Isolation and characterisation of starch biosynthesis genes from cassava (*Manihot esculenta* Crantz)

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Stellingen

1. Winter in The Netherlands is so cold that were it not for the central heating all the Dutch may have migrated back to Africa, the cradle of all human kind.

2. The transfer of information from nucleic acid to nucleic acid, or from nucleic acid to protein, may be possible, but transfer from protein to protein, or from protein to nucleic acid, is impossible (Francis Crick, 1958 in Levin, B. (1994) Genes V).

3. Knowledge of the proverbs of a people can often reveal their history, and culture.

4. *Murumirwo ndamenyaga igithira.*
One who is fed by somebody else does not realise when food runs out (Kikuyu proverb).

5. The revival period for cassava research has coincided with great leaps in the field of plant molecular biology with gene cloning and transfer being readily applicable techniques (This thesis).

6. It is important that some form of international legal arrangements are made to ensure that resource poor farmers in developing countries benefit from crops, like cassava, which after all they have nurtured over the centuries (This thesis).

7. The history of genetics has shown that progress in understanding the functions and functioning of genes has depended on framing the right questions and using the right organism to answer them (Watson, J.D., 1989).

8. To believe in evolution is to believe that a whirlwind passing through a junkyard can give rise to a fully functional motor vehicle.

9. The genetic message, the programme of the present organism, therefore resembles a text without an author, that a proof -reader has been correcting for more than two billion years, continually improving, refining and completing it, gradually eliminating all imperfections (François Jacob, 1973 in Levin, B. (1994) Genes V).

10. The listener is the clever one (Shona proverb).

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For my parents who provide
the firm foundation of my life

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General Introduction

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Munyikwa, T.R.I., Langeveld, S., Jacobsen, E. & R.G.F. Visser

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General Introduction

Cassava (*Manihot esculenta* Crantz) of the family Euphorbiaceae is a tropical crop grown mostly for its starchy thickened roots in the lowlands of Asia, Africa, and South America. It is an important crop for over 500 million people (Cock, 1985). The annual world production of cassava roots was about 158 million metric tons in 1994 (FAO, 1994). Of this amount about 21% was produced in Latin America, 33% in Asia, and 46% in Africa. The main producing countries are Thailand, Indonesia, Nigeria, Brazil and Zaire which together produce over two thirds of the total world output. Most of the cassava produced is consumed as food or feed in the producing countries. About 20% of the Asian production (mainly from Thailand) is exported to Europe for use as a cheap source of carbohydrates for animal feed.

The importance of cassava is increasing, especially in Africa, owing to its tolerance to drought, soil acidity and low soil nutrients, all of which hinder the production of cereals (Hahn & Keyser, 1985). One of the African countries which has embarked on cassava production as a way of feeding its people during times of famine as well as a source of income for resource poor farmers located in marginal areas, is Zimbabwe.

Cassava in Zimbabwe

Zimbabwe is a sub-tropical country situated in central Southern Africa, between the Limpopo and Zambezi rivers. Agriculture is the mainstay of the economy of the country with over three-quarters of the population deriving its livelihood from agriculture and related activities. In terms of contribution to total output (Gross Domestic Output) agriculture at 14% ranks only second to manufacturing which contributes 23% (Anon, 1996).

Due largely to the colonial history of the country over 60% of Zimbabwe's 11 million people are crammed in the poorer agro-ecological zones, natural ecological regions (NR), NRIV and NRV (Fig 1). These are low lying areas (average 800m above sea level) typified by poor sandy soils, scanty and unpredictable rainfall (less than 800 mm per annum) and mid season dry spells all of which adversely affect the cultivation of maize, the staple food.

