6-linkages of pullulan (pullulan is an extracellular bacterial polysaccharide produced from starch by *Aureobasidium pullulans*. It is a linear polysaccharide made up of α–1, 6-linked maltotriose residues and amylopectin (Nakamura et al., 1996; Ball and Morell, 2003). Isoamylases on the other hand digest amylopectin but don’t normally digest pullulan (Doehlert and Knutson; 1991, Nakamura et al., 1996; Ball and Morell, 2003). Isoamylases are plastidial enzymes whereas pullulanases are located inside the plastid and outside the plastid (Zhu et al., 1998).

The final structure of amylopectin is determined by a balance between the activities of starch branching enzyme and starch debranching enzymes. The starch debranching enzyme functions to reduce the branching of glucan polymers (Martin and Smith, 1995). The maize endosperm mutant *sugary 1 (su1)*, the rice endosperm mutant *sugary 1 (su1)*, and the *Chlamydomonas reinhardtii* mutant *sta7* synthesize a highly branched, soluble α-
1,4, α-1,6-linked glucan polymer called phytoglycogen in addition to or instead of starch (Nakamura et al., 1996; Zhu et al., 1998). These mutants have reduced debranching enzyme activity, an indication that debranching enzymes are required for amylopectin synthesis (Nakamura et al., 1996; Ball and Morell, 2003).

1.10 ADP-GLUCOSE PYROPHOSPHORYLASE

1.10.1 Introduction

Sucrose plays a very important role in plant growth and development both as a free molecule and as the sugar nucleotide, ADP-glucose, which is the substrate for starch synthesis. The most important scenario for starch biosynthesis is the ability to convert triose phosphates into hexose phosphates which are required for starch synthesis. Most starch biosynthesis in roots and stems takes place in heterotrophic plastids which do not have fructose-1, 6- bisphosphatase required for the interconversion of triose phosphates to hexose phosphates (Entwistle and ap Rees, 1990; Neuhaus et al.; 1993). ADP-glucose has been identified as a precursor for starch biosynthesis and is not always made at the organelle of starch synthesis. Due to lack of fructose-1, 6- bis phosphatase in heterotrophic plastids there has to be another way to supply substrates for starch synthesis (Entwistle and ap Rees, 1990; Neuhaus et al.; 1993), hence the cytoplasmic synthesis of ADP-glucose. In cereals and tomato fruits ADP-glucose is transported into the plastid in exchange for AMP (Mohlmann et al.; 1997). The ADP-glucose is transported into the plastid by the adenylate translocator (Figure 1.4). In other plants AGPase activity is localized to the plastid (Denyer et al., 1996). It has also been reported that in tomato, the localization of starch biosynthesis is the same as that of cereals.
Figure 1.4: Carbon and adenylate metabolism in maize endosperm amyloplasts during starch synthesis (Mohlmann et al., 1997).
ADP-glucose pyrophosphorylase is a heterotetrameric enzyme comprising a large subunit (shrunken2 locus) and a small subunit (brittle2 locus). The large subunit is 54 - 60 kDa while the small subunit is 51 - 55 kDa. The subunits are also referred to as S and B subunits from the name of the loci from which the first AGPase cDNAs were cloned from maize (Bae et al., 1990; Preiss, et al., 1990). The two subunits have catalytic activity independent of each other, but activity is optimal in the hetero-enzyme complex of the two subunits. A 10 - 70-fold increase in activity has been observed with the complex versus individual subunits. In comparison, the bacterial AGPase is a homodimer which is activated by fructose-6-phosphate and inhibited by AMP. ADP-glucose pyrophosphorylase is the only known starch biosynthetic enzyme under allosteric regulation. In plants it is enhanced by 3-PGA and inhibited by Pi, facilitating its high activity during photosynthesis. AGPase is sensitive to the metabolites both in photosynthetic and non-photosynthetic tissues, however, barley AGPase is not sensitive to 3-PGA and Pi demonstrating that there are differences in allosteric regulation of different plant AGPases (Sowokinos and Preiss, 1982; Kleczkowski et al., 1993).

1.10.3 Gene cloning

The first plant AGPase genes cloned were from maize (Bae et al., 1990; Preiss, et al., 1990). Genes encoding both subunits have been cloned from rice (Anderson et al., 1989), maize (Bae et al., 1990; Bhave et al., 1990), barley (Villand et al., 1992), and wheat (Ainsworth et al., 1993). AGPase has also been cloned from some dicot plants including potato (Muller-Rober et al., 1990) and spinach (Smith-White and Preiss, 1992).
The small (*CagpS*1) and large (*CagpL*1) subunits of AGPase have also been cloned from chickpea (*Cicer arietinum* L.) (Singh *et al.*, 2002). Two gene family members were identified for the small and large subunits of chickpea AGPase. *CagpL*1 (chickpea large subunit) is expressed strongly in the leaf with reduced expression in the stem and no detectable expression in the seed and roots. In turn, *CagpL*2 is expressed in the seeds with little expression elsewhere. *CagpS*1 has higher expression in the seeds than *CagpS*2 (Singh *et al.*, 2002).

Additional isoforms of AGPase have been reported from other plants. AGPase small subunit isoforms have been found in Arabidopsis (Villand *et al.*, 1993) and potato (La Cognita *et al.*, 1995). AGPase isoforms and subunits have also been cloned from cassava including, AGPase B, AGPase S2 and AGPase 3 (Munyikwa *et al.*, 1994). The genes encoding AGPase small subunits are generally more conserved than those encoding the large subunit. The AGPase small subunits of potato, rice and spinach are 84 - 93% identical in amino acid sequence compared to 35% identity between potato and spinach large subunits (Nakata *et al.*, 1991 and Preiss *et al.*, 1991).

### 1.10.4 Importance

The importance of AGPase in starch biosynthesis was confirmed by antisense inhibition of its expression or by mutation of the subunits. Mutations of the *sh2* and *br2* genes of maize led to reduction in starch levels (Hannah *et al.*, 1980). In *Arabidopsis thaliana*, a mutant containing 0.2% of wild-type AGPase activity had up to 98% reduction in leaf starch without affecting the activities of other enzymes involved in starch biosynthesis.
(Lin et al., 1988a and b). Muller-Rober et al. (1992) reported that antisense inhibition of the expression of the gene encoding the small subunit of AGPase led to the reduction of starch and increase in soluble sugars in potato. More recently, Rolletschek et al., (2002) reported that anti-sense inhibition of AGPase expression in Vicia faba seeds increased soluble sugars and decreased the amount of starch mostly by reducing the available amylose. In contrast, an increase in expression of AGPase due to introduction of a mutant bacterial glgC gene into potato resulted in 35% increase of starch in the tuber (Stark et al., 1992).

1.10.5 Regulation of AGPase

Starch biosynthetic enzymes are not known to be under allosteric regulation except AGPase. Furthermore, the enzymes are not apparently regulated by phosphorylation. However, other kinds of regulation have been reported for AGPase such as reductive activation by thioredoxin and dithiothreitol (Ballicora et al., 2000; Tiessen et al., 2002).

Mutation of Gly336 in E. coli inhibits allosteric feedback regulation by fructose-1,6-bisphosphate (FBP). Residue Lys296 is involved in binding the allosteric activator, FBP, in E. coli AGPase (Kumar et al., 1989). Later on, Frueauf et al., (2001) identified other important and conserved amino acids involved in AGPase catalysis through out the super family of AGPases (Frueauf et al., 2001). Asp142 in the homotetrameric bacterial (E. coli) AGPase enzyme has been demonstrated to be crucial in catalysis of the enzyme. The residue is highly conserved in plant AGPases. Asp142 in the small subunit of plants corresponds to the bacterial Asp142 (Frueauf et al., 2003). Mutation of the Asp 142 in
the small subunit of potato led to a four-fold decrease in specific activity. Mutation of
the large subunit Asp160, the presumed equivalent of Asp142 in the small subunit, of
potato did not affect the specific activity, however, the apparent activity for the activator
3-phosphoglycerate was altered. These results demonstrate that the two subunits have
different roles in AGPase catalysis.

Other conserved amino acids are also important in the functioning of the enzyme. Lys
404 and Lys441 are important in the regulation of potato small subunit AGPase
(Ballicora et al., 1998). Mutation of Lys404 and Lys441 to alanine in the small subunit
AGPase of potato decreased the apparent affinity for the activator, 3-PGA, by more than
3,000-fold and 50-fold, respectively and also decreased the apparent affinity for the
inhibitor Pi by 400-fold. Mutation to glutamic acid at both sites led to greater effects.
Mutation of these conserved lysine residues in the potato large subunits (Lys 417 and Lys
455) did not alter phosphate inhibition and affinity for the activator (3-phosphoglyceric
acid, 3-PGA) decreased only 3-fold (Lys 417 →Ala) and 9-fold (Lys455 →Ala),
respectively (Ballicora et al., 1998, Figure 1.5). The equivalent mutation on potato small
subunit AGPase (Lys401→Ala and Lys441→Ala) decreased the apparent affinity for the
phosphate inhibitor by 400-fold while the apparent affinity for the activator 3-PGA by
3,090-fold (Lys401→Ala) and 54-fold (Lys441→Ala) (Ballicora et al., 1998).
Cys-12 is conserved in AGPase small subunits of all plants studied to date except for the monocot endosperm enzymes (Ballicora et al., 2000). Cys-12 forms an intramolecular disulfide linkage in the catalytic subunit and its reduction functions in activation of the enzyme. Proof of this came from the work of Ballicora et al., (2000) who reported that reduced thioredoxin f and thioredoxin m from spinach leaves reduced and activated AGPase even at low (10 µM) 3-phosphoglyceric acid concentrations. This activation was reversed by oxidized thioredoxin (Figure 1.6).
Figure 1.6: The activation of AGPase by thioredoxin (adapted from Ballicora et al., 2000). Ox, oxidized; red, reduced.
The activity of AGPase increased by more than 2-fold when assayed in the absence of allosteric activator, 3-PGA when it was reduced by thioredoxin.

Experiments with potato tubers detached from the mother plant showed an inhibition of starch synthesis even though AGPase protein levels were unchanged when there was increase in hexose-phosphates and 3-PGA (Tiessen et al., 2002). This inhibition was shown to be a result of oxidation and dimerization of the small subunit of AGPase. Reduction of the enzyme with DTT released the inhibition.

The N-terminus of the small subunit of AGPase plays a significant role in the catalytic properties and heat stability of the potato enzyme (Ballicora et al., 1995). A ten-amino acid truncated small subunit potato AGPase expressed alone in E. coli had 10-fold less activity than the full length clone (Ballicora et al., 1995). A ten amino acid extension to complement the original potato sequence of the small subunit increased its enzyme activity but the enzyme had lower affinity for 3-PGA and higher sensitivity to phosphate inhibition. Together with the large subunit, the extended small subunit AGPase expressed in potato had the same catalytic properties as the potato enzyme (Ballicora et al., 1995). This shows that the N-terminus of the small subunit AGPase is important in regulating catalysis.

The work of Wingler et al., (2000) indicates that trehalose, a disaccharide structurally similar to sucrose, induces the expression of ApL3, an Arabidopsis large subunit AGPase.
gene. Trehalose also induced the expression of β-amylase gene, AT-β-Amy, indicating that trehalose can effect sugar-mediated gene expression. It is speculated that trehalose interferes with carbon allocation to the sink by inducing starch synthesis in the source tissues (Wingler et al., 2000). Tiessen et al. (2002) also reported a correlation between the redox state of AGPase and the supply of sucrose. High levels of sucrose reduced dimerization of AGPase, increased AGPase activation and stimulated starch synthesis.

1.10.6 Regulation of sucrose and starch biosynthesis

Sucrose and starch biosynthesis are two important processes in plant growth and development. The two processes depend on photosynthesis as a source of substrates (Figure 1.7). It is therefore important that the two processes are temporally and spatially separated in plant organs that make storage carbohydrate.
Figure 1.7: Starch and Sucrose Synthesis Interactions (Buchanan et al., 2000).

3-PGA, 3-phosphoglyceric acid; F1,6bP, fructose-1,6-bisphosphate; F2,6bP, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; Pi, inorganic ortho-phosphate; PPI, inorganic pyrophosphate
Plants have mechanisms to make sure that the two metabolic processes are not equally active at the same time. When the rate of photosynthesis is high, the concentration of dihydroxyacetone increases leading to a rise in the ratio of 3-phosphoglycerate: Pi in the cytoplasm. This results in the export of 3-phosphoglycerate out of the chloroplast in exchange for Pi via an antiporter system. The high concentration of 3-phosphoglycerate in the cytosol inhibits fructose-6-phosphate-2 kinase thereby decreasing the concentration of fructose-2, 6-bisphosphate. An increase in photosynthesis, a consequence of higher light intensity and CO₂, decreases fructose-2, 6-bisphosphate levels and reduces inhibition of fructose-2,6-phosphatase. The increase in fructose-2, 6-phosphatase activity increases sucrose synthesis (Stitt, 1995). The activity of fructose-2, 6-phosphatase is also enhanced by an increase in the ratio of ATP: ADP. On the other hand, when photosynthesis is in abeyance, there is a sharp drop in triose phosphate for exchange with Pi in the cytosol. This leads to accumulation of Pi in the cytosol leading to inhibition of fructose-2, 6-phosphatase. This in turn leads to an inhibition of sucrose synthesis. Also the reduction of triose phosphate in the cytosol reduces inhibition of fructose-6-phosphate, 2-kinase which synthesizes fructose-2, 6-bisphosphate. The increase in the concentration of fructose-2, 6-bisphosphate inhibits fructose-2, 6-phosphatase, an important component in sucrose synthesis (Stitt and Krapp, 1999).

1.10.7 AGPase in transgenic plants

Plants have been transformed with AGPase genes from other sources. Potato has been transformed with the *E. coli* AGPase (*glgC*) gene (Stark et al., 1992). Due to the fact that AGPase is allosterically regulated by different metabolites in bacteria and plants (Stark et
the bacterial AGPase allosteric regulation sites were modified before transformation into potato. This resulted in increased starch biosynthesis in potato because the bacterial enzyme, despite mutations, had a higher \( V_{\text{max}} \) than the plant enzyme (Stark et al., 1992). Sweetlove et al., (1996), reported the transformation of potato with \( glgC16 \) gene under the control of the patatin promoter and demonstrated an increase in tuber AGPase activity of 200%-400%. But on the other hand, Sweetlove et al., (1996), found that the increase in enzyme activity (200-400%) actually did not lead to an increase in starch synthesis. Wheat has also been transformed with the maize AGPase gene. In this case the gene was expressed in the endosperm (Smidansky et al., 2002). The seed weight of AGPase transformed wheat seeds increased by 38% per plant. Also, Smidansky et al., (2003) reported that there was more than a 20% increase in rice seed weight, a consequence of an increase in starch due to expression of a modified maize AGPase gene (\( Sh2r6hs \)).

### 1.11 SUCROSE SYNTHESIS

Sucrose is important in starch synthesis because it is the main source of glucose-1-phosphate required for starch synthesis (Denyer et al., 1996; Huber and Huber, 1996). Sucrose synthesis is catalyzed by two enzymes in higher plants, sucrose-phosphate synthase (EC 2.4.1.14) and sucrose synthase (EC 2.4.1.13) (Huber and Huber, 1996). The reactions of both enzymes are reversible but the product of sucrose phosphate synthase (sucrose-6-phosphate) is rapidly removed by sucrose phosphatase rendering the overall reaction irreversible and making sucrose phosphate synthase the more important
of the sucrose-synthesizing enzymes (Huber and Huber, 1996). Sucrose phosphate synthase is allosterically regulated by metabolites and by reversible protein phosphorylation. Phosphorylation in leaves controls the sucrose phosphate synthase activity in response to light/dark signals and end product accumulation (Huber and Huber, 1996). Phosphorylation occurs at multiple serine residues and the major site of phosphorylation may vary from plant to plant: serine 158 in spinach leaves and serine 162 in maize leaves. Incremental changes in the activity of sucrose phosphate synthase has been achieved in several plants by over-expression of the gene that encodes sucrose phosphate synthase. Tomato plants that were transformed with maize sucrose phosphate synthase had elevated leaf sucrose phosphate synthase activity. The maize enzyme is not regulated in response to normal light/dark modulation (Galtier et al., 1993; Galtier et al., 1995; Worrell et al., 1991). Tomato plants expressing the maize sucrose phosphate synthase gene, driven by the leaf-specific Rubisco small subunit promoter, had increased total numbers of fruits, fruits matured earlier, and total fruit dry weight increased (Micallef et al., 1995). Sucrose phosphate synthase transformants also had higher rates of photosynthesis compared to control plants (Huber and Huber, 1996).

1.12 HYPOTHESES

1. Different cassava varieties will respond differently to different somatic embryo induction media.

2. Maturation of somatic embryos from different cultivars will also require different media for optimization.
3. There may be varietal differences in requirements for germination of somatic embryos.

4. Cassava has a high rate of photosynthesis and one of the highest known rates of sucrose synthesis. This implies that cassava has a high source potential for reduced carbohydrate. By increasing sink strength in cassava roots we hypothesize that we can increase root biomass.

5. By increasing source strength (sucrose production) we also may be able to increase sink biomass allocation.

6. It is hypothesized that by transforming cassava with a modified *E.coli* *glgC* gene expressed in roots and having enhanced enzyme kinetics relative to the plant AGPase we will increase sink strength in roots and increase root biomass (starch) production.

7. It is hypothesized that by transforming cassava with a maize *sps* gene expressed in leaves we will increase source strength in leaves and increase root biomass (starch) production.