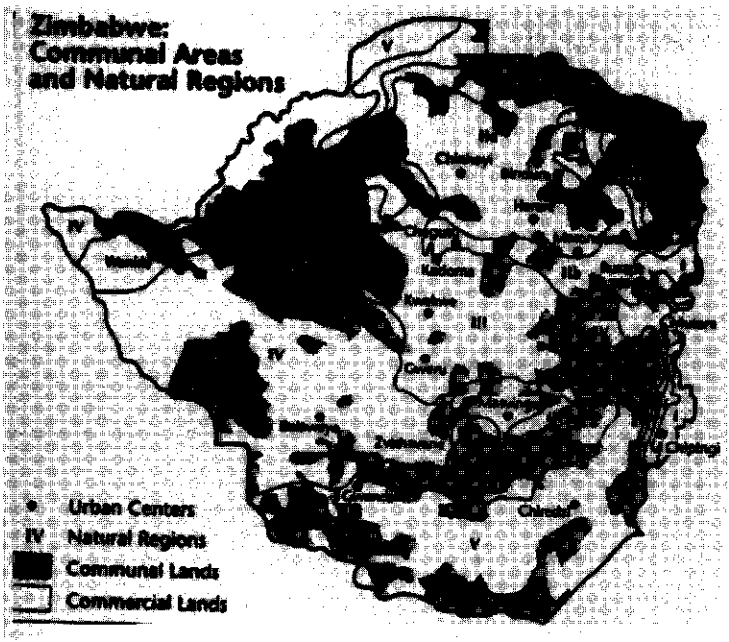

Fig 1: Map of Zimbabwe showing the location of communal areas in relationship to the natural agro-ecological regions (NR) [after Mehretu, (1994)]

This in addition to the recurrent droughts which the country experiences, five out of every ten years are drought years (C. Chasi personal communication), reduces food security and makes cassava the crop of choice within these areas.

Cassava or "mufarinya" as it is called in Zimbabwe is not a staple food for Zimbabweans as it is in neighbouring Zambia, Malawi and Mozambique. The crop is thought to have been largely introduced into Zimbabwe by migrant workers from these countries. However, the rise to prominence of maize as a cash crop led to the demise of sorghum, millet and cassava production and the loss of the traditional knowledge to process the cassava crop. In view of the cyanogenic potential of cassava (Rosling, 1988) and the lack of traditional knowledge to process it, many Zimbabweans are reluctant to grow the plant. At present cassava is grown largely as hedges around homesteads and sometimes farmers fields in the areas bordering the afore mentioned countries (Mharapara and Nzima, 1985).

Between 1979 and 1985 surveys as well as field trials were initiated by the

Zimbabwean Ministry of Agriculture, University of Zimbabwe and Delta Corp. (a private company) to determine the feasibility of producing cassava in Zimbabwe. Using planting material from the International Institute of Tropical Agriculture (IITA) as well as material collected in Zimbabwe, selection and breeding programmes to determine cassava varieties best suited for the local agro-ecological zones were set up. Market surveys showed a high potential for income generation by communal farmers in Zimbabwe as they would be able to sell excess cassava roots to the starch industry (Brian et al., 1989).

Cassava in the Modern Economy

World wide cassava has entered the modern market economy and there is a growing demand for its use in processed food and feed products. Increasingly cassava starch and starch derivatives such as dextrins, glucose, and high fructose syrups have become the main products of the cassava agro-industry. Cassava starch has also found uses in the paper and textile industry as well as in the manufacture of alcohol and adhesives (Kay, 1987).

The various uses of starch require different sorts of starch with distinct physical and chemical properties. Generally these properties are produced by various techniques which are largely based on chemical modification of the extracted starch. The inherent environmental pollution and cost of such modifications make the genetic modification of cassava, to produce cultivars with starch of differing physico-chemical properties, a viable option.

Information on the process of starch biosynthesis, particularly for cassava, is largely inadequate. In this thesis the present state of knowledge of cassava starch biosynthesis and its regulation is described. In addition it is shown how genetic modification of cassava could lead to the production of starch with distinct physical and chemical properties.