### 1.12 GOALS AND OBJECTIVES

The overall objectives of this study are to increase the starch production potential in transgenic cassava. We also will identify cassava varieties from Africa for their ability to be, cultured *in vitro*, regenerated from tissue culture, and for their ease of genetic transformation. We will also use the Colombian cultivar MCol 2215 as the positive control for transformation. The cultivar MCol 2215 is the model cassava cultivar in our lab having been transformed with a binary plasmid carrying the HNL gene expressed by CaMV 35S promoter (White *et al.*, 1998, Siritunga *et al.*; 2003) and antisense expression
of cytochrome P450 genes (CYP79D1/D2) involved in cyanogenic glycoside synthesis (Siritunga and Sayre, 2003) in the lab. These varieties will be transformed with binary plasmids containing the maize sucrose-phosphate synthase (sps) gene, which is insensitive to phosphorylation driven by the CAB1 promoter, and a modified *E.coli* glgC gene driven by the patatin promoter and targeted to the chloroplast. Cassava plants will be transformed individually with these constructs to determine the impact of the genes on carbohydrate storage in roots.

### 1.13 JUSTIFICATION

While several laboratories have been pursuing cassava regeneration and transformation systems, the focus has been largely limited to cassava varieties from South America. Less effort has been placed on developing regeneration and transformation systems for African cassava varieties (Taylor and Henshaw, 1993; Konan *et al.*, 1994). This is surprising since cassava is the major source of caloric input for the sub-Saharan African peoples. Since environmental (e.g. soil and water), biological (e.g. disease and insect pressure) and human (e.g. selection by farmers for desirable traits) demands for African cassava varieties are undoubtedly different from those for South American varieties, it is expected that African cassava varieties have genetically diverged from their South American progenitors (Kawano, 2003; Dr. Sayre personal communication). In order to exploit the desirable traits of African varieties as well as to increase the genetic diversity of transformable cultivars, it is important to identify and develop strategies for regenerating and transforming African cassava varieties. This is
important since the traditional breeding methods have been difficult in developing cassava varieties with improved traits due to a lack of flowering, its consequent fruiting problems and heterosis, as well as the long duration for generating new clones in Africa.

In collaboration with Dr. William Roca (CIAT, Cali, Colombia) and Dr. Stan Gelvin (Purdue University, W. Lafayette, IN, USA) we have developed *Agrobacterium tumefaciens* Ti plasmid vectors which give high rates of *in vitro* transformation. Using the IITA cassava cultivars which we have identified as responsive in culture, we will introduce foreign genes into cassava to increase starch quantity.

Cassava has a long growing season, taking about 12 months to maximize yield. This is too long considering the population explosion in Africa and loss of arable land to desert-encroachment. We propose that enhancing the activity of AGPase, the most critical enzyme involved directly in starch biosynthesis as well as sucrose phosphate synthase, which is involved in producing substrates for AGPase, could produce a cassava that could potentially have reasonable yields in six months, half the time in nature. Relief would come to African farmers when we meet our goals.
CHAPTER 2

SOMATIC EMBRYOGENESIS AND REGENERATION IN RECALCITRANT CASSAVA VARIETIES FROM AFRICA.

2.1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important food crops for sub-Saharan African peoples. Its starchy tuberous roots provide a valuable source of cheap calories for about 500 million people in the developing world where food deficiency and malnutrition are common. In addition, the leaves and tender shoots are eaten in many parts of Africa as a source of vitamins, minerals and protein (Cock, 1982). Cassava tolerates low fertility and drought and for this reason it is popular among small-scale farmers in places with infertile soils and adverse climates (Koch *et al.*, 1994). In spite of these good qualities, cassava contains cyanogenic glycosides which when not effectively removed may cause a variety of health problems in nutritionally-compromised peoples (Rosling *et al.*, 1993). In addition, African cassava may frequently be infected with African Cassava Mosaic Virus. These and other traits are potentially amenable to genetic engineering techniques particularly since genetic improvement through traditional
breeding has been problematic because seed production is low and the generation time (one year) is long.

One of the requirements for the generation of transgenic cassava is an efficient and reproducible plant regeneration system. Somatic embryos have been induced from cassava young leaf lobes and cotyledons leading to plant regeneration (Stamp and Henshaw, 1982, 1986, 1987a,b; Stamp, 1987; Szabados et al., 1987; Taylor and Henshaw, 1993; Mathews et al., 1993; Raemakers et al., 1993; Konan et al., 1994). The focus of most cassava tissue culture regeneration efforts, however, has largely been on cassava varieties from South America. Apart from a few studies, (Taylor and Henshaw, 1992; Konan et al., 1994), little if any efforts have been placed on developing regeneration and transformation systems for African cassava varieties. Furthermore, reports show that cassava varieties from Africa respond differently in culture from South American varieties. Environmental (e.g. soil and water), biological (e.g. disease and insect pressure) and human (e.g. selection by farmers for desired traits) demands for African varieties, which are undoubtedly different from those of South American varieties, may have lead to genetic divergence of African cassava from their South American progenitors potentially accounting for the different responses in culture (Sayre, personal communication).

In this chapter, we have developed an improved method of somatic embryogenesis and plant recovery for African cassava varieties.
2.2 MATERIALS AND METHODS:

2.2.1 Plant material

In vitro-established plants of the following cassava cultivars were obtained from Dr. S.Y.C. Ng at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria: TMS 30555, TMS 60142, TMS 4 (2) 1425, TMS 71173, TMS 30786, TMS 4488, TMS 60444, TMS 30395, TMS 30572, TMS 30337, TMS 30001. The plants were multiplied in vitro through nodal segments on standard cassava micropropagation medium (Roca, 1984) comprising MS salts (Murashige and Skoog, 1962), 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 2 % (w/v) sucrose, 0.02 mg/L α-naphthalene acetic acid (NAA), 0.04 mg/L 6-benzylaminopurine (BA) and 0.05 mg/L giberellic acid (GA₃). The medium was solidified with 0.8 % (w/v) Difco-Bacto agar. Shoots were maintained on a 12 hr/day photoperiod at 28 °C with a light intensity of 20 µmol photons m⁻² s⁻¹.

2.2.2 Induction of somatic embryogenesis

Young leaf lobes (1-5 mm long) from in vitro-grown plants were cultured on MS basal medium supplemented with 2 % (w/v) sucrose, B5 vitamins (Gamborg et al., 1968), 50 mg/L casein hydrolysate, 0.5 mg/L additional copper in the form of CuSO₄ (Schopke et al., 1992) and 4 - 16 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D); (b) substituting 2,4-D in (a) with picloram; (c) MS medium supplemented with 2 % (w/v) sucrose and 4 - 16 mg/l picloram; (d) factors affecting embryogenesis were studied with 0 - 8% sucrose, 0 - 1.0 mg/L additional CuSO₄ and keeping the embryogenesis cultures in darkness or on 12 hr/day light; the media pH was adjusted to 5.7 with 1.0 N KOH and the medium
solidified with 0.8 % (w/v) Difco-Bacto agar. The cultures were maintained in the dark by covering with cheese cloth at 28 °C. The explants were left in the induction medium for 3-4 weeks.

2.2.3 Maturation of somatic embryos

Maturation in this sense, describes the development of globular stage embryos into green cotyledonary embryos with defined shoot and root axes (Mathews et al., 1993). Globular stage somatic embryos were subcultured on maturation media, MS salts (Murashige and Skoog, 1962) supplemented with 2 % (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA, and 0.5 mg/L GA₃; MS medium supplemented with 2 % (w/v) sucrose, 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA₃; or MS basal medium supplemented with 2 % (w/v) sucrose. The media pH was adjusted to 5.7 with 1N KOH and solidified with 0.8 % (w/v) Difco-Bacto agar. The embryos were maintained in the maturation medium mentioned above for 3-4 weeks.

2.2.4 Repetitive embryogenesis

Green cotyledonary embryos were cultured on cassava embryo induction medium comprising: MS (Murashige and Skoog, 1962) basal medium supplemented with 2 % (w/v) sucrose, B5 vitamins (Gamborg et al., 1968), 50 mg/L casein hydrolysate, 0.5 mg/L additional copper in the form of CuSO₄ (Schopke et al., 1992) and 8 mg/L 2,4-D. The cultures were maintained in the dark by covering with cheese cloth at 28 °C. The explants were left in the induction medium for 3-4 weeks.
2.2.5 Desiccation of somatic embryos

Some mature somatic embryos were subjected to desiccation by enclosing them in sealed sterile petri dishes and incubating them at 28 °C for 7 days in Percival Scientific incubator (Boone, IA) (Mathews et al., 1993). At the end of this period the embryos were transferred to basal MS medium supplemented with 2 % (w/v) sucrose; or MS medium supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA and 0.5 mg/L GA₃; or MS basal salt supplemented with 2 % (w/v) sucrose and 0.8 % (w/v) activated charcoal. All the media were solidified with 0.8 % (w/v) Difco-Bacto agar for germination and plant recovery.

2.2.6 Germination and plant recovery

Mature somatic embryos (having distinct root and shoot axes) with or without desiccation treatment were transferred to basal MS medium supplemented with 2 % (w/v) sucrose; or MS medium supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA and 0.5 mg/L GA₃; or MS basal salt supplemented with 2 % (w/v) sucrose and 0.8 % (w/v) activated charcoal. All the media were solidified with 0.8 % (w/v) Difco-Bacto agar for germination and plant recovery. Germination and conversion rates were recorded after four weeks in culture.

The maturation and desiccation cultures were kept in the dark, while the germination and conversion experiments were exposed to a daily photoperiod of 12 hours with General Electric, cool white fluorescent tubes (40 µmol photon m⁻² s⁻¹ at the point of culture). All cultures were kept at 28 °C.
2.2.7 Statistical analysis

Analysis of variance (ANOVA) and other statistical analysis were done with the SAS software (SAS, 1985) at the Horticulture and Crop Science Department, The Ohio State University, Columbus, OH. Samples were evaluated using analysis of variance (ANOVA) for a randomized complete block-design. Duncan’s multiple range test was used to separate treatment means found significantly different by ANOVA. All analyses were performed at $P \leq 0.05$ confidence level.
2.3 RESULTS

2.3.1 Effects of plant growth regulators on somatic embryogenesis

Embryogenesis has been induced in all African cassava varieties tested, using either 2, 4-D or picloram in the induction medium.

Four concentrations of 2, 4-D and picloram (4, 8, 12 and 16 mg/L) were tested for their ability to induce somatic embryos. The different 2, 4-D treatments were used to determine which concentration was best for somatic embryo induction of the cassava cultivars. All the treatments produced somatic embryos. The best treatment in either 2, 4-D or picloram was 12 mg/L with the exception of TMS 4 (2) 1425 which produced more embryos on 4 mg/L 2, 4-D medium than in the other treatments (Table 2.1). Embryogenesis starts as swollen regions at the cut ends and mid-veins on the adaxial surface of the explant. The swollen segments then give rise to two kinds of calli namely: a loose non-embryogenic friable white callus and a translucent gelatinous embryogenic callus that formed globular stage embryos.

When using 2, 4-D, the best response with regards to number of embryos per explant was observed with cultivar TMS 30555 followed by TMS 60142 and TMS 4 (2) 1425 which produced more than 80 somatic embryos per explant. The next response group
Table 1: Effects of 2,4-D on the induction of embryos from leaf lobes of cassava. Medium, MS + 2% sucrose + 50 mg/l casein hydrolysate + B5 vitamins + 0.5 mg/l additional CuSO₄ + 12 mg/l 2,4-D. Numbers with the same letter superscript are not statistically different. Numbers with different letter superscripts are statistically different.
<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TMS 30555</td>
<td>0.0^z</td>
<td>40.2 ± 2.1^l</td>
<td>84.6 ± 2.9^c</td>
<td>100.6 ± 3.4^a</td>
<td>44.2 ± 2.5^h</td>
</tr>
<tr>
<td>TMS 60142</td>
<td>0.0^z</td>
<td>34.0 ± 3.8^jkl</td>
<td>67.7 ± 4.1^e</td>
<td>84.7 ± 3.5^c</td>
<td>31.2 ± 1.5^l</td>
</tr>
<tr>
<td>TMS 4(2) 1425</td>
<td>0.0^z</td>
<td>91.6 ± 4.4^b</td>
<td>73.0 ± 3.0^d</td>
<td>60.8 ± 2.4^l</td>
<td>36.8 ± 1.8^l</td>
</tr>
<tr>
<td>TMS 71173</td>
<td>0.0^z</td>
<td>32.5 ± 1.9^l</td>
<td>40.0 ± 3.2^l</td>
<td>52.7 ± 3.8^g</td>
<td>22.3 ± 1.2^no</td>
</tr>
<tr>
<td>TMS 4488</td>
<td>0.0^z</td>
<td>19.6 ± 1.6^opqr</td>
<td>24.8 ± 2.7^mn</td>
<td>33.3 ± 3.2^kl</td>
<td>18.3 ± 1.6^qr</td>
</tr>
<tr>
<td>TMS 30395</td>
<td>0.0^z</td>
<td>21.3 ± 2.2^opq</td>
<td>25.0 ± 3.0^mn</td>
<td>31.6 ± 3.4^l</td>
<td>17.5 ± 1.5^r</td>
</tr>
<tr>
<td>TMS 30786</td>
<td>0.0^z</td>
<td>20.3 ± 3.1^opqr</td>
<td>26.1 ± 2.1^m</td>
<td>32.5 ± 4.8^l</td>
<td>18.0 ± 1.5^r</td>
</tr>
<tr>
<td>TMS 60444</td>
<td>0.0^z</td>
<td>21.6 ± 3.9^op</td>
<td>27.5 ± 2.5^m</td>
<td>35.6 ± 2.5^jk</td>
<td>18.8 ± 1.6^pqr</td>
</tr>
<tr>
<td>TMS 30572</td>
<td>0.0^z</td>
<td>13.6 ± 2.4^s</td>
<td>19.3 ± 2.2^opqr</td>
<td>20.5 ± 4.6^opqr</td>
<td>11.0 ± 1.3^st</td>
</tr>
<tr>
<td>TMS 30337</td>
<td>0.0^z</td>
<td>3.3 ± 0.9^wxy</td>
<td>8.3 ± 1.9^uv</td>
<td>9.3 ± 1.5^tu</td>
<td>7.0 ± 1.3^uv</td>
</tr>
<tr>
<td>TMS 3001</td>
<td>0.0^z</td>
<td>2.2 ± 1.2^yz</td>
<td>5.5 ± 1.7^vwx</td>
<td>6.1 ± 1.1^vw</td>
<td>3.0 ± 1.3^vwy</td>
</tr>
</tbody>
</table>
in the hierarchy comprised TMS 71173, TMS 4488, TMS 30395, TMS 30786, TMS 60444, and TMS 30572 which produced more than 30 somatic embryos per explant. The third group comprised TMS 30337 and TMS 30001, which produced less than 10 somatic embryos per explant (Table 2.1). The number of explants undergoing somatic embryogenesis in response to 2, 4-D (12 mg/L) treatment was more than those on picloram medium (12 mg/L) (Table 2.2).

Four cultivars (TMS 30555, TMS 60142, TMS 71173 and TMS 4 (2) 1425) were used in studying the effect of picloram on embryogenesis. Compared to 2,4-D, the supplementation of the embryogenesis media with picloram resulted in cultivar-dependent response to somatic embryogenesis. While TMS 30555 (100 somatic embryos per explant) was best with regards to number of embryos produced per explant on 2, 4-D medium, TMS 4 (2) 1425 (95 somatic embryos per explant) produced the most embryos on embryogenesis media supplemented with picloram. The number of embryos produced on picloram and 2, 4-D media were high for TMS 4 (2) 1425 (60 versus 95 embryos) and TMS 71173 (52 versus 56 embryos). However, the number of embryos produced on picloram medium plummeted relative to 2,4-D with regards to TMS 30555 (10 versus 100) and TMS 60142 (7 versus 84) (Table 2.2 and Table 2.3).
<table>
<thead>
<tr>
<th>Number of somatic embryos and percent of embryogenic explants regenerated</th>
<th>2, 4-D (12 mg/L)</th>
<th>Picloram (12mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMS 30555</td>
<td>32/35 (91.4)</td>
<td>13/30 (44.4)</td>
</tr>
<tr>
<td>TMS 60142</td>
<td>29/32 (90.6)</td>
<td>12/27 (44.4)</td>
</tr>
<tr>
<td>TMS 4(2) 1425</td>
<td>20/25 (80)</td>
<td>26/28 (92.8)</td>
</tr>
<tr>
<td>TMS 71173</td>
<td>22/25 (88)</td>
<td>21/25 (84)</td>
</tr>
<tr>
<td>TMS 4488</td>
<td>26/30 (86.7)</td>
<td>NT¹</td>
</tr>
<tr>
<td>TMS 30395</td>
<td>19/26 (73.1)</td>
<td>NT</td>
</tr>
<tr>
<td>TMS30786</td>
<td>18/25 (72)</td>
<td>NT</td>
</tr>
<tr>
<td>TMS 60444</td>
<td>25/32 (78.1)</td>
<td>NT</td>
</tr>
<tr>
<td>TMS 30572</td>
<td>25/32 (78.1)</td>
<td>NT</td>
</tr>
<tr>
<td>TMS 30337</td>
<td>12/25 (48)</td>
<td>NT</td>
</tr>
<tr>
<td>TMS 30001</td>
<td>11/27 (40.7)</td>
<td>NT</td>
</tr>
</tbody>
</table>

Table 2.2: Effect of picloram and 2,4-D on the induction of somatic embryogenesis from cassava leaf lobes. NT, not tested.
<table>
<thead>
<tr>
<th>Cultivars</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMS 30555</td>
<td>0.0</td>
<td>2.6 ± 1.5</td>
<td>7.6 ± 2.1</td>
<td>9.8 ± 1.7</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>TMS 60142</td>
<td>0.0</td>
<td>2.5 ± 0.9</td>
<td>6.2 ± 1.3</td>
<td>7.0 ± 1.3</td>
<td>1.2 ± 1.1</td>
</tr>
<tr>
<td>TMS 4(2) 1425</td>
<td>0.0</td>
<td>68.5 ± 2.1</td>
<td>77.6 ± 3.9</td>
<td>95.0 ± 4.2</td>
<td>40.0 ± 3.0</td>
</tr>
<tr>
<td>TMS 71173</td>
<td>0.0</td>
<td>38.0 ± 2.6</td>
<td>41.5 ± 1.7</td>
<td>56.2 ± 3.2</td>
<td>26.7 ± 2.1</td>
</tr>
</tbody>
</table>

Table 2.3: Effects of picloram on the induction of embryos from leaf lobes of cassava.

Medium, MS + 2% sucrose + B5 vitamins + 50 mg/l casein hydrolysate + 0.5 mg/l additional CuSO$_4$ + 12 mg/l picloram.
2.3.2 Effect of light on embryogenesis

The cultivar TMS 30555 was used to study the effect of exposing the somatic embryo cultures to 12 hr./day photoperiod versus dark incubation (covering the cultures with four layers of cheese cloth). Exposing the explant to 12 hr./day photoperiod *ab initio* affected the process of embryogenesis. After four weeks in culture, the light-treated explants formed more non-embryogenic calli than those kept in the dark (data not shown). More than half of the surface area of the light-treated explants did not produce callus. Overall, only a few embryos were produced when the cassava leaf lobe explants were given 12 hours of light followed by 12 hours of darkness (Table 2.4). With dark incubation, 92% of the explants produced somatic embryos compared to 60% of explants given 12 hours of dark and light cycles (Table 2.4). However, light is required for germination of embryos (Raemakers *et al.*, 1997).

2.3.3 Effect of sucrose

Callus formation and embryogenesis did not take place with the exclusion of sucrose from the induction medium. The explants lost color after three weeks and subsequently died. The number of embryos produced in 2-8% sucrose media was comparable. However, the embryos produced on 2% sucrose medium were healthier than the others. On 4% sucrose medium, half of the embryos were green, while the others were cream-colored. The embryos grown on 8% sucrose were all cream-colored and most turned brown after 4 weeks in culture. The browning of the calli and embryos on embryogenesis media supplemented with 8% sucrose is a sign of cell death caused by formation of phenolic compounds in the cultures.
<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Photoperiod/day</th>
<th>Total number of cultured explants</th>
<th>Number and (percent) of explants with developed embryos</th>
<th>Mean number of somatic embryo/explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMS 30555</td>
<td>12 hr</td>
<td>25</td>
<td>15(60)</td>
<td>$3 \pm 2.5^b$</td>
</tr>
<tr>
<td>TMS 30555</td>
<td>0</td>
<td>25</td>
<td>23(92)</td>
<td>$95 \pm 3.8^a$</td>
</tr>
</tbody>
</table>

Table 2.4: Effect of light on embryogenesis. Medium, MS + 12 mg/L 2,4-D.
The color difference between the embryos cultured on 2% and 4-8% sucrose also suggests that high levels of sucrose in embryo induction medium could lead to stress (Table 2.5).

2.3.4 Effects of copper sulphate

The effect of additional copper in the form of CuSO₄ was studied with cultivars TMS 30555, TMS 30786, TMS 4488, TMS 4 (2) 1425 by the inclusion of 0.25, 0.5, 0.75 and 1.0 mg/L additional CuSO₄ in the induction medium. The embryos produced on media lacking additional copper were difficult to distinguish from the calli. Previously, Schopke et al., had demonstrated that regeneration of South American cassava responded positively to additional copper in MS media (Schopke et al., 1992). These results were cultivar-dependent, however. With cultivar 30555, there was no significant difference among the plus and minus additional copper treatments, it produced similar number of embryos at all copper concentrations tested. However, TMS 30786 had an increase in the number of embryos from 0.25 to 0.5 mg/L copper (data not shown). The number of somatic embryos was reduced at higher (0.5 mg/L) copper concentrations. Cultivars TMS 4488 and TMS 4 (2) 1425 produced embryos in all treatments but had a peak at 0.5 mg/L. Additional copper did not play a significant role in the germination of the embryos. TMS 4488 embryos from all the treatments converted to plantlets at the same frequency (data not shown).
<table>
<thead>
<tr>
<th>Cultivar</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMS 30555</td>
<td>0.0 ±0.0a</td>
<td>95.5 ±2.6b</td>
<td>93.0 ±3.1b</td>
<td>92.8 ±7.0b</td>
</tr>
</tbody>
</table>

Table 2.5: Effect of sucrose concentration on somatic embryogenesis. Medium, MS + 12 mg/L 2,4-D.
2.3.5 Repetitive embryogenesis

The maintenance of mature somatic embryos on fresh induction medium, under dark incubation at 28°C led to the induction of somatic embryos in numbers comparable to the induction of embryogenesis when young leaf lobes were used as explants. These began as small swelling on the cotyledons of the embryos which produced callus while proliferating embryos.

2.3.6 Maturation of embryos

The early stage somatic embryos were transferred to the maturation medium after 3 - 4 weeks of culture with adjoining callus. More than 90% of the embryos grew to cotyledonary embryos with distinct root and shoot axes. The maintenance of the embryos on MS medium supplemented with 2% sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA and 0.5 mg/L GA3 was superior to MS medium supplemented with 2% sucrose, 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA3 and MS medium supplemented with 2% sucrose was superior in the sense that culture on this media resulted in 30% larger embryos that had a 50% higher rate of germination than the others on the germination medium and were separated easily from the aggregate (data not shown). In the medium supplemented with 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA3, 50% of the mature embryos failed to germinate (did not turn green). On the other hand, the embryos maintained on MS basal medium germinated but were smaller than those on maturation medium supplemented with 0.01 mg/L 2,4-D.
Three types of embryos were observed in the maturation media, embryos with two distinct cotyledons; embryos with one cotyledon; and embryos with fused cotyledons. The embryos with two cotyledons and the embryos with fused cotyledons occurred at the same frequency and made up about 90% of the total embryo population. These embryos were produced on somatic embryogenesis media supplemented with 4 – 16 mg/L 2, 4-D. All the treatments had the same frequency of producing embryos with one cotyledon, embryos with fused cotyledon and embryos with two cotyledons. Germination and conversion to plantlets was not a function of the morphology of the embryos, embryos with two distinct cotyledons, embryos with one cotyledon, and embryos with fused cotyledons all had the capacity to convert to plantlets.

2.3.7 Germination of somatic embryos and plant recovery

The frequency of embryo germination was enhanced significantly by desiccation treatment before culturing on regeneration medium. Desiccated embryos germinated in 10-14 days, while embryos excluded from the desiccation treatment germinated in 4 - 6 weeks. The germination medium comprising MS salts, 2 % sucrose, and 0.8 % activated charcoal was superior to basal MS medium plus 2 % sucrose, and MS supplemented with 2 % sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA and 0.5 mg/L GA₃ in terms of germination time of desiccated embryos (Table 2.6).

Non-desiccated embryos from MS medium supplemented with 2 % sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2, 4-D, 1.0 mg/L BA, 0.5 mg/L GA₃ had a shorter germination time (data not shown) when transferred on fresh medium than
when maintained on activated charcoal medium or MS basal medium. The embryos germinated in 20 - 30 days of culture. The plantlets regenerated on the 2, 4-D medium without desiccation grew faster (data not shown) than those from activated charcoal medium with prior desiccation treatment. The plantlets from the 0.01 mg/L 2, 4-D germination medium reached a height of 9 - 12 cm in one month as compared to 7 - 10 cm in plantlets recovered from the activated charcoal medium via desiccation.

Single embryos did not always give rise to single plantlets. About 20 % of the embryos converted to multiple plantlets. In some instances up to five plantlets arose from one embryo.

Finally, the recovery of plantlets from embryos was dependent on the cultivar in question. To date, we have recovered plantlets from eight of the eleven cultivars, including TMS 71173, TMS 30786, TMS 60142, TMS 4488, TMS 30555, TMS 60444, TMS 30001 and TMS 30395. There was no success in the conversion of embryos to plantlets with the embryos from TMS 30372, TMS 30337 and TMS 4 (2) 1425. The best results were obtained with TMS 71173 which had more than 90 % germination and conversion to plantlets (Table 2.6).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial number of embryos</th>
<th>Number of germinated embryos</th>
<th>Number of regenerated embryos</th>
<th>Dormancy (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without desiccation</td>
<td>50</td>
<td>3 (6%)</td>
<td>1 (2%)</td>
<td>20 - 30</td>
</tr>
<tr>
<td>With desiccation</td>
<td>50</td>
<td>46 (92%)</td>
<td>41 (82%)</td>
<td>10 - 14</td>
</tr>
<tr>
<td>Without desiccation (0.01 mg/L 2,4-D)</td>
<td>50</td>
<td>45 (90%)</td>
<td>39 (78%)</td>
<td>20 - 30</td>
</tr>
<tr>
<td>Without desiccation (Basal medium)</td>
<td>50</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

Table 2.6: Effect of desiccation on germination and plant regeneration in TMS 71173. The standard medium for plant regeneration was MS medium supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 1.0 mg/L BA and 0.5 mg/L GA$_3$. 
2.4 DISCUSSION

We report an improvement in somatic embryogenesis and plant recovery for African cassava varieties. Contrary to the report of Taylor et al., 1992, we observed that 12 mg/L 2, 4-D was better than 12 mg/L picloram for embryogenesis induction for most of the cultivars. Picloram (12 mg/L) was better with regards to frequency of leaf lobe embryogenesis than 2,4-D (12 mg/L) only in one cultivar, 4 (2) 1425 (93% compared to 80%, Table 2.2). Embryogenesis was induced at high 2, 4-D (16 mg/l) concentrations in all the cultivars that we tested. Finer and Nagasawa (1988), reported successful somatic embryogenesis in *Glycine max* on medium supplemented 40 mg/L 2, 4-D. However, Konan et al., 1994, reported an inhibition of embryogenesis in cassava by the supplementation of the induction-medium with more than 12 mg/L 2, 4-D. The difference in our results could have been the result of different explants; while we used young leaf lobes, Konan’s group used the cotyledons of zygotic embryos.

The role of copper in somatic embryogenesis seems to be enhancement of the growth of the embryos. Somatic embryogenesis experiments performed with media lacking additional copper produced embryos difficult to distinguish from the non-embryogenic callus because they were too small. However, the inclusion of additional copper made the embryos distinct at the early stage of embryogenesis.

Somaclonal variation has been a problem with many plant species regenerated *in vitro* through callus. This problem could be the result of the high rate of cell division during
callus formation induced by high auxin levels in the induction medium. This problem is reduced in plants recovered through somatic embryogenesis. The incidence of somaclonal variation in cassava regenerated by somatic embryogenesis with 4 – 16 mg/L 2, 4-D is negligible (Konan et al., 1994). Normally, embryogenesis in most plants starts with callus induction, followed by transfer to another medium for embryo emergence. In cassava, the explant on the induction medium proliferates to globular stage embryos in about two weeks of culture, hence reducing the length of time the regenerative tissues stay on high auxin (8-12 mg/L) medium. This presumably reduces the incidence of somaclonal variation further in cassava plants recovered from somatic embryos. Recently, there have been more reports of somaclonal variation in cassava regeneration systems (Raemakers et al., 1997). The drive to improve on the efficiency of cassava transformation has led to the development of additional techniques for plant regeneration. One of those is the use of friable embryogenic callus to regenerate somatic embryos. This technique requires the callus to be on the high auxin medium (50 mg/L) for six or more months resulting in high rates of somaclonal variation (Raemakers et al., 1997).

The organogenesis method of cassava regeneration involves the induction of shoots from young leaf lobes and embryo cotyledons on MS medium supplemented with cytokinins (23 μM zeatin and 44 μM benzylaminopurine) (Mussio et al., 1998; Guohua, 1998). This method results in the regeneration of multiple shoots but the origin of each shoot is doubtful making it suspect for plant transformation purposes.
Embryo development was achieved in medium supplemented with low levels of BA (1.0 mg/L), GA₃ (0.5 mg/L) and either NAA (0.01 mg/L) or 2, 4-D (0.01 mg/L) and also in medium devoid of growth regulators. Mathews’ et al., 1993, cultured globular stage embryos of cassava on medium without plant growth regulators and achieved the highest rate of regeneration yet in cassava. These results indicate that the presence of growth regulators is only critical in the induction medium for embryogenesis to take place.

Exposing the explants in the induction medium to light at the beginning of the induction process lead to vegetative growth, hence, the drastic reduction in the number of embryos formed. Also the light-intensity given to the parent plants could affect embryogenesis. The explants we used were harvested from plants grown under 20 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) light intensity. Raemakers et al., 1992 reported enhancement of embryogenesis in cassava by lowering the light intensity (10 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) during the growth of the donor plants. Lowering the light intensity of the cultures could have accounted for the high rates of embryogenesis in some of the cultivars we reported.

The induction of somatic embryogenesis and plant recovery in the African cassava cultivars provides a leeway for the introduction of foreign beneficial genes to enhance the root quality of cassava.
CHAPTER 3

GENETIC MODIFICATION OF CASSAVA FOR ENHANCED STARCH PRODUCTION

3.1 INTRODUCTION

The starchy tuberous roots of Cassava (*Manihot esculenta* Crantz; Euphorbiaceae) are a valuable source of cheap calories for about 500 million people in the developing tropical countries where food deficiency and malnutrition are common (Figure 3.1). The leaves and tender shoots of cassava also are eaten in many parts of Africa as a source of vitamins, minerals and proteins (Cock, 1982). Though cassava serves primarily as a food crop, it is also used industrially in the production of ethanol, mannitol, glucose, sorbitol, as animal feed, and as a raw material for the starch industry (Cock, 1982).

Cassava tolerates low soil fertility and drought well and therefore is the crop of choice for many small-scale farmers, particularly in the tropics. Cassava thrives on eroded soils and on marginal lands including those abandoned because other crops fail to grow on them. Cassava is resistant to locust damage and to many generalist pests (Koch *et al.*, 1994).
Figure 3.1: Cassava’s root is consumed by about 500 million people worldwide. Figures showing the processing of cassava for use as food (IITA website, www.iita.org).
In addition, cassava adapts easily to traditional farming methods and can be intercropped with maize, yam, beans, and melon. The roots can be left in the ground without harvest for up to three years. This makes it a very important crop for security against famine (Lynam, 1993).

Cassava has a high efficiency of photosynthetic conversion of carbon dioxide into assimilates (Hunt et al., 1977). It has a high rate of photosynthesis (43 µmol CO$_2$/m$^2$/s) and is photosynthetically efficient at temperatures as high as 45°C (Angelov, et al., 1993, Edwards., et al., 1990; Hunt et al., 1977). It also has chlorenchymatous bundle sheath cells and is able to carry out photosynthesis at high light intensities. These C4-like photosynthetic characteristics notwithstanding, cassava is a C3 plant because the first product in photosynthetic carbon reduction is 3-PGA and not a C4 compound (Angelov et al., 1993). Cassava also is prone to photorespiration like C3 plants although at reduced rates. The CO$_2$ compensation point (65 µl/L) is lower than that of C3 plants but definitely higher than that of C4 plants (Angelov et al., 1993; Edwards et al., 1990). Cassava also has one of the highest rates of carbon dioxide conversion to sucrose of any plant measured (Hunt et al., 1977; Angelov et al., 1993). For these reasons, cassava is potentially an excellent candidate for increased starch production in sink tissues.

Since cassava is typically clonally propagated by stem cutting rather than by seeds it is a prime candidate for genetic modification by genetic transformation. Reports on stable

In cassava, carbohydrates are translocated mainly to the stems and storage roots. The accumulation of carbohydrates in the leaf decreases during the crop cycle. In the first 75 days after planting, the accumulation of dry matter is mainly in the leaves rather than in the stems and roots. Afterwards, the allocation of carbohydrates is mainly to the storage roots reaching 60% of the total dry matter after 4 months (*Howeler and Cadavid*, 1983). The period of maximal rates of dry matter accumulation of cassava in the tropics, where it is normally grown, is 3 - 5 months after planting.

The photoassimilate partitioning from leaves to roots increases between 6 – 10 months after planting facilitating the bulking of storage roots. At this time, starch synthesis in the root (sink) is very active generating a carbohydrate gradient that presumably facilitates the translocation of sucrose to the roots for starch biosynthesis (*Stitt et al.* 1995). The highest rate of root dry matter accumulation occurs within this period (*Tavora et al.*, 1995).
AGPase plays a critical role in the regulation of starch synthesis in plants not only because it is the first committed enzyme in starch synthesis but it is also the rate-limiting step in starch synthesis. In addition, the potato AGPase activity is regulated allosterically by 3-PGA (enhances) and inorganic phosphate (Pi; inhibits) (Plaxton and Preiss, 1987; Preiss, 1991; Okita, 1992; Martin and Smith, 1995). Antisense-mediated inhibition of AGPase expression and/or mutagenesis of glgC gene to eliminate or reduce its enzymatic activity leads to severe reductions in starch production (Muller-Rober et al., 1992; Denyer et al., 1996; Munyikwa et al., 1998).

The plant AGPase holoenzyme is a hetero-tetramer and is formed from two distinct polypeptides which comprise the large and small units (Copeland and Preiss, 1981). The large subunit is 54 to 60 kD in size while the small subunit is 51 to 54 kD depending on the species (Copeland and Preiss, 1981; Martin and Smith, 1995). Multiple isoforms of the subunits have been found in plants (Okita, 1992; Martin and Smith, 1995). Interestingly, both subunits have been reported to be enzymatically active and both subunits have to be present for maximal activity. (Frueauf, et al., 2001; Tiessen et al., 2002). This makes the genetic manipulation of the plant AGPase difficult because it potentially requires incorporation of more than one gene into the plant.