Occurrence of Starch

Starch is stored in the form of osmotically inactive, water-insoluble granules in amyloplasts (storage starch) and chloroplasts (transitory starch). In cassava most of the starch is stored within amyloplasts in the thickened roots commonly known as tubers. The starch content in tubers varies from 73.7% to 84.9% on dry weight basis (Rickard et al., 1991).

Cassava starch granules are round structures, flat on one side and containing a

conical pit that extends to a well defined eccentric hilum. They have a size range of 5-40 μm (Moorthy, 1994). An eighteen month investigation into granule size variation with age showed an increase in size of the granules up to the 6th month. Thereafter the granules remained a constant size throughout the study period (Moorthy & Ramanujan, 1986).

Structure and Composition of Starch

In common with other plant starches, cassava starch can be fractionated into two types of polymers namely amylose and amylopectin. Amylose consists essentially of linear chains of 100-10000 α (1-4) linked glucose residues. There is a low degree of α (1-6) branching within the amylose chains. Amylose imparts definite characteristics to starch and is thus an important factor in starch quality. The amylose content of cassava starch ranges from 13.6% to 23.8% (Ketiku & Oyenuga, 1972; Kawabata et al., 1984; Moorthy & Ramanujan, 1986). Insignificant differences were found in the amylose content when various varieties of cassava were compared during their growth period (Moorthy, 1985; Moorthy, 1994). The soluble amylose (which is thought to be mainly responsible for cohesiveness in cooked starch) content of cassava was determined to range from 10-40% of total amylose (Moorthy, 1994). Purified amylose forms stiff gels due to hydrogen bonding between molecules. It may also undergo retrogradation (shrinking and crystallisation) after heating.

Amylopectin is made up of much shorter chains of α-D glucopyranose units. These are primarily linked by α (1-4) bonds with α (1-6) branches. The outer regions of the amylopectin molecules which are short and unbranched are called the A chains, whereas the B chains exhibit multiple branching, the C chains have a single non reducing end (Guilbot & Mercier, 1985). The short nature of the A chains in cassava is apparent when cassava starch is debranched with isoamylase and analysed for its chain length distribution by Dionex. A peak corresponding to single glucose units thereby indicating short chain length is visible for cassava but not for potato, after 10 minutes retention time (Fig. 2). Cassava amylopectin has a molecular weight of 4.5×10^8 (Banks et al., 1972).

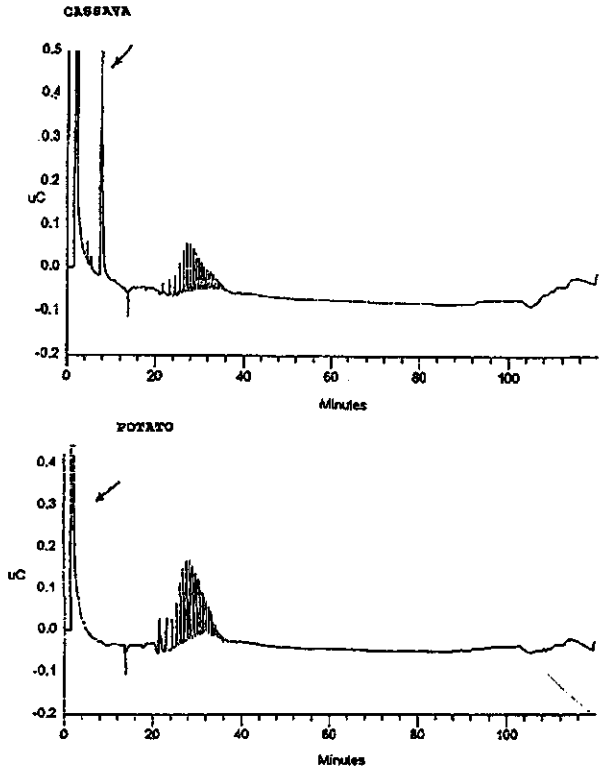

Figure 2: Distribution of chain length in cassava and potato using Dionex-HPLC after isoamylase treatment. A peak corresponding to single glucose units (an indication of short chain length) is clearly visible for cassava at retention time 10 minutes (marked by the arrow)