In contrast, the bacterial AGPase is a single gene (glgC) product. In addition, the enzyme is allosterically regulated by different regulators. The bacterial AGPase is activated by fructose-1, 6-bisphosphate (FBP) and is inhibited by adenosine monophosphate (AMP)
The bacterial AGPase has two important allosteric regulation sites which include the following amino acids: Gly336 and Lys296 (G336 and K296). Mutation of Gly336 to Asp inhibits allosteric feedback regulation by FBP, although the activity of the modified enzyme is reduced by more than 90% compared to the wild type (Kumar et al., 1989). In spite of this reduction in activity the introduction of the mutant glgC16 gene having an N-terminal chloroplast transit peptide (TP) into potato via nuclear transformation resulted in a 36% increase in enzyme activity and a 35% increase in tuber starch content (Stark et al., 1992). This increase in tuber starch production occurred only when the glgC16 gene was expressed in the tuber driven by the patatin promoter. Such an increase was lacking when the glgC16 gene was expressed in all tissues (Stark et al., 1992). In fact the plants struggled to survive. However, Stark et al., (1992), did not report AGPase activity in the tubers. Subsequently, Sweetlove et al., (1996), reported the transformation of potato with glgC16 gene under the control of patatin in the tuber and demonstrated the increase in AGPase activity of 200%-400% as a function of glgC16 gene expression.

The need for modification of the bacterial glgC gene to enhance starch production arises from the fact that the bacterial AGPase is regulated by different metabolites from the plant AGPase. While the site-specific mutation Gly336→Asp removes the feedback regulation by FBP, it greatly reduces the AGPase activity compared to wild type (Kumar et al., 1989). A second site-specific mutation Lys296Glu was necessary to enhance the AGPase activity. The K296E/G336D (Lys296Glu/Gly336Asp) double mutant form of bacterial AGPase lacks allosteric regulation and has the same activity as the wild-type
bacterial enzyme. Also the $V_{\text{max}}$ of the bacterial AGPase double mutant is higher than the wild-type plant enzyme enzyme (Kumar et al., 1989). We hypothesized that an enhanced production of ADP-glucose catalyzed by the double mutant $glgC$ gene product lacking the allosteric regulation and targeted to root amyloplasts would increase cassava root starch production. As described below, we have generated transgenic plants expressing the bacterial $glgC$ gene which have elevated levels of AGPase activity and enhanced root as well as top (stem and leaf) biomass production. We hypothesized that the enhanced top production represents a release of feedback inhibition on photosynthesis (dry matter accumulation) in tops similar to that observed in transgenic potato expressing the $glgC$ gene in tubers (Stark et al., 1992).
3.2 MATERIALS AND METHODS

3.2.1 Plant material

Cassava cultivar TMS 71173 and TMS 60444, from the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria was used for transformation purposes. In vitro plants were generated from apical meristems that were cultured on MS basal medium (Murashige and Skoog, 1962). Somatic embryogenesis was induced with apical leaves cultures on MS basal medium (Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose, 8 mg/L 2,4-D, 10mg/L 100X Gamborg’s B-5 vitamins (Gamborg et al., 1968), 50 mg/L casein hydrolysate and 0.5 mg/L CuSO₄, pH 5.7. Cultures were maintained at 12 h/day photoperiod at 28 °C at a light intensity of 50 μmol photons/m²/s. Somatic embryos were transferred to germination medium (MS basal medium supplemented with 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 2% (w/v) sucrose, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA, pH 5.7) after 4 weeks to generate embryo cotyledons.

3.2.2 Site-directed mutagenesis of E. coli glgC gene

Site-directed mutagenesis of glgC gene (Gly296→Asp and Lys 336→Glu) was done as described by Sayers et al., (1988). Because this method requires a single-stranded DNA template, bacterial glgC gene was subcloned into the phage M13mp18. Two primers
designed for Gly296→Asp: 3’ CACGGCCTTGACCTATACATG 5’ and 3’
CAAAAGCTGCAACA designed for Lys 336→Glu were used on the single stranded
phage DNA for the site-directed mutagenesis (Sayers et al., 1988). Once the mutations
were confirmed by sequencing, the gene fragment was returned to the original plasmid
pMON17336. To verify that there were no unintended mutations, the entire coding
region of the plasmid was sequenced. These mutations were generated by Dr. Diana
Arias-Garzon, a former student in Dr. R.T. Sayre’s lab.

3.2.3 Construction of vector

The backbone of the plasmid used in making the construct for the patatin/glgC
(G296D/K336E) gene is the pBI121 plasmid (Strategene). The construct was made by
substituting the CAMV 35S promoter with the 1.2 kb patatin promoter (Rosahl et al.,
1986) a β-Glucuronidase gene, and a 400 bp pea chloroplastic transit peptide (TP)
(Anderson and Smith, 1986) fused to the 5’ end of the 900 bp mutated E. coli glgC gene
(K296E/G336D) (Baecker et al., 1983, Kumar et al., 1989). The origin of the
chloroplastic transit peptide is the leader sequence of the small subunit of the ribulose-
bisphosphate carboxylase from pea (Pisum sativum) (Anderson and Smith, 1986). The
T-DNA region of the plasmid also contains the nptII gene for the resistance to kanamycin
and its analogue paromomycin. The patatin promoter was cloned into pBI121 with
HindIII and Smal restriction enzymes following restriction of the plasmid with the same
enzymes to cut out CAMV 35 S promoter.
The TP/glgC piece was cloned into pBI121 with ClaI and SstI restriction enzymes following restriction of pBI121 with ClaI and SstI to remove β-Glucuronidase. (Figure 3.2). The terminator for TP/glgC gene is the *Agrobacterium* nos terminator (Bevan, 1984).
Figure 3.2: T-DNA region of the binary vector 3D containing the glgC gene with the chloroplast transit peptide (TP) driven by the patatin promoter. \( GlgC = \) ADP-glucose pyrophosphorylase gene. NptII = neomycin phosphotransferase. Nos-pro = nopaline synthase promoter. Nos-ter = nopaline synthase terminator.
In each instance, the digested plasmid DNA was separated on a 1% agarose gel and the target piece isolated using a Qiagen Qiax II Gel Extraction Kit (Qiagen Inc., Valencia, CA). For ligating the promoter into the pBI121 plasmid, approximately 300 ng of the plasmid and 800 ng of patatin promoter and TP-\textit{glgC} gene were used. Ten units of T4 DNA ligase was used for each ligation carried out over 12 hours at 14 °C. Ten percent of the ligation mixture was used to transform DH5α \textit{E.coli} competent cells (Life Technologies, Grand Island, NY). The recombinant DNA and \textit{E. coli} competent cells mixture were incubated on ice for 30 minutes followed by a 42 °C heat shock for 30 seconds. The mixture was incubated on ice for 2 minutes. This was followed by the addition of 900 μL of SOC media (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, and 2.5 mM KCl) prewarmed to 42 °C. The cell suspensions were incubated for 1 hr at 37 °C and 250 rpm on a shaker. The cells were then spread on LB medium supplemented with 100 mg/L ampicillin. Colonies carrying the recombinant DNA grew on the selection medium and were used to grow 250 mL liquid cultures containing 100 mg/L ampicillin used to purify the plasmid with Qiagen Plasmid Midi Kit (Qiagen Inc., Valencia, CA). The recombinant plasmid is called 3D.

PCR analysis was carried out to confirm the transformation of \textit{E. coli} competent cells with \textit{glgC} specific primers. PCR was done in 50 μL containing 0.4X PCR buffer (8 mM Tris-HCl, pH 8.4, 20 mM KCl), 20-100 ng leaf DNA, 0.1 mM dNTP, 0.2 mM each primer (IDT, Coralville, IA), 1 unit Taq polymerase (Life Technologies, Grand Island, NY), 1 mM MgCl₂ (Life Technologies, Grand Island, NY). The forward primer was TTCTCGCGCGTTCGCGTGAATT and reverse primer was
ATCGCACGACCAGCAACAGGATTC. The PCR conditions were as follows: 3 minutes at 94 °C, 30 seconds at 94 °C, 45 seconds at 58 °C and 30 seconds at 72 °C, for 30 cycles. The PCR product was run on 0.8% agarose gel, 70V, using 1X TAE (0.04M Tris-acetate, 0.01 M EDTA pH 8.0). Control experiments were carried out with untransformed competent cells (negative control).

### 3.2.4 Agrobacterium transformation

One hundred ng of plasmid 3D (Patatin/glgC) was used to transform LBA4404 strain of *Agrobacterium tumefaciens* from Life Technologies Inc (Rockville, MD). The transformation was performed by electroporating 20 µL of ElectroMAXX LBA4404 cells (Life Technologies Inc., Grand Island, NY) with 100 ng 3D vector with a Bio Rad electroporator (Bio-Rad Laboratories, Hercules, CA). The electroporator was set at 1.8 kV, 25 uF, 200 ohms and 5 milliseconds. Electroporation was carried out in Bio-Rad Gene Pulser/E.coli Pulser Cuvette (0.2 cm electrode gap Bio-Rad, Hercules, CA). This was followed by incubation in 1mL YM medium at 250 rpm for 3 hours at 28 °C. Subsequently, 100 mL of cell suspension was inoculated on solid YM medium (0.04% (w/v) yeast extract, 1% (w/v) mannitol, 1.7 mM NaCl, 0.8 mM MgSO₄·7H₂O and 2.2 mM K₂HPO₄·3H₂O) supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin for 2 days at 28 °C. The colonies that grew on the selection media were screened by PCR with *nptII* specific primers to amplify an 800 bp piece to confirm transformation. The forward primer was CTTCGTGGCCGTGACCCGCGCGGC and the reverse primer was CCGAATTTCATAGATGACCCGCGC. DNA amplification was carried out for 30 PCR cycles of 94 °C, 50 seconds, 55 °C, 60 seconds (annealing temperature), 72 °C, 4 minute
(extension temperature). PCR was performed in 50 μL containing 0.4X PCR buffer (8 mM Tris-HCl, pH 8.4, 20 mM KCl), 20-100 ng leaf DNA, 0.1 mM dNTP, 0.2 mM each primer (IDT, Coralville, IA), 1 unit Taq polymerase (Life Technologies, Grand Island, NY), 1 mM MgCl₂ (Life Technologies, Grand Island, NY).

3.2.5 Bacterial strain and constructs

Cassava somatic embryos were transformed by Agrobacterium-mediated transformation using strain LBA4404 harboring plasmid 3D (containing patatin/TPglgC) (Figure 3.2). The vector contains bacterial AGPase gene (gglgC) modified by site-directed mutagenesis of the following residue, K296E/G336D whose expression is driven by the potato patatin promoter. *Agrobacterium* strains harboring the plasmid were started on YM medium (0.04% Yeast Extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM MgSO₄·7H₂O and 2.2 mM K₂HPO₄·3H₂O) supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin and grown for two days at 28 °C. Liquid cultures then were started from the solid cultures by inoculating 5 mL YM medium containing 100 mg/L streptomycin and 50 mg/L kanamycin with a single colony of Agrobacterium harboring the 3D plasmid and grown at 28 °C on gyratory shaker (250 rpm) for 2 days. This is followed by spectrophotometric reading at OD₅₆₀ and dilution to OD₅₆₀ 0.5. The bacteria were then pre-induced for Ti-plasmid transfer by incubation in 5-10 mL liquid MS (Murashige and Skoog, 1962) media supplemented 2% (w/v) sucrose and 200 μM acetosyringone for 2-5 hr (Li *et al*., 1996).
3.2.6 Transformation with *Agrobacterium* and selection of putative transformants

The cotyledons of germinated somatic embryos were used for the transformation experiment (Arias-Garzon, 1997). *Agrobacterium* was co-cultivated with cassava somatic embryo cotyledons on MS basal medium plus 100 µM acetosyringone for two days. The tissues were then transferred to MS medium containing 75 mg/L paromomycin and 500 mg/L carbenicillin to kill *Agrobacterium* and to select for transformants by incubation for using a 12 h/day photoperiod at a light intensity of 50 µmol photons/m²/s grown at 28 °C. Clumps of somatic embryos formed after four weeks of culture and were transferred to cassava germination medium containing 75 mg/L paromomycin and 500 mg/L carbenicillin for four more weeks. On germination, individual somatic embryos were transferred to cassava micropropagation medium (MS salts plus 2% (w/v) sucrose, 0.04 mg/L benzylamino purine, 0.05 mg/l giberellic acid, 0.02mg/L NAA, 1 mg/L thiamine-HCl, 100mg/L myo-inositol, pH 5.7) without antibiotics for root induction. Wild-type plants used for biochemical experiments were regenerated from somatic embryos using the above protocols used to regenerate transgenic plants. Embryo cotyledons cultured on the selection medium lacking antibiotics and without *Agrobacterium* co-cultivation were used as negative controls.

3.2.7 PCR verification of transformants

PCR analysis for identification of transformed plant material was carried out using genomic DNA isolated from leaves of *in vitro*-grown putative transformed cassava and wild-type cassava according to Dellaporta et al., (1983) and Soni and Murray (1994). One hundred grams of leaf tissue was ground with extraction buffer comprising 50 mM
Tris-HCl (pH 8.0), 10 mM EDTA, 2% (w/v) SDS, 100 mM LiCl, 10 µg/mL proteinase K. The ground tissue was incubated for 15 minutes and vortexed occasionally. This was followed by 15 minute centrifugation at 2800 g. RNase (200 µg) was added to the supernatant followed by 30 minutes incubation at 37 °C. The sample was extracted with an equal volume of phenol, phenol/chloroform and phenol/chloroform/isoamyl alcohol, respectively. The aqueous phase of the extraction was incubated with 0.25 X volume of 10 M ammonium acetate and 2X volume of ethanol for 10 minutes at room temperature. The DNA was pelleted for 20 minutes at 13,000 rpm (10,400g). The DNA pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended with 0.1 mL TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). PCR analysis was performed to verify the presence of patatin promoter and the bacterial \textit{glgC} gene. PCR was done in 50 µL containing 0.4X PCR buffer (8 mM Tris-HCl, pH 8.4, 20 mM KCl), 20-100 ng leaf DNA, 0.1 mM dNTP, 0.2 mM each primer (IDT, Coralville, IA), 1 unit Taq polymerase (Life Technologies, Grand Island, NY), 1 mM MgCl$_2$ (Life Technologies, Grand Island, NY). The transgenes were identified with primers specific for patatin promoter (PatatinF1: CTACCGTGCAATCGCCGCGGTG) and \textit{glgC} gene (glgCR1: GCAGCGGCGCAGACGGCACGAGCG). DNA amplification was carried out for 30 PCR cycles of 94 °C, 50 seconds, 58 °C, 45 seconds (annealing temperature), 72 °C, 1 minute (extension temperature). The 850 bp PCR product was sequenced to prove the fidelity by the Ohio State University Plant Microbe Genomics Facility (PMGF).
3.2.8 Southern blot analysis

Genomic DNA was isolated from greenhouse grown plants according to the methods of Soni and Murray (1994). One gram of leaf tissue was ground with 10 mL of extraction buffer comprising 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2% (w/v) SDS, 100 mM LiCl, 10 µg/mL proteinase K). The ground tissue was incubated for 15 minutes with occasional vortexing. This was followed by 15 minute centrifugation at 3,500 rpm (2,800 g). RNase (200 µg) was added to the supernatant followed by 30 minutes incubation at 37 °C. The sample was extracted sequentially with an equal volume of phenol, phenol/chloroform and phenol/chloroform/isoamyl alcohol, respectively. The aqueous phase of the extraction was incubated with 0.25 X volume 10 M ammonium acetate and 2X volume of ethanol for 10 minutes at room temperature. The DNA was pelleted for 20 minutes at 13,000 rpm (10,400g). The DNA pellet was washed with 500 µl 70 % (v/v) ethanol, air-dried and resuspended with 0.5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Southern blot analysis of wild-type and transgenic cassava was carried out according to the methods of Sambrook et al. (1989). Genomic DNA (20 µg) was restricted with KpnI followed by gel separation of the restriction fragments using 0.8% (w/v) agarose gel run at 70 V using 1X TAE (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0) buffer. KpnI does not cut the T-DNA region of the vector carrying Patatin:TP/glgC. The gel was depurinated with 250 mM HCl for 10 minutes while shaking slowly. This was followed by 1 hr denaturation with 1.5 M NaCl, 0.5 M NaOH, then 1 hr neutralization with 1 M Tris-HCl (pH 8.0), 1.5 M NaCl with slow shaking. The genomic DNA transfer to
ZetaBind nylon membrane (Life Science Products, Inc., Denver, CO) was done overnight with 20 X SSC (3M sodium chloride, 0.3M sodium citrate buffer, pH 7.0). Following transfer, DNA was cross-linked to the dry membrane by UV cross-linker for 2 minutes. The membrane is then stored dry at room temperature until prehybridization with CHURCH buffer (0.5 m NaHPO₄ (pH 7.2), 1.0 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS), 50°C for 3 hr. The probe for the Southern blot was made by PCR amplification of glgC/nos terminator fragment: glgC forward primer
(TTCTCGCGCGTGTCGTAATT) and nos terminator reverse primers
(ATCGCACGACCAGCAACAGGATTC) in a total volume of 50 µL 5 µL 10X PCR buffer (200 mM Tris-HCl, pH8.4, 500 mM KCl), 100 ng 3D vector, 0.1 mM of dATP/dTTP/ dGTP each, 100 µCi 32P-dCTP (Amersham, Piscataway, NJ), 2.5 units Taq polymerase (Life Technologies, Grand Island, NY), 0.4 µM each of the primers, (IDT, Coralville, IA) 1.5 mM MgCl₂) with Perkin Elmer Cetus thermal cycler. The PCR was run with the following conditions: 3 minutes at 94°C, 30 seconds at 94°C, 45 seconds at 58°C and 30 seconds at 72°C, 30 cycles. The probe was purified with Qiagen PCR kit (Qiagen In, Valencia, CA), boiled at 100°C for 5 minutes, quickly cooled on ice and added to the hybridization buffer (same as prehybridization buffer) overnight. Subsequently, the membrane was washed with 1 X SSC, 0.1% (w/v) SDS for 30 minutes, 50°C and two washes with 0.1 X SSC, 0.1% (w/v) SDS for 30 minutes, 50°C. The membrane was wrapped with Saran wrap and incubated overnight with a phosphor-imager that was bleached 30 minutes with light.
3.2.9 Reverse transcriptase-PCR (RT-PCR) analysis

Total RNA was extracted from 100 mg of in vitro putative transformed root material using a Qiagen Plant RNA Extraction Kit (Qiagen Inc., Valencia, CA). The extract was treated with 1 unit DNase (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature to eliminate DNA contamination. The DNase was inactivated according to manufacturer’s instructions. The first strand cDNA synthesis was carried out with 10 µg of total RNA using 1X reverse transcription buffer (50 mM Tris-HCl pH 8.0, 75 mM KCl, 3 mM MgCl₂) 0.3 mM dNTP, 0.5 µg oligo-dT₁₄₋₁₈ primers, and 200 units of SuperScript II reverse transcriptase (Life Technologies, Rockville, MD). The mixture was incubated at 65 °C for 5 minutes without reverse transcriptase followed by incubation at 42 °C for 1 hour with reverse transcriptase.

The cDNA from above was amplified by PCR using 1X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5mM MgCl₂, 0.1 mM dNTP, 2.5 units of Taq polymerase, 0.4 µM of each primer specific for glgC gene. The forward primer sequence is TTCTCGCGCGTTCGCGTGAATT and the reverse primer sequence is ATCGCACGACCGGCAACAGGATTC. The PCR conditions were as follows: 3 minutes at 94 °C, 30 seconds at 94 °C, 45 seconds at 58 °C and 30 seconds at 72 °C for 30 cycles. The PCR product was run on 0.8% (w/v) agarose gel at 70V in 1X TAE buffer. Control experiments were carried out without cDNA (negative control) and by amplification of the CYP79D1 gene, that encodes a cytochrome P450 enzyme that catalyzes the first-dedicated step in linamarin synthesis (Anderssen et al., 2000). The
RT-PCR was non-quantitative, the bands from first amplification were used to repeat the PCR analysis

3.2.10 Greenhouse establishment
One month-old plantlets with well-established roots were transferred to the greenhouse for root growth measurements. Plantlets were removed from test tubes, washed with running tap water and planted in sterile soil. Plantlets are covered with transparent waterproof bags to increase humidity. The plantlets were maintained in a growth chamber at 28 °C at a light intensity of 50 μmol photons m⁻² s⁻¹. After 2 weeks, the bags were punctured to reduce humidity. The bags are finally removed after 4 weeks. Plantlets were watered every other day and grown from May 2000 to January 2002 in 11” X 12” plastic pots filled with Scott’s MetroMix soil (The Scott’s Company, Marysville, OH) to generate stakes for greenhouse growth experiments. One foot long stakes were cut with a machete from 3D and wild-type TMS 71173 plants and planted in 11” X 12” plastic pots filled with Scott’s MetroMix soil (The Scott’s Company, Marysville, OH). The plants were grown from January 23 2002, to August 27, 2002 (7 months). Roots were also harvested from 6 month old plants for AGPase assays.

For the confirmatory experiment, one foot stakes were generated from both transformed and wild type TMS 71173 plants that grew from the stomps of plants harvested in January 2002 from the first yield experiment. Both transformed and wild-type plants were grown in 11” X 12” in plastic pots filled with Scott’s MetroMix soil (The Scott’s Company, Marysville, OH) from February 27, 2003, to July 3, 2003 (4 months). The
plants for the growth experiment were harvested short of 7 months because some of the plants died following toxic pesticide treatment to control green mite infestation. Each treatment had 3 – 4 plants. Each treatment came from a tissue culture clone of the original transformed plant.

Following the harvest of the transformed and wild-type plants, the leaves, stem, roots, root-stem junction were separated by treatments and put in the brown paper bags. The samples were weighed to obtain the fresh weight and then put in an incubator at 80°C to dry them. The samples were weighed every two days until a constant dry weight was obtained. The weights were then recorded. Each treatment had a minimum of three replicates.

**3.2.11 AGPase enzyme extraction and assays**

All steps were conducted at 4°C unless indicated otherwise. Five grams of each root sample was sliced and placed into a Waring Blender containing 10 mLs of 0.05 M Tris-HCl, (pH 7.5), 10 mM glutathione, 1.0 mM EDTA and 0.04 mL of 10% (w/v) sodium bisulfite, pH 6.0 for each g of tissue. The tissue was homogenized by two 15 s slow speed periods followed by two 15 s fast-speed blending period. The suspension was filtered through miracloth and the extract centrifuged for 20 min. at 27,000 g (Sowokinos, 1976; Kalt-Torres and Huber, 1987). The supernatant was used for all enzyme assays. Reaction assays for ADP-glucose pyrophosphorylase contained in 1 mL, 2.0 µmol ADP-glucose, 5 µmol MgCl₂, 80 µmol glycylglycine, 10 µmol NaF, 1 unit of P-glucomutase (Sigma-Aldrich, St. Louis, MO) (4.4 µg), 20 µmol cysteine, 0.02 µmol glucose-1,6-diP,
0.75 unit glucose-6-P dehydrogenase (Sigma-Aldrich, St. Louis, MO) (2.1 µg), 0.6 µmol NADP, and 0.005-0.05 units of pyrophosphorylase, in 50 mM Tris-HCl pH 7.5. The reaction is initiated by the addition of 1.5 µmol PPI. The production of NADPH (molar extinction coefficient is $6.22 \times 10^6$ M$^{-1}$cm$^{-1}$ at 340 nm) is monitored spectrophotometrically at 340 nm for 10 minutes at 25°C (Sowokinos, 1976; Kalt-Torres and Huber, 1987). Quantification of crude protein was done with BCA protein assay kit (Pierce, Rockford, IL) according to manufacturer’s specifications with BSA protein as a standard.

3.2.12 Statistical analysis

Analysis of variance (ANOVA) and other statistical analysis were done with the SAS software (SAS, 1985) at the Horticulture and Crop Science Department, The Ohio State University, Columbus, OH. Samples were evaluated using analysis of variance (ANOVA) for a randomized complete block-design. Duncan’s multiple range test was used to separate treatment means found significantly different by ANOVA. Pearson’s correlation coefficient was used in determining the correlation coefficient. All analyses were performed at $P \leq 0.05$ confidence level.
3.3 RESULTS

3.3.1 Construction of vectors and transformation of bacteria.

The patatin promoter was cloned into pBI121 plasmid with HindIII and Smal restriction enzymes while the TP/glgC gene fusion was cloned into the same pBI121 vector with ClaI and SstI restriction enzymes giving raise to the vector plasmid, 3D. The 3D plasmid was then transformed into DH5α competent cell and confirmed by PCR screening for the glgC gene (Figure 3.3). Agrobacterium strain LBA 4404 was transformed with plasmid 3D. Transformants were confirmed by PCR screening for the presence of the nptII gene product (800 bp) using whole cell DNA extracts as template (Figure 3.4).

3.3.2 Production of transgenic cassava

Cassava cultivar TMS 71173 was transformed via Agrobacterium-mediated transfer of the T-DNA carrying the modified glgC gene. Transformed cells underwent somatic embryogenesis in 4-6 weeks on MS8 medium supplemented with 75 mg/L paromomycin and 500 mg/L carbenicillin. Paromomycin, a less toxic analogue of kanamycin, ensures that most tissues that regenerate are transgenic. Paromomycin resistant embryos converted to plantlets in 1-2 months. Plantlets from each embryo were then transferred to individual test tubes after 1 month. The individual plants in test tubes were grown for 4-6 weeks and were then transferred to the greenhouse. (Figure 3.5, Table 3.1). We recovered 26 paromomycin resistant embryos from TMS 71173 explants. Eleven of the embryos converted to plants.
Figure 3.3: Gel analysis of PCR products of glgC gene with DH5α competent cells transformed with 3D plasmid. E, untransformed competent cell (negative control); P, 3D plasmid (positive control); M, marker DNA
Figure 3.4: Gel of PCR products from *Agrobacterium* cells transformed with 3D plasmid screening for *glgC* gene. P, 3D plasmid; E, negative control untransformed E. coli cells carrying 3D; M, marker DNA.
Fig. 3.5: Regeneration of cassava on selective regeneration medium containing 75 mg/L paromomycin, 500 mg/L carbenicillin (A-C). Putative transformed plants growing in the green house, 1 month (D).
Table 3.1: Response of cassava somatic embryo cotyledons to Agrobacterium transformation. Somatic embryos were induced from cotyledon explants on MS medium supplemented with 8 mg/L 2, 4-D, 75 mg/L paromomycin, 500 mg/L carbenicillin.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Number of explants</th>
<th>Cultivar</th>
<th>Putative transformed embryos</th>
<th>Embryos converted to plants</th>
<th>Percent of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$glgC/TP (3D)$</td>
<td>872</td>
<td>TMS 71173</td>
<td>26</td>
<td>11</td>
<td>1%</td>
</tr>
<tr>
<td>$glgC/TP (3D)$</td>
<td>474</td>
<td>MCol 2215</td>
<td>15</td>
<td>7</td>
<td>1%</td>
</tr>
</tbody>
</table>
The efficiency of the transformation of cassava cultivar TMS 71173 with the 3D plasmid was 1% of the total embryos treated with Agrobacterium. (Table 3.1). The wild-type plants used in parallel analysis with the transformed plants were regenerated from somatic embryo cotyledons following the same tissue culture practices as that for transformed plant except that there was no antibiotic selection involved.

3.3.3 PCR analysis: PCR experiments were carried out to determine if the putatively transformed plants were carrying the transgenes located in the T-DNA. Four of the 23 putatively transformed K3D cassava plants showed positive amplification of an 850 bp patatin promoter–glgC gene fragment by PCR. The wild-type plants did not amplify the 850 bp piece (Figure 3.6A). The transformed plants were negative for the vir gene PCR analysis, showing that the glgC gene amplification was not because of Agrobacteria contamination of the tissue culture plants (Figure 3.6B). All PCR products were sequenced and their identity confirmed.

3.3.4 Southern blot:
To further verify the integration of the T-DNA in the putative transformants, Southern blot analysis was carried out using genomic DNA digested with KpnI, which does not restrict the T-DNA, and probed with the radiolabeled glgC gene. The copy number of glgC genes integrated into the cassava genome varied from one to two copies (Figure 3.7). The banding pattern was slightly different between the different transgenic lines suggesting independent transformation events. Expectedly, the bacterial glgC probe did not hybridize to wild-type DNA.
Figure 3.6: A, Gel analysis of PCR products of patatin/TP/glgC gene border from transformed plants and untransformed plants and K3D plasmid control. PCR was performed with 5’ primers from the glgC gene and 3’ primers from the nos terminator. B, PCR analysis with Agrobacterium vir gene primers to show that the DNA bands in A are not from Agrobacterium.
Fig. 3.7: Southern blot of wild-type and transgenic cassava lines. Cassava DNA probed with a $^{32}$P-labeled fragment of the $glgC$ gene-nos terminator.
3.3.5 RT-PCR analysis

To demonstrate expression of the *glgC* gene, RT-PCR was performed using root and leaf total RNA (of the transformed plants that were positive for PCR amplification of *glgC* gene) as a template. The cDNA was amplified using *glgC* and *nos* terminator specific primers. Five of the 23 paromomycin-resistant 3D transformed cassava plants were positive for expression of the *glgC* gene by RT-PCR screening (Figure 3.8). The transformed plants and the 3D plasmid control had a 350 bp band whereas wild-type cassava plants had no band. The RT-PCR was non-quantitative, the bands from first amplification were used to repeat the PCR analysis. The tissue specific expression of the patatin promoter, which is tuber-specific in potato and regulated by carbohydrate as well (Raemekkers et al., 1996) was verified by the RT-PCR analysis of leaf RNA. The results showed no expression of the bacterial *glgC* gene in the leaves of cassava plants transformed with *glgC* gene driven by the patatin promoter. Verification of the success of the RT-PCR reaction was confirmed by RT-PCR amplification of CYP79D1 transcripts (Figure 3.9). All PCR products were sequenced and their identity confirmed.

3.3.6 Enzyme assay

Greenhouse grown plant material from 6 month old plants was used for AGPase enzyme assay studies (Figure 3.10). The result of AGPase activity assays shows that the different transformed lines had between a 0 to 95% increase in AGPase enzyme activity relative to wild-type when assayed under pH and temperature conditions optimal for plant enzyme activity. The root AGPase activity (0.48 µmol ADP-glucose/mg protein/hour) of the strain 3D-1 was 95% greater than that of the wild-type (0.23 µmol ADP-glucose/mg protein/hour), while 3D-3 (0.36 µmol ADP-glucose/mg protein/hour) was 65% more than the wild-type.
Figure 3.8: RT-PCR analysis of \textit{glgC} gene expression in transgenic and wild-type cassava roots
Figure 3.9: RT-PCR products for *glgC* gene and CYP79D1 transcripts using leaf RNA isolated from wild-type and transformed plants. P, plasmid DNA used only for PCR; W, wild-type; 3D1-3 transformed plants; M, marker
Figure 3.10: Transformed plants growing in the greenhouse (a) early stage of growth, one month (b) later stage of growth, 6 months.
3D-2 (0.23 μmol ADP-glucose/mg protein/hour) had approximately the same AGPase activity as the wild-type cassava plant (Figure 3.11). Importantly, the variance in the enzyme assays was not great suggesting that the tissue assayed was uniformly expressing AGPase and was not chimeric for its expression.

### 3.3.7 Analysis of cassava yield

Transformed plants expressing the TP/glgC constructs had a higher total number of leaves per plant, stem fresh weight, root/stem junction fresh weight, root fresh weight and number of roots per plant than wild-type plants (Table 3.2). The fresh weight yield of the tuberous roots of the transformed cassava plants was 50% to 160% more than the wild-type plants (Figure 3.12). The average number of tuberous roots per transformed plant was also higher than that of the wild type, ranging from 8 – 12 compared to 7 for wild-type plants. The transformed plants were also taller (>1.9 m), plants were measured against a 1.9 m man) than the wild-type plants (<1.9 m) as seen in the photograph (Figure 3.13). The statistical analysis of the root yields of cultivar 3D-1 (198 g), 3D-3 and wild-type cassava (74 g) demonstrated that the differences in fresh weight were statistically significant at the 95% confidence interval (Table 3.2). Total plant leaf weight of the 3D-1, 3D-3 and wild-type cultivars was 335 g, 299g, and 220g, respectively. Statistical analyses of the fresh weight of leaves of cultivars indicated that the observed values were also significantly different between 3D-1 and 3D-3 versus wild type. There was no difference between 3D-1 and 3D-3 while 3D-2 was not statistically different from 3D-1 and 3D-3.
Fig 3.11: Comparative AGPase enzyme activities for 6-month old wild type and transformed cassava roots grown in the greenhouse. Assays were repeated three times for each plant with extracts from root. AGPase activity is measured as the generation of NADPH
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>3D-1</th>
<th>3D-2</th>
<th>3D-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf (g)</strong></td>
<td>220.67+/−45.7^b (100)</td>
<td>335+/−26.9^a (156)</td>
<td>284.33+/−4.9^ab (127)</td>
<td>299+/−38.4^a (135)</td>
</tr>
<tr>
<td><strong>Number of Leaves</strong></td>
<td>92.3+/−9.4^b (100)</td>
<td>123+/−10.3^a (134)</td>
<td>108.6+/−18.5^ab (116)</td>
<td>114+/−13.1^ab (124)</td>
</tr>
<tr>
<td><strong>Stem (g)</strong></td>
<td>374+/−75.8^c (100)</td>
<td>584+/−47.5^ab (156)</td>
<td>431.67+/−85^b (116)</td>
<td>529+/−60.6^ab (144)</td>
</tr>
<tr>
<td><strong>Stem-Root Junction (g)</strong></td>
<td>59+/−7.5^a (100)</td>
<td>86.33+/−5.4^a (146)</td>
<td>81.33+/−12.7^a (137)</td>
<td>63.67+/−15.2^a (106)</td>
</tr>
<tr>
<td><strong>Root (g)</strong></td>
<td>74.33+/−19^c (100)</td>
<td>198.67+/−29.6^a (266)</td>
<td>113.33+/−31^bc (152)</td>
<td>123+/−23.4^b (166)</td>
</tr>
<tr>
<td><strong>Number of Roots/Plant</strong></td>
<td>7+/−0.8^b (100)</td>
<td>12+/−3.3^a (174)</td>
<td>8+/−2.0^b (114)</td>
<td>11+/−0.8^a (157)</td>
</tr>
</tbody>
</table>

Table 3.2: Fresh weight comparison of transformed and wild-type plants. 3D-1, 3D-2, 3D-3, wild type. *Numbers with same letter represent no statistical difference, different letters means statistical difference at 95% confidence level. Values in parentheses are % of wild-type values.
Fig. 3.12: The yield of *glgC transgenic* and wild-type plants following 7 months of growth in the greenhouse
Figure 3.13: Comparison of 7 month old glgC transformed and wild-type cassava to show differences in bulk. A-C, transformed plants: D, wild-type.
The stem weight of the transformed plants ranged from 584g (3D-1) to 431g (3D-2) versus 374 g for wild–type plants. The transformed plants also produced more leaves than the wild type. The average number of leaves produced by the transformed cassava plants ranged from 108 (3D-2) to 123 (3D-1). The number of leaves produced by 3D-1 and 3D-3 was significantly different from the average number of leaves produced by 3D-2 (108) and wild-type (92) plants. (Table 3.2). The number of leaves per plant also correlated with the root mass of the plants (WT, 92 and 74g; 3D-1, 123 and 198g; 3D-2, 108 and 113g; 3D-3, 114 and 123 g). It was also observed that the transformed plants produced more tuberous roots per plant than wild–type plants.  3D-1 produced an average of 12 tuberous roots per plant and 3D-3 produced 11. Wild-type plants produced 7 tuberous roots per plant. The 3D-1 and 3D-3 plant tuberous root number was statistically different from 3D-2 and wild type. However, there was no statistical difference in the weight of the stem-root junction between the transformed and wild-type plants. It was also observed that there was no significant difference between the transformed plants and the wild type plant regarding the fresh weight of the root-stem junction (Table 3.2). This is an important observation since it indicates that the performance of transgenic plants was not biased by the size of the planting stake.