The amylopectin molecules are highly organised and have a definite crystalline nature. According to Blanshard (1987) amylopectin chains are arranged radially into alternating semi-crystalline and amorphous lamellae growth rings. The semi-crystalline region consists of domains of parallel packed double helices which also have disordered regions within or between the crystalline areas. The disordered regions between the crystallites may contain amylose or non organised regions of amylopectin chains. Three main types of X-ray diffraction crystalline pattern have been described, namely; A, B, and C starch patterns (Gallant et al., 1982; Guilbot & Mercier, 1985). Cassava has been found to consist largely of the A type pattern which is characteristic of cereal starches. This arrangement reflects a closely packed array of double helices in contrast to the more open B

arrangement of crystallites of the maize endosperm and potato tuber starches. The latter have considerably more water incorporated into their structures (Gallant et al., 1982; Guilbot & Mercier, 1985; Moorthy, 1994)

Extracted amylopectin is more stable than amylose due to limited hydrogen bonding. This enables it to remain fluid and confers high viscosity and elasticity to pastes and thickeners. Cassava starch also contains crude fat (0.08-1.54%), crude protein (0.03-0.6%), ash (0.02-0.33%) and very low phosphorous levels (0.75-4%) (Rosenthal et al., 1974; Soni et al., 1985). A better understanding of the process of starch biosynthesis is essential if any alteration of starch production in plants, in both nutritionally and commercially useful ways, is to be considered.

Biosynthesis of starch

Several pathways of starch biosynthesis have been proposed. These have been based on *in vitro* studies of isolated enzymes and increasingly on molecular analysis of the genes which give rise to these proteins. It is generally held that starch is synthesised from sucrose. Most cytosolic sucrose is converted to hexose phosphates which are then transported to the amyloplast via a hexose translocator. The glucose-6-phosphate formed is then converted into glucose-1-phosphate by the enzyme phosphoglucomutase (Viola et al., 1991)

The key step in starch biosynthesis in plants takes place inside the amyloplast where the enzyme adenosine diphosphate glucose pyrophosphorylase (AGPase; EC2.7.7.23) catalyses the synthesis of ADP-glucose and pyrophosphate from ATP and glucose-1-phosphate (Espada, 1962; Preiss, 1982. The pyrophosphate is removed by inorganic alkaline phosphatase thereby driving the reaction in the direction of ADP-glucose synthesis. ADP-glucose is the primed glucose molecule which functions as the glucosyl donor for α-glucan synthesis by various starch synthases.

The starch synthases (SS; EC 2.4.1.21), mainly granule bound starch synthase (GBSS), catalyse the conversion of ADP-glucose into amylose through (1-4) linkage of a ADP glucose to a pre-existing glucan chain. In vitro SS are able to utilise both amylose and amylopectin as substrates. How the initial primers for glucan chain formation *in vivo* are produced remains unclear. However, in bacteria, which synthesise glycogen through a process similar to starch biosynthesis in plants, like maize, priming proteins termed

glycogenin and amylogenin respectively, have been identified (Hengge-Aronis & Fisher, 1992; Singh et al., 1995).

Amylopectin is thought to be formed mainly due to the action of Branching Enzyme (BE; EC 2.4.1.18) and Soluble Starch Synthase (SSS). BE introduces branch points in the amylose molecules by hydrolysis of the α (1,4) glucan chains at 15 - 20 units from the non-reducing end. It then catalyses the formation of an α (1,6) cross linkage between the reducing end of the cleaved chain and another glucose residue (Smith & Martin, 1993).