The dry weight yields of the transformed and wild-type plants followed the same trend as the fresh weight yields except for the fact that there was no statistical difference between the dry weights of the leaves of the different cultivars (WT, 55 g, 3D-1, 68g, 3D-2, 60 g and 3D-3, 62g) (Table 3.3).
Table 3.3: Dry weight comparison of transformed and wild-type plants.

Harvested materials were dried in the oven at 80°C for blocks of 3 days until constant weight was attained. *Numbers with same letter represent no statistical difference, different letters means statistical difference. 95% confidence level. Values in parentheses are % of wild-type values.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>3D-1</th>
<th>3D-2</th>
<th>3D-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf (g)</td>
<td>55.1+/−8.3ᵃ(100)</td>
<td>68.55+/−4.0ᵃ(123)</td>
<td>60.4+/−0.8ᵃ(109)</td>
<td>62.45+/−9.4ᵃ(112)</td>
</tr>
<tr>
<td>Stem (g)</td>
<td>89.69+/−5.0ᶜ(100)</td>
<td>147.78+/−8.8ᵃ(165)</td>
<td>114.02+/−25.3ᵇᶜ(128)</td>
<td>134.4+/−13.8ᵇᶜ(150)</td>
</tr>
<tr>
<td>Stem-Root Junction (g)</td>
<td>16.66+/−1.5ᵇ(100)</td>
<td>25.36+/−2.5ᵃ(156)</td>
<td>25.16+/−2.7ᵃ(156)</td>
<td>23.1+/−3.6ᵇᶜ(143)</td>
</tr>
<tr>
<td>Root (g)</td>
<td>18.9+/−4.3ᶜ(100)</td>
<td>48.23+/−9.3ᵃ(266)</td>
<td>24.89+/−7.8ᵇᶜ(133)</td>
<td>28.52+/−8.7ᵇ(155)</td>
</tr>
</tbody>
</table>
The dry weight of the stem of the transgenic lines 3D-1 (147 g) and 3D-3 (134g) were not significantly different from each other, but differed significantly from WT (89 g).

The root-stem junction of 3D-1 (25 g) and 3D-2 (25 g) were significantly different from the wild type (16g) while 3D-3 overlapped with 3D-2. However, the dry weight of the roots, 3D-1 (48 g) and 3D-3 (28 g) were significantly different and both were significantly different relative to wild type (18 g). There was no significant difference between the roots of 3D-3 (28 g) and 3D-2 (24 g).

The repeat of the growth experiment of the \textit{glgC} transformed and wild-type plants in the greenhouse corroborated the earlier experiment. 3D-1 plants had the highest fresh weight root yield (69g) followed by 3D-3 (40g), then 3D-2 (29g) (Table 3.4). The average fresh weight yield of the wild-type plants was 11g (Table 4). The dry weight of the roots were significantly different between all cultivars (3D-1>3D-3>3D-2>WT). However, the plants were harvested after only 4 months of growth compared to 7 months in the earlier trial. This was a consequence of plants being sprayed with toxic pesticide application by the Kottman Hall greenhouse management to control green mite infestation. The pesticide ended up killing some of the plants leading to the immediate harvest of the plants. The average dry weight yield of roots for the 3D-1, 3D-3 and 3D-2 plants was 8 g, 7g, and 5 g, respectively. There was no significant difference among the transformed plants regarding the root dry weight. The wild type root dry weight yield was 2g and was significantly different from the transformed plants. This corroborates the trend of the first experiment.
Table 3.4: Root fresh and dry weight measurements of the \( glgC \) transformed plants (3D1-3) and wild-type cassava from second greenhouse trial. Plants were grown for four months. *Numbers in parenthesis represent percent of wild type. *Numbers with same letter represent no statistical difference, different letters means statistical difference at the 95% confidence level.

<table>
<thead>
<tr>
<th></th>
<th>Fresh weight (g)</th>
<th>Dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>11.0+/-2.5 (^d) (100)</td>
<td>2.0+/-0.3 (^b) (100)</td>
</tr>
<tr>
<td>3D-1</td>
<td>69.2+/-7.9 (^a) (627)</td>
<td>8.79 +/-.2.2 (^a) (435)</td>
</tr>
<tr>
<td>3D-2</td>
<td>29.46+/-4.1 (^c) (263)</td>
<td>5.12+/-0.9 (^a) (255)</td>
</tr>
<tr>
<td>3D-3</td>
<td>40.56+/-7.4 (^b) (363)</td>
<td>7.04+/-1.1 (^a) (350)</td>
</tr>
</tbody>
</table>
where 3D-1 and 3D-3 produced more root biomass mass than the 3D-2 or wild-type plants (Table 3.2, 3.3).

The Pearson correlation coefficient analysis (P<0.05) (SAS, 1985) showed that there is a correlation between the leaf mass and the root mass (r = 0.73), weight of stems and root mass (r = 0.59) and number of leaves and root mass, but no interaction between root-stem junction and root mass and also no interaction between root number and root mass in the glgC transformed cassava plants (Table 3.5A).

There was negative correlation between the stem weight and root mass (r = 0.91), stem-root junction and root mass (r = 0.05). However, there was correlation between number of leaves and root mass (r = 0.67), and number of roots and root mass (r = 0.69) in the wild type cassava plants (Table 3.5B).

The transformed cassava plants had better correlation between leaf weight and root mass (r = 0.73 versus 0.57), and stem mass and root mass (r = 0.59 versus -0.91) than the wild type cassava. On the other hand, wild type cassava had better correlation between root number and root weight (r = 0.69 versus 0.19), and leaf number and leaf weight (r = 0.67 versus 0.52). These results are indications that the higher yield of the glgC transformed cassava plants compared to the wild type is a function of the higher leaf mass and stem mass than the wild type cassava (Table 3.5).
Table 3.5: Correlation coefficient (P<0.05) of the different organs of cassava A, transformed with modified bacterial *glgC* gene; B, wild type plant.

<table>
<thead>
<tr>
<th></th>
<th>Roots (g)</th>
<th>Stems (g)</th>
<th>Leaves (g)</th>
<th>Stem-Root Junction (g)</th>
<th>Number of Leaves</th>
<th>Number of Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>glgC</em> cassava</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stems (g)</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves (g)</td>
<td>0.73</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem-Root Junction (g)</td>
<td>0.19</td>
<td>0.67</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of leaves</td>
<td>0.52</td>
<td>0.89</td>
<td>0.74</td>
<td>0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Roots</td>
<td>0.19</td>
<td>0.55</td>
<td>0.40</td>
<td>0.60</td>
<td>0.38</td>
<td></td>
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</tbody>
</table>

| **B**              |           |           |             |                        |                  |                |
| Wild type cassava  |           |           |             |                        |                  |                |
| Stems (g)          | -0.91     |           |             |                        |                  |                |
| Leaves (g)         | 0.57      | -0.91     |             |                        |                  |                |
| Stem-Root Junction (g) | -0.05 | -0.34     | -0.84       |                        |                  |                |
| Number of Leaves   | 0.67      | -0.33     | 0.99        | -0.76                  |                  |                |
| Number of roots    | 0.69      | -0.35     | 0.98        | -0.75                  | 0.99             |                |
3.4 DISCUSSION:

We were able to demonstrate integration of the patain/TP/glC T-DNA into cassava plants by PCR (Figure 3.6) and Southern blot analysis (Figure 3.7). Three transgenic lines expressing the TP/glC gene were identified by RT-PCR (Figure 3.8) and shown to express the TP/glC gene only in root and not in leaves (Figure 3.9). AGPase enzyme activity analyses indicated that the transformed plants had between a 65% and 95 % increase in AGPase activity relative to wild type (Figure 3.11). The relative differences in AGPase activity between transformants could be the result of position effects or the site of insertion of the transgene into the genome (Zhang et al., 2000a; Sarria et al., 2000) or variation in copy numbers of the TP/glC copies inserted into the cassava genome. The transformed plants (3D-1 and 3D-3) having two copies of the glC gene had higher AGPase enzyme activity and higher root yields compared to the transformed plant (3D-2) with only one copy of bacterial glC gene (Fig. 3.7, 3.11 and Table 3.2). The relative increase in AGPase activity compared to wild type was less than that observed for expression of the HNL gene in transgenic cassava roots (also driven by the patatin promoter) (Siritunga et al., 2003). However, the base-line level of AGPase activity in wild type cassava roots is substantially higher than that of HNL. The absolute AGPase activity of wild-type and transgenic cassava was similar to that reported for other species. In transgenic rice carrying the modified maize large subunit AGPase gene (Sh2r6hs), Smidansky et al., (2003), reported 42% increase in AGPase activity of transgenic rice over the wild type. In a similar transformation with wheat, Smidansky et al., (2002) observed only 24% increase. On the other hand, Sweetlove et al., (1996), reported 400 %
increase of transgenic AGPase over the wild type in potato. Also, Stark et al., (1992), reported 36% increase in AGPase activity in potato following transformation with bacterial \( glgC \) gene. While Sweetlove et al (1996) report was high, our observed AGPase activity of up to 95% increase over the wild type seems reasonable considering the fact that we used the bacterial \( glgC \) double mutant (K296E/G336D) which has a gene product with higher \( V_{\text{max}} \) than the single mutant \( glgC16 \) used by Stark et al., (1992).

As predicted, the transformed plants expressing greater AGPase activity produced more roots with greater biomass than wild-type plants (Table 3.2). Transformed plants also had a greater total biomass and were taller than wild–type plants (Figure 3.13), which was also reported for rice (Smidansky et al, 2003), and wheat (Smidansky et al., 2002). This may reflect the higher rates of starch synthesis and greater rates of sucrose metabolism. The greater rates of sucrose utilization by roots may in turn reduce feed-back inhibition on photosynthesis resulting in bigger plant tops (Smidansky et al., 2002; Worrell et al., 1991; Martin and Smith, 1995). Plants with high rates of starch biosynthesis generally have greater photosynthetic capacities and less feedback inhibition of photosynthesis due to an increased potential to utilize starch as a transient sink (Sun et al., 2002). Moreover, these plants are also characterized by higher rates of starch turnover during the diurnal cycle. These properties, cumulatively, translated to an increase in growth and root mass in our transformed plants compared to the wild type (Sun et al., 2002).
The increase in root numbers in TP/glC transformed plants (Table 3.2) was similar to that reported for potatoes transformed with a cytosolic invertase construct (Sonnewald et al., 1997). The transformed potatoes, however, had smaller potatoes per unit than the wild type. However, the transformed potatoes made up for the paucity of size with an increase in number of tubers per plant, resulting in more root mass than the wild type plant. In our experiments, the transformation of cassava with TP/glC gene led to an increase of root number from 7 to 8-12 in the case of the best transformant 3D-1, representing an increase of 71%. The cassava wild type plants produced bigger single roots than the transgenic plants. However, the transgenic plants produced more root biomass than the wild type as a result of production of more roots (Table 3.2).

The over expression of a modified endosperm-specific shrunklen2 (Sh2), which encodes the large subunit of the heterotetrameric AGPase in maize led to an increase of 18% in maize seed weight (Giroux et al., 1996). Increased AGPase activity has also been achieved in the endosperm of transgenic wheat using a modified form of maize Shrunken2 gene (Sh2r6hs) which encodes an altered AGPase large subunit (Smidansky et al., 2002). The seed weight of AGPase transformed wheat seeds increased by 38% per plant. This increase wheat seed weight was a consequence of 24% increase in AGPase activity compared to the wild type wheat plant. Also, Smidansky et al., (2003) reported that there was more than 20% increase in seed weight of transgenic rice, a consequence of increase in starch due to expression of a modified maize large subunit AGPase gene (Sh2r6hs). The two subunits have catalytic activity independent of each other, but activity is optimal in the hetero-enzyme complex of the two subunits (Smidansky et al., 2003).
An increase of 10 - 70 fold in activity has been observed with the complex (Ballicora et al., 1998). The use of the modified large subunit AGPase in rice transformation resulted in a 49% increase in AGPase activity compared to the wild type plant. Normally transforming a plant with a single gene encoding only one subunit of a multimeric complex may reduce overall enzyme activity, but in the case of transforming rice with maize large subunit AGPase gene, the increase in enzyme activity could have resulted from the modification of the large subunit AGPase to decrease sensitivity to the inhibitor, (Pi) and to have a more stable interaction with the small subunit. Since AGPase functions in an enzyme complex, what happened in the rice (Sh2r6hs) is that the maize large subunits could have formed a complex with the rice small subunits because both are cereal plants, thereby forming a fully functional enzyme (Smidansky et al., 2003).

Increases in storage in potato tubers has also been achieved by expression of cytosolic invertase under the control of the root specific potato B33 promoter (Sonnewald et al. 1997). The transgenic potatoes had a 10-fold increase in invertase activity relative to wild type and a 2.4-fold increase in tuber number relative to wild type. The increased invertase activity presumably facilitated sucrose unloading in the tuber and conversion to hexoses which are the substrate for starch synthesis.

In summary, we have transformed cassava with E. coli glgC gene modified to remove allosteric regulation by glucose-6-phosphate (enhancer) and Pi (inhibitor) and having higher enzyme activity. As a result, AGPase activity increased leading to overall increase in plant biomass and most importantly, increase in root yield. These results
provide an important insight into the biochemical mechanism that underlies root yield and total plant development. This will enable farmers in the poor continents to maximize crop production.
CHAPTER 4

TRANSFORMATION OF AFRICAN CASSAVA WITH MAIZE SPS GENE.

4.1 INTRODUCTION

Sucrose, a non-reducing sugar, is the form of carbohydrate which is transported in many plants including cassava (Cheng et al.; 1996, Huber and Huber, 1996; Lunn 2002). Sucrose synthesis depends on the Calvin cycle for substrates. Triose phosphates, produced in the chloroplast, are transported into the cytoplasm in exchange for pyrophosphate leading to synthesis of hexose phosphates and subsequently sucrose (Salerno and Curatti, 2003). Sucrose has also been implicated in cellular metabolism by affecting gene expression (Leon and Sheen, 2003). Its wide distribution and utilization among higher plants has been attributed to its non-reducing nature (Huber and Huber, 1996). The only other known non-reducing disaccharide, trehalose plays similar roles in fungi and insects leading to speculation that it could have evolved as a carbohydrate transport molecule earlier than sucrose (Salerno and Curatti, 2003).
Sucrose synthesis can be catalyzed by two different enzymes in higher plants namely, sucrose phosphate synthase (SPS; EC 2.4.1.14) and sucrose synthase (SuSy; EC 2.4.1.13). Sucrose phosphate synthase produces sucrose-P:

$$\text{UDP-Glucose} + \text{Fructose-6-P} \rightarrow \text{Sucrose-P} + \text{UDP} + \text{H}^+$$

Whereas SuSy produces sucrose directly:

$$\text{UDP-Glucose} + \text{Fructose} \rightarrow \text{Sucrose} + \text{UDP} + \text{H}^+.$$ 

Both reactions are essentially reversible, but in the case of SPS, sucrose phosphate phosphatase (SPP; EC 3.1.3.24) prevents the reversibility of the reaction by removing the phosphate from the sucrose (Figure 4.1). This series of reactions involving SPS makes the sucrose-phosphate synthesis pathway more efficient than the SuSy catalyzed pathway (Huber and Huber, 1996). Importantly, there is evidence that SPS and SPP can form a complex in vivo. This makes the dephosphorylation of sucrose-P inevitable (Huber and Huber, 1996, Salerno and Curatti, 2003).

Sucrose phosphate synthase and SuSy are soluble in the cytosol. The rapid dephosphorylation of sucrose-P by SPP makes SPS the main enzyme for sucrose synthesis. Given the low equilibrium constant for SuSy and its localization largely in sink tissues its role is largely to catalyze sucrose hydrolysis (Huber and Huber, 1996). Invertases are the other important enzymes involved in sucrose hydrolysis (Figure 4.1). Unlike SuSy, the hydrolysis of sucrose by invertase is not reversible. Invertases play an important role when there is a high demand for carbon and energy. Invertases are also important in sucrose unloading from the phloem which is crucial in the supply of substrates for starch biosynthesis (Sturm, 1999; Lunn 2002). There are two classes of
Figure 4.1: Sucrose metabolism in higher plants (Salerno and Curatti, 2003)
invertases that can be distinguished by their pH optima for catalysis. The neutral and alkaline invertases (pH optima between 6.5 – 8.0) are localized in the cytosol and the acid invertases (pH optimum 5.0) are extracellular or vacuolar (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999). The localization of SPS is chiefly in the photosynthetic parts of the plants, although it can be found in the non-green parts too (Salerno and Curatti, 2003). SuSy is localized mainly in the non-green parts of plants. In the case of cassava, which synthesizes starch in the roots, SPS is likely localized in leaves and not roots whereas SuSy likely has the opposite tissue-specific localization. The localization of SPS in the leaf would help separate the source and sink tissues generating a strong gradient between them.

Sucrose phosphate synthase is regulated by allosteric effectors (mostly in cereals). It is enhanced by glucose-6-phosphate and inhibited by inorganic phosphate (Huber and Huber, 1996). It is also under a reversible seryl phosphorylation control. The reversible seryl phosphorylation of SPS works hand in hand with the regulation by allosteric effectors. An increase of glucose-6-P and the decrease of Pi that takes place with dark-to-light transition favors dephosphorylation and activation of SPS. It also increases the catalytic activity as a result of allosteric regulation.
When darkness falls, the reverse is the case (Figure 4.2). The regulation by effectors and seryl phosphorylation coincide with the availability of substrates for sucrose synthesis which depends on photosynthesis. When photosynthesis is in abeyance, triose phosphates are in short supply for exchange with Pi (Huber and Huber, 1996). This leads to accumulation of Pi in the cytosol triggering the synthesis of fructose, 2, 6- diphosphate which inhibits fructose-1, 6-phosphatase, a key enzyme in sucrose synthesis (Huber and Huber, 1996, Stitt, 1995, Stitt and Krapp, 1999).
Figure 4.2. Schematic representation of allosteric regulation and seryl phosphorylation regulation of sucrose phosphate synthase (Huber and Huber, 1996)
On the other hand, darkness shuts down photosynthesis and activates SPS-kinase which phosphorylates and inactivates SPS (Huber and Huber, 1996). These processes ensure that the enzymes of sucrose synthesis are inactive during periods of low substrate availability.

There are three types of SPS which vary with respect to allosteric regulation and their ability to be phosphorylated or not (Huber and Huber, 1992; Huber and Huber 1996). As discussed above, class I SPS (exemplified by maize SPS) is allosterically regulated by glucose-6-phosphate and inorganic phosphate and is prone to dephosphorylation in the light which leads to 2-3 fold increase in $V_{\text{max}}$ (Huber and Huber, 1996). Class II SPS (e.g. spinach SPS) are also allosterically regulated, but dephosphorylation has no effect on its $V_{\text{max}}$. Class III SPS (e.g. soybean SPS) is weakly regulated by effectors and there is no evidence of dephosphorylation. In our experiments with transgenic cassava we used a maize SPS gene. The significance of the maize (a monocot) SPS in cassava is that monocot SPS is not under regulation by dephosphorylation/phosphorylation due to a difference in the properties of SPS-kinases between monocots and dicots (Huber and Huber, 1996).

Transgene expression of SPS has been reported in several different plants. Transgenic tomato plants over-expressing maize SPS had increased leaf SPS enzyme activity (Galtier et al., 1993; Worrell et al., 1991). Leaf-specific expression of SPS did not improve overall growth of the transgenic plants. However, the total number of fruits increased, the fruits matured earlier and there was an increase in total fruit dry weight. (Micallef et al., 1995; Worrell et al., 1991). Tomato overall plant growth enhancement occurred with
the constitutive expression of SPS gene (Foyer et al., 1994). Transformation of tobacco and potato with spinach SPS led to an increase in SPS protein, but the SPS activity was down-regulated by phosphorylation, thereby leading to no change in metabolism between transgenic and non-transgenic plants (Stitt and Sonnewald, 1995).

The importance of SPS in sucrose synthesis has been studied by inhibiting the expression of SPS by antisense techniques. This strategy led to reduction of sucrose synthesis and an increase in starch and amino acid synthesis in potato (Geigenberger et al., 1995).

In our study, we express the maize SPS gene in leaves of transgenic cassava and characterize its effects on growth.
4.2 MATERIALS AND METHODS

4.2.1 E. coli and Agrobacterium strains

*Escherichia coli* strain DH5α (Life Technologies, Grand Island, NY) was used for isolation of plasmid constructs. Luria-Beritani (LB) media supplemented with 100 mg/L ampicillin was used to select transformed *E. coli* carrying the modified plasmid of choice having an amp' gene. *Agrobacterium tumefaciens* strain LBA4404 obtained from Life Technologies (Grand Island, NY) was used in cassava transformation. Transformed (see below) Agrobacterium cells were selected on YM medium containing 50mg/L kanamycin and 100 mg/L streptomycin.

4.2.2 Plant material

Cassava cultivar TMS 71173, from the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria, was used for transformation purposes. *In vitro* plants were generated from apical meristems that were cultured on MS basal medium (Murashige and Skoog, 1962). Somatic embryogenesis was induced using apical leaves cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose, 8 mg/L 2, 4-D, 10mg/L 100X Gamborg’s B-5 vitamins (Gamborg *et al.*,1968), 50 mg/L casein hydrolysate and 0.5 mg/L CuSO4, pH 5.7. Cultures were maintained on a 12 h/day photoperiod at 28 °C at a light intensity of 50 μmol photons/m²/s. Somatic embryos were transferred to germination medium (MS basal medium supplemented with 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 2% (w/v) sucrose, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA, pH 5.7) after 4 weeks to generate embryo cotyledons for use in cassava transformation experiments.
4.2.3 Construction of vector

The backbone of the plasmid used in making the construct for CAB1/sps gene is the pBI121 plasmid (Strategene). The construct was made by substituting the CAMV 35S promoter with the 400 bp CAB1 promoter (Sheen, 1994) and the β-Glucuronidase gene with the 3.5 kb maize sps gene (Calgene, Inc, Davis, CA, Worrell et al., 1991). The T-DNA region of the plasmid also contains the nptII gene for the resistance to kanamycin and its analogue paromomycin. The CAB1 promoter was cloned into pBI121 with HindIII and BamHI restriction enzymes following restriction of the plasmid with the same enzymes to cut out CAMV 35 S promoter. The CAB1/sps fragment was cloned into BamHI and EcoRI restriction sites following restriction of pBI121 with BamHI and EcoRI to remove β-Glucuronidase. In each instance, the plasmid was run on 1% agarose gel with 1X TAE (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0) buffer to separate the fragments. Qiagen Qiax II Gel Extraction Kit (Qiagen Inc., Valencia, CA) was used to purify the DNA from the gel. For ligating the promoter into the pBI121 plasmid, approximately 300 ng of the plasmid and 800 ng of CAB1 promoter and sps gene were used. Ten units of T4 DNA ligase was used for each ligation carried out over 12 hours at 14 °C. Ten percent of the ligation mixture was used to transform DH5α E.coli competent cells (Life Technologies). The recombinant DNA and E. coli competent cells mixture were incubated on ice for 30 minutes followed by a 42 °C heat shock for 30 seconds. The mixture was then incubated on ice for 2 minutes. This was followed by the addition of 900 µl of SOC media (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, and 2.5 mM KCl) prewarmed to 42 °C. The cell suspensions were incubated for 1 hr at 37 °C and 250 rpm on a shaker. The cells were then spread on LB medium supplemented with 100
mg/L ampicillin. Colonies carrying the recombinant DNA grew on the selection medium and were used to grow 250 mL liquid cultures containing 100 mg/L ampicillin. Plasmid DNA was purified with a Qiagen Plasmid Midi Kit (Qiagen Inc., Valencia, CA). The recombinant plasmid is called PCS.

PCR analysis was carried out to confirm the transformation of *E. coli* competent cells using *sps* gene specific primers. The forward primer was (CTGCTAGCATCAAGGTCACAGG) and the reverse primer was (GATTGATACATTGAGAAATGGA) from the 3'terminator end *sps*. PCR was performed in 50 µL containing 0.4X PCR buffer (8 mM Tris-HCl, pH 8.4, 20 mM KCl), 20-100 ng DNA, 0.1 mM dNTP, 0.2 mM each primer (IDT, Coralville, IA), 1 unit Taq polymerase (Life Technologies, Grand Island, NY), 1 mM MgCl₂ (Life Technologies, Grand Island, NY). The PCR conditions were as follows: 3 minutes at 94 ºC, 30 seconds at 94 ºC, 45 seconds at 58 ºC and 30 seconds at 72 ºC, 30 cycles. The PCR product was run on 0.8% agarose gel at 70V using 1X TAE buffer. Control PCR experiments were carried out using template DNA isolated from untransformed competent cells (negative control).

4.2.4 *Agrobacterium* transformation

One hundred ng of CAB1: *sps* plasmid DNA was used to transform LBA4404 strain of *Agrobacterium tumefaciens* from Life Technologies Inc. (Rockville, MD). The transformation was performed by electroporating 20 µl of ElectroMAXX LBA4404 cells (Life Technologies Inc., Grand Island, NY) with 100 ng of CAB1: *sps* vector using a Bio Rad electroporator (0.2 cm electrode gap Bio-Rad, Hercules, CA). The electroporator
was set at 1.8 kV, 25 uF, 200 ohms and 5 milliseconds. This was followed by incubation in 1.0 mL YM medium at 250 rpm for 3 hours at 28 °C. Subsequently, 100 mL of cell suspension was inoculated on solid YM medium supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin for 2 days at 28 °C. The colonies that grew on the selection media were screened with sps specific primers: 5’ end (CTGCTAGCATCAAGGTCACAGG) and reverse primers (GATTGATACATTGAGAAATGGA) from sps terminator 3’ end to confirm transformation. DNA amplification was carried out for 30 PCR cycles of 94 °C, 50 seconds, 58 °C, 45 seconds (annealing temperature), 72 °C, 1 minute (extension temperature). The PCR primers and buffer composition are the same as described in section 4.2.4. The PCR product was sequenced to determine the fidelity of the PCR product by the automated dideoxy sequencing at the Ohio State University Plant Microbe Genomics Facility (PMGF).

4.2.5 Bacterial strain and constructs

Cassava somatic embryos were transformed by Agrobacterium-mediated transformation using strain LBA4404 harboring plasmid PCS: CAB1/sps (Figure 4.3). The vector contains maize sucrose phosphate synthase (sps) gene (with the nos terminator) whose expression is driven by the Arabidopsis thaliana CAB1 promoter, kindly supplied by Dr. JC Jang of the Horticulture and Crop Science Department of the Ohio State University, Columbus, OH (Figure 4.3). The cassette was verified by DNA sequencing at the Ohio State University Plant Microbe Genomics Facility (PMGF).
Figure 4.3: T-DNA region of the binary vector pCS containing the *sps* gene from maize.
Agrobacterium strains harboring the plasmid were started on YM medium (0.04% (w/v) yeast extract, 1% (w/v) mannitol, 1.7 mM NaCl, 0.8 mM MgSO₄·7H₂O and 2.2 mM K₂HPO₄·3H₂O) supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin and grown for two days at 28 °C. Liquid cultures then were started from the solid cultures by inoculating 5 mL YM medium containing 100 mg/L streptomycin and 50 mg/L kanamycin with single colony and then grown at 28 °C on gyratory shaker (250 rpm) for 2 days. This is followed by spectrophotometric reading at OD₅₆₀ and dilution to OD₅₆₀ of 0.5. The bacteria were then pre-induced for Ti-plasmid transfer by incubation in liquid MS (Murashige and Skoog, 1962) media supplemented 2% sucrose and 200 µM acetosyringone for 2-5 hr (Li et al.; 1996, White et al.; 1998).

4.2.6 Transformation with Agrobacterium and selection of putative transformants

The cotyledons of cassava germinated somatic embryos were used for the transformation experiment (Arias-Garzon et al., 1997). Agrobacterium harboring the PCS: CAB1: sps plasmid were co-cultivated with cassava somatic embryo cotyledons on MS basal medium plus 100 µM acetosyringone for two days at 28°C in the dark. The tissues were then transferred to MS medium containing 75 mg/L paromomycin and 500 mg/L carbenicillin to kill Agrobacterium and to select for transformants and incubated at 12 h/day photoperiod of 28 °C at a light intensity of 50 µmol photons/m²/s. Clumps of somatic embryos formed after four weeks of culture and were transferred to cassava germination medium (MS medium supplemented with 2 % (w/v) sucrose, 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA₃) containing 75 mg/L paromomycin and 500 mg/L carbenicillin for four more weeks. Following germination, individual somatic embryos
were transferred to cassava micropropagation medium (MS salts plus 2% (w/v) sucrose, 0.04 mg/L benzylamino purine, 0.05 mg/L giberellic acid, 0.02mg/L NAA, 1 mg/L thiamine-HCl, 100mg/L myo-inositol, pH 5.7) without antibiotics for root induction. Wild-type plants used for biochemical experiments were regenerated from somatic embryos using the above protocols to regenerate transgenic plants. Embryo cotyledons cultured on the selection medium without incubation with *Agrobacterium* were used as negative controls. Positive controls included plants regenerated in the absence of antibiotics which were not transformed with Agrobacterium.

### 4.2.7 PCR verification of transformants

PCR analysis for the integration of the transgene was carried out with genomic DNA isolated from leaves of *in vitro*-grown putative transformed cassava and wild-type cassava according to Dellaporta *et al.*, (1983) and Soni and Murray (1994). The PCR analysis was performed to verify the presence of the maize *sps* gene. PCR was done in 50 µL containing 0.4X PCR buffer, 20-100 ng leaf DNA, 0.1 mM dNTP, 0.2 mM each primer (IDT, Coralville, IA), 1 unit Taq polymerase, 1 mM MgCl₂. The transgenes were identified with primers specific for the 5’ end of the *sps* gene (CTGCTAGCATCAAGGTCACAGG) and reverse primers (GATTGATACATTGAGAAATGGA) from the 3’ end of the *sps* terminator. DNA amplification was carried out for 30 PCR cycles at 94 °C, 50 seconds, 58 °C, 45 seconds (annealing temperature), and 72 °C, 1 minute (extension temperature). The PCR product was sequenced to prove the fidelity of the PCR product by the Ohio State University Plant Microbe Genomics Facility (PMGF).
4.2.8 Greenhouse establishment of PCS plants

One month-old plantlets with well-established roots were transferred to the greenhouse for root growth measurements. Plantlets were removed from test tubes washed with running tap water and planted in sterile soil. Plantlets are covered with transparent waterproof bags to increase humidity. The plantlets were maintained in a growth chamber at 28 °C and 50 μmol photons/m²/s⁻¹. After 2 weeks, the bags are punctured to reduce humidity. The bags are finally removed after 4 weeks. Plantlets were watered every other day and grown from May 2003 to date in 11” X 12” in plastic pots filled with Scott’s MetroMix soil (The Scott’s Company, Marysville, OH) to generate stakes for the greenhouse growth experiment. The leaves of transgenic plants that have been fully certified by DNA analysis were harvested from the greenhouse for SPS enzyme assay.

4.2.9 Southern blotting

Genomic DNA was isolated from greenhouse grown plants according to the methods of Soni and Murray (1994). One gram leaf tissues was ground with 10 mL of extraction buffer comprising 50 mM Tris-Cl (pH8), 10 mM EDTA, 2% SDS, 100 mM LiCl, 10 μg/mL proteinase K. The ground tissue was incubated for 15 minutes with occasional vortexing. This was followed by 15 minute centrifugation at 2800 g (3500 rpm). RNase (200 μg) was added to the supernatant followed by 30 minutes incubation at 37 °C. The sample was purified with equal volume of phenol, phenol/chloroform and phenol/chloroform/isoamyl alcohol respectively. The liquid phase of the extraction was incubated with 0.25 X volume 10 M ammonium acetate and 2X volume of ethanol for 10
minutes at room temperature. The DNA was pelleted for 20 minutes at 10,400 g (13,000 rpm). The DNA pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended with 0.5 mL TE buffer (10 mM Tris-Cl, 1 MM EDTA pH 8.0). The DNA concentration was measured at 260, 270 and 280 nm using a Cary 3E UV-Visible Spectrophotometer (Walnut Creek, CA) with a computer-based DNA measurement program.

Southern blot analysis was carried out according to the methods of Sambrook et al., (1989). Genomic DNA (20 µg) was restricted with Kpn1 followed by gel analysis on 0.8% agarose gel, 70V, 1X TAE. Kpn1 does not cut the T-DNA region of the vector carrying Patatin: glgC. The gel was depurinated with 250 mM HCl for 10 minutes on a slow shaker. This was followed by 1 hr denaturation with 1.5 M NaCl, 0.5 M NaOH, then 1 hr neutralization with 1 M Tris-HCl, 1.5 M NaCl, (pH 8) on a slow shaker. The genomic DNA was transferred to ZetaBind nylon membrane (Life Science Products, Inc., Denver, CO) overnight with 20 X SSC. Following transfer, the DNA was cross-linked to the membrane using a UV cross-linker. The membrane was then stored dry until prehybridization with CHURCH buffer (0.5 M NaHPO4 (pH 7.2), 1.0 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS) at 50 °C for 3 hr. The probe for the Southern blot was made by PCR amplification of 450 bp piece of the sps gene, in a total volume of 50 µl (5X PCR buffer, 100 ng CAB1-sps vector, 0.1 M of dATP/dTTP/ dGTP each, 100 µCi 32P-dCTP (Amersham, Piscataway, NJ), 2.5 units Taq polymerase Life Technologies, Grand Island, NY, 0.4 µM each of the primers, IDT, Coralville, IA, 1.5 mM MgCl2) with a Perkin Elmer Cetus thermal cycler. The PCR amplification was run with the following conditions: 3 minutes at 94 °C, 30 seconds at 94 °C, 45 seconds at 58 °C and 30 seconds
at 72 °C, 30 cycles. The probe was then purified using a Qiagen PCR kit (Qiagen Inc, Valencia, CA), boiled at 100 °C for 5 minutes, quickly cooled on ice, and added to the hybridization buffer (same as prehybridization buffer) overnight at 50°С. Subsequently, the membrane was washed with 1 X SSC, 0.1% (w/v) SDS for 30 minutes at 50 °C followed by two washes with 0.1 X SSC, 0.1% (w/v) SDS for 30 minutes at 50 °C. The membrane was wrapped with Saran wrap and exposed overnight to a phosphor imager that was bleached prior to use for 30 minutes with light.

**4.2.10 RT-PCR analysis of plants**

Leaf tissues (100 mg) of *in vitro* putative transformed and control plant material was used to extract total RNA with Qiagen Plant RNA Extraction Kit (Qiagen Inc., Valencia, CA). The leaf tissue was ground with mortar and pestle using liquid nitrogen. Followed by the addition of 450 μL RLT extraction buffer. The mixture was vortexed and transferred into Qiagen’s QIAshredder spin column and centrifuged at 13,000 rpm (Eppendorf Centrifuge 5415 D, Hamburg Germany) for 2 minutes. The supernatant was transferred to a new microfuge tube containing 0.5 volumes of 96% ethanol (Pharmco Products, Inc. Brookfield, CT). The mixture was mixed by pipetting with a wide-bore pipette tip. The whole sample was then applied to Qiagen’s RNeasy mini column and the eluent was collected in a 2 mL collection tube and centrifuged for 15 seconds at 8000 g (10,000 rpm). The flow-through was discarded and 700 μL Qiagen’s buffer RW1 was added to the column and centrifuged for 15 seconds at 8000 g (10,000 rpm). The RNeasy column was transferred to a new collection tube. 500 μL Qiagen’s RPE buffer was added to the column and centrifuged 15 seconds at 8,000 g (10,000 rpm). The process was repeated
and the column was spun for 2 minutes. The RNeasy column was put in a new empty collection tube and centrifuged for 1 minute at 8000 g (10,000 rpm). For RNA elution, the RNeasy column was transferred to a new 1.5 mL collection tube and 50 µL RNase-free water was added to the RNeasy column (directly onto the RNeasy silica-gel membrane) and centrifuged for 1 minute at 8000 g (10,000 rpm). The eluent was used immediately for cDNA synthesis or stored at –20°C. The extract was treated with 1 unit DNase (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature to eliminate DNA contamination. The DNase was inactivated according to manufacturer’s instructions.

The first strand cDNA synthesis was carried out with 10 µg of total RNA using 1X reverse transcription buffer (50 mM Tris-HCl pH 8.0, 75 mM KCl, 3.0 mM MgCl2) 0.3 mM dNTP, 0.5 µg oligodT(12-18) primer, 200 units of SuperScript II reverse transcriptase (Life Technologies, Rockville, MD). The mixture was incubated at 65 °C for 5 minutes without reverse transcriptase followed by incubation at 42 °C for 1 hour with reverse transcriptase.

The cDNA was amplified with 1X PCR buffer, 1.5mM MgCl2, 0.1 mM dNTP, 2.5 units of Taq polymerase, 0.4 µM of each primer specific for the sps gene (CTGCTAGCATCAAGGTCACAGG, 5’ primer) (GATTGATACATTGAGAAATGGA, 3’ primer). The PCR conditions were as follows: 3 minutes at 94 °C, 30 seconds at 94 °C, 45 seconds at 58 °C and 30 seconds at 72 °C for 30 cycles. The PCR product was run on 0.8% agarose gel at 70V using 1X TAE. The tissue specific expression of the CAB1 promoter, which is normally leaf-specific, was verified by the RT-PCR analysis using root RNA as well. The RT-PCR experiments were
carried out without cDNA (negative control) and using primers for CYP79D1 gene that
encodes a cytochrome P450 enzyme which catalyzes the first-dedicated step in linamarin
synthesis (Anderssen *et al*., 2000).

The primers used in the PCR amplification of *CYP79D1* gene are as follows:

Forward primer: GCTAAATCAACCAGAAATCCTGAAG
Reverse primer: TGCAAGAGAAACAAGATAACCCC

4.2.11 Extraction of sucrose phosphate synthase

All steps are conducted at 4 °C unless indicated otherwise. Leaf samples (1 g) were
sliced and placed in a Warring Blender containing 0.05 M Tris-HCl, 10 mM glutathione
(GSH), 1.0 mM EDTA buffer (pH 7.5). Sodium bisulfite (0.04 mL of 10% (w/v), pH 6)
was added for each gram of tissue homogenized. Two 15 s slow speed blending were
followed by two 15 s fast-speed blending. The suspension was then filtered through
miracloth and the extract was centrifuged for 20 min. at 27,000 g (Sowokinos, 1976).
The supernatant was used for all enzyme assays. Quantification of crude protein was
done with BCA Protein assay Kit (Pierce, Rockford, IL) according to manufacturer’s
specifications with BSA protein standard.

4.2.12 Sucrose phosphate synthase

Sucrose phosphate synthase is measured by quantification of sucrose formation using the
resorcinol method. Assays (350 µl) contained 3.5 µmol fructose 6-P and 8.75 µmol
UDP-glucose dissolved in (100 X stock each was made) 50 mM Hepes-NaOH (pH 7.5),
15 mM MgCl₂ plus 135 µl plant extract. The reaction was terminated after 0, 5, 10, and
20 min. by removing a 70 µl aliquot of the reaction mixture and placing it in a tube containing 70 µl 1N NaOH. Tubes were boiled for 10 min. to destroy any remaining fructose, then 250 µl 0.1% (w/v) resorcinol in 95% ethanol plus 750 µl 30% (v/v) HCl was added. Tubes were incubated at 80°C for 8 min. and after cooling the A₅₂₀ was measured (Kalt-Torres and Huber, 1987). Sucrose formation was quantitated by comparison to a sucrose standard curve after subtraction of A₅₂₀ at 0 min. (background).

4.2.13 Statistical analysis

Analysis of variance (ANOVA) and other statistical analysis were done with the SAS software (SAS, 1985), at the Horticulture and Crop Science Department, The Ohio State University, Columbus, OH. Samples were evaluated using analysis of variance (ANOVA). Duncan’s multiple range test was used to separate treatment means found significantly different by ANOVA. All analyses were performed at P ≤ 0.05 confidence level.
4.3 RESULTS

4.3.1 Construction of vectors and transformation of bacteria

The CAB1 promoter was cloned into pBI121 plasmid with HindIII and BamHI restriction enzymes while *sps* gene was cloned into the same pBI121 vector with BamHI and EcoRI restriction enzymes. The recombinant plasmid was then transformed into DH5α competent cell and analyzed by PCR screening for the *sps* gene piece. Gel analysis of PCR products confirmed that the *sps* gene had been inserted into the pBI121 plasmid (Figure 4.4). Plasmid DNA isolated from confirmed transformed cells was transformed into Agrobacteria strain LBA 4404. Positive transformants were confirmed to be carrying the recombinant plasmid by PCR screening for the *nptII* gene (Figure 4.5).

4.3.1 Production of transgenic cassava

Cassava cultivar TMS 71173 was transformed via Agrobacterium-mediated transfer of the T-DNA carrying the maize *sps* gene. Transformed cells underwent somatic embryogenesis in 4-6 weeks. The embryos were transferred to MS regeneration media containing 0.01 mg/L 2, 4-D, 75 mg/L paromomycin and 500 mg/L carbenicillin for development and production of fully developed embryos with green cotyledons in 4-6 weeks. The mature embryos converted to plantlets in 1-2 months. Plantlets from each embryo were transferred to individual test tubes from which they were transferred to the greenhouse after 4-6 weeks where they generated materials for enzyme assay and molecular analysis of the plants (Table 4.1). We recovered 21 paromomycin resistant embryos from the TMS 71173 explants and 9 of the 21 embryos converted to plants (43% efficiency). Further analysis was carried out with three of the transformed plants because of the labor involved.
Figure 4.4: Gel analysis of sps PCR products of DH5α competent cells transformed with PCS plasmid. C, untransformed competent cell (negative control), M, marker DNA.
Figure 4.5: Gel analysis of sps PCR products of Agrobacterium competent cells transformed with PCS plasmid. C, untransformed competent cell (negative control), M, marker DNA.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Cultivar</th>
<th>Number of explants</th>
<th>Putative transformed embryos</th>
<th>Embryos converted to plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAB1-<em>sps</em> (sucrose phosphate synthase)</td>
<td>TMS 71173</td>
<td>653</td>
<td>21</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 4.1: Recovery of cassava somatic embryo cotyledons transformed with CAB1-*sps* gene.
4.3.3 Southern blot analysis

Genomic DNA from wild-type and transformed cassava plants was used for Southern Blot analysis using the PCR product of the *sps* gene (described above) as a probe. The copy numbers of the *sps* genes integrated into the transformed plants varied from two to three (Figure 4.6). The probe did not hybridize to the wild-type cassava plant DNA. The SPS-1 and SPS-2 plants had two copies of the *sps* gene, while SPS-3 had three copies of the gene.

4.3.2 RT-PCR analysis

To show that the putatively transformed cassava plants were expressing the transgene, RT-PCR analysis was done. The transformed plants had positive amplification of a 450 bp *sps* reverse transcriptase product while the wild-type did not show any bands (Figure 4.7). The maize *sps* gene was expressed in the leaf under control of the CAB1 promoter. We showed the tissue specificity of gene expression by carrying out RT-PCR analysis using root RNA from transgenic and wild-type cassava. There was no RT-PCR product recovered from total root RNA isolated from either wild-type or transgenic cassava lines. The control, amplification of cDNA from root of transgenic and wild-type plants using CYP79D1 primers (which encodes cassava cytochrome P450 oxygenase) was positive (Figure 4.8). This proves that the CAB1 promoter is not leaky and is effective only in the leaf and green tissues of plants (Siritunga and Sayre, 2003).
Figure 4.6: Southern blotting of CAB1-SPS plants showing multiple SPS integrations for transformed plants. P, plasmid DNA; W, wild type; PCS1-3, sps transformed plants.
Figure 4.7: RT-PCR analysis of CAB1-sps plants checking for 400 bp of maize sps gene and CYP79 gene as control using leaf RNA. WT, wild type cassava; PCS1-3, sps transformed plants; PCS, CAB1-sps plasmid; D1, CYP79 gene.
Figure 4.8: RT-PCR with root RNA to prove the fidelity of $sps$ gene expression in the leaf of cassava. M, marker DNA, PCS-1-3, transformed plants, WT, wild type cassava.
4.3.4 Sucrose phosphate synthase enzyme assay

The enzyme assay for SPS was done to check for the activity of the expressed proteins. Sometimes, transgenic proteins are made but are not functional due to a myriad of factors in the new environment. The enzyme assay shows that the transgenic plants had higher SPS activity than the untransformed cassava plants. The transformed plants had between a 58% to 82% increase in SPS activity relative to wild type (Figure 4.9). The activity of PCS-1 plant was 65% higher than the wild-type plant, PCS-2 had 58% higher activity than wild-type plants while PCS-3 had 82% more activity than the wild-type cassava plants. These SPS activities (168 – 193 micromole sucrose/hr/g FW) are higher than those reported for tomato (80 micromole sucrose/hr/g FW, Worrell et al., 1991), maize (150 micromole sucrose/hr/g FW, Lunn et al., 2003). Replicates of the wild type and transformed plants used for the assay gave similar results and the statistical variation between plant assays was low. These results suggest that the transgenic plants were not chimeric, otherwise the standard deviation in the assays would be expected to be large.
<table>
<thead>
<tr>
<th>Plants</th>
<th>Micromole Sucrose/hr/g FW</th>
<th>Percent of Wildtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>106±12.47c</td>
<td>100</td>
</tr>
<tr>
<td>PCS-1</td>
<td>175±9.3ab</td>
<td>165</td>
</tr>
<tr>
<td>PCS-2</td>
<td>168±18.6b</td>
<td>158</td>
</tr>
<tr>
<td>PCS-3</td>
<td>193±12.45a</td>
<td>182</td>
</tr>
</tbody>
</table>

Figure 4.9: SPS enzyme assay
4.4 DISCUSSION:

We have transformed cassava with a maize cDNA for sucrose phosphate synthase (Table 4.1, Figure 4.6, Figure 4.7). We used maize sps gene because no others were available for cassava or other root crops at the time of our experiments. Maize SPS belongs to the Class I SPS enzymes which are allosterically regulated by glucose-6-phosphate as an enhancer and inhibited by pyrophosphates (Huber and Huber, 1996). This class of SPS enzymes is post-translationally modified (phosphorylation) in the dark and dephosphorylated in the light. This regulation is important in regulating carbohydrate metabolism to guard against concomitant full activation of starch and sucrose biosynthesis. This semi-temporal demarcation is essential because the plant cannot support both biosynthesis pathways at the same time as they are highly demanding of energy and also they utilize the same substrates (Stitt, 1992; Salerno and Curatti, 2003).

Our main objective of transformation of cassava for enhanced SPS activity was to generate cassava plants that have enhanced sucrose production (Figure 4.9) in the leaf which would serve as a substrate for starch synthesis in cassava roots when hydrolyzed to hexoses by either root sucrose synthase or invertase(s). The overexpression of the sps gene in the leaf of tomato was shown to (Worrell et al.; 1991) increase the sucrose to starch ratio in the leaf. These results support the hypothesis that SPS regulates intracellular carbon partitioning (Worrell et al.; 1991). We therefore, speculated that enhanced SPS activity in cassava leaves would tilt the balance of carbon partitioning in the leaf towards sucrose production.
We have been able to transform the African cassava cultivar TMS 71173. There has been no report elsewhere of the transformation of the cultivar. To date, only the transformation of a few cassava cultivars has been reported in literature including TMS 60444 (Zhang et al., 2000 a, b; Gonzalez et al., 1998); TMS 71173 (Ihemere et al., this thesis), MCol 2215 (White et al., 1998; Siritunga and Sayre, 2003), MCol 122 (Zhang et al., 2000 a, b), MPer183 (Sarria et al., 2000). The efficiency of transformation was low as is the case with all cassava transformation, being under 2%. The reason for the low efficiency might be attributed to the selection pressure of transformed tissues. The antibiotics typically used in selection of transformed tissues in plants namely, hygromycin and kanamycin are all very toxic to cassava tissues (Shopke et al., 1996). This led to the use of the kanamycin analogue, paromomycin, in selecting the transformed cassava tissues (Shopke et al., 1996). Transformation efficiency increased but was still low in comparison to other crops.

We determined by enzyme assay that the transgenic plants had higher sucrose phosphate synthase activity than the untransformed cassava plants, bettering the wild-type cassava SPS activity rate by 58 – 82% (Figure 4.9). An increase in SPS activity has also been reported for other crops transformed with sps gene exemplified by a six-fold increase in tomato leaf SPS activity (Worrell et al.; 1991). In this case a maize sps gene was transformed into cassava, a dicotyledonous plant. We speculate that the SPS enzyme in monocots is regulated differently from that the dicotyledonous plant. Worrell et al.; 1991 attributed the six-fold increase of transgenic tomato SPS activity to be the result of being uncoupled from tomato regulation system. This difference in regulation (especially
phosphorylation by SPS-kinase) might have reduced the constraints on the maize SPS enzyme expressed in cassava (Huber and Huber, 1996). This probable release from phosphorylation regulation may have produced a more active enzyme.

The choice of SPS enzyme over sucrose synthase in cassava transformation for enhanced sucrose synthesis is a good one, even though both enzymes have similarities. Both enzymes use the same substrate, UDP-Glucose and are involved in sucrose synthesis. Further proof of similarity between SPS and sucrose synthase is given by the fact that their C-termini are similar in sequence (Huber and Huber, 1996; Lunn et al., 2002). However, SPS is thought to be more important than sucrose synthase because its substrate for reverse activity is removed by dephosphorylation by sucrose phosphatase with which SPS forms a complex (Huber and Huber, 1996, Cheng et al.; 1996). The dephosphorylation of sucrose-phosphate makes it impossible for SPS to recognize sucrose as a substrate making the reaction irreversible. This ensures that the reaction favors sucrose production which is needed in other carbohydrate metabolism. Comparatively, sucrose synthase reaction is reversible depending on the need of the plant. Sucrose synthase does not form a complex with other enzymes so its substrate is not immediately modified hence the reversibility of the reaction (Lunn et al., 2002).

The variation of SPS enzyme activity between transgenic lines could be a function of the number of integration events (Figure 4.6) as well as the site of insertion of the transgene into the genome (Zhang et al., 2000a; Sarria et al., 2000). The transformed plant (PCS-3) with three copies of the sps gene (PCS-3) had higher SPS enzyme activity than PCS-1.
and PCS-2 which both had two copies of the gene. It is hypothesized that the increase in SPS enzyme activity would translate to a stronger source for translocation of sucrose to the sink. This would result in a more substrate for AGPase in the root. This turn of events would also lead to a faster downloading of sucrose from the symplast. We speculate that this would lead to even a still more active SPS in the leaves. The active translocation of the products of SPS to the sink removes any feedback inhibition on SPS, giving it more room for maximal functioning (Worrell et al., 1991).

In summary, we have transformed cassava with maize sps gene. The transformation event resulted in increase of SPS activity. However, further experiments are to be carried out to study the impact of the transgene on cassava root yield. It will also be worthwhile to perform carbon assimilation experiments to find out if the transgene affected that as was reported for tomato (Worrell et al., 1991).
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