It would, therefore, seem as if the postulated pathway for starch biosynthesis is relatively simple involving only three committed enzymes namely; AGPase, SS and BE. However in all species investigated it has been shown that the participating enzymes have several isoforms that are involved in the process of starch biosynthesis. These isoforms differ in their tissue specificity, timing of expression, kinetic properties and products. The existence of these isoforms is thought to provide the plants with an ability to adjust the process of starch formation depending on the prevailing conditions and stage of growth. Investigation of the role and function of these "committed" enzymes through biochemical and molecular analysis enables us to understand the molecular control of the process of starch biosynthesis. Our research has been focused on identifying and characterising the genes and enzymes involved in cassava starch formation.

ADP Glucose Pyrophosphorylase

Plant AGPases exist as heterotetrameric proteins (ca. 210-240 kDa) composed of two small (ca. 50-55 kDa) and two large (ca. 51-60 kDa) subunits, and their size depends on the plant species (Preiss et al., 1990; Kleczkowski et al., 1991). These subunits are also called AGPase B and S respectively, from the *Brittle* and *Shrunken* loci in maize, from which the first AGPase cDNAs were cloned (Bae et al., 1990; Bhave et al., 1990; Preiss et al., 1990). In contrast, AGPase in bacteria, from which the plant AGPase is thought to have evolved, consists of four equally-sized subunits (Preiss et al., 1990). Genes encoding both subunits of AGPase have been cloned from several species, e.g. the monocotyledons maize (Bae et al., 1990; Bhave et al., 1990), barley (Villand et al., 1992), wheat (Ainsworth et al., 1993) and rice (Anderson et al., 1989) and the dicotyledons potato (Müller-Röber et al., 1990), and spinach (Smith-White & Preiss, 1992). Isoforms of AGPase S have been found in Arabidopsis (Villand et al., 1993) and in potato (La Cognita et al., 1995).

In general there is greater homology between genes encoding small subunits from

different species than large and small subunits in the same species. The potato small subunit has 84% amino acid identity to the rice endosperm small subunit and 93% amino acid identity to the spinach leaf small subunit. This is in contrast to the 52% and 35% amino acid identity between small and large subunits of potato and spinach leaf respectively (Nakata et al., 1991; Preiss et al., 1991).

Most plant AGPases are allosterically regulated. The metabolite 3-phosphoglyceric acid (PGA) activates, and ortho-phosphate (Pi) inhibits the enzyme (for reviews see Preiss, 1984; Okita et al., 1993). Sensitivity to these metabolites has been demonstrated in photosynthetic as well as non photosynthetic tissues (Sowokinos & Preiss, 1982). According to Kleczkowski et al. (1993) barley AGPase is insensitive to PGA and Pi regulation. This demonstrates the heterogeneity in the allosteric response of the plant enzyme.

An abundance of evidence demonstrates the prominent role of AGPase in the biosynthesis of starch in plants. A dramatic reduction of maize endosperm AGPase activity, leading to reduced starch levels, was shown to be caused by mutations at the independent loci *Shrunken-2* and *Brittle-2* (Hannah et al., 1980). In *Arabidopsis thaliana*, a mutant containing less than 0.2% of the leaf starch content observed in the wild type, showed only 0.2% of the wild type AGPase activity in leaf without alterations in the activities of the other enzymes involved in starch biosynthesis (Lin et al., 1988a and b). Furthermore, by using kinetic models, Petterson & Ryde-Petterson (1989) showed the importance of AGPase in starch biosynthesis. More recently, Müller-Röber et al. (1992) showed a decrease in starch production and the accumulation of soluble sugars in potato tubers caused by the antisense inhibition of the expression of the gene encoding for the small subunit of the AGPase.

In addition, a mutant *glgC* gene from *Escherichia coli* which caused a 33% increase in glycogen content in *E.coli*, was fused to a chloroplast transit peptide and a patatin promoter and introduced into potato. This resulted in an increase in tuber starch content of 35% compared to the control tubers (Stark et al., 1992).

Starch Synthases

Starch synthases, which make amylose, can be sub-divided into two groups based on their location. Granule bound starch synthase (GBSS) is found tightly bound to the starch granule, whereas soluble starch synthase (SSS) is located in the stroma of the amyloplast or chloroplast as a soluble form (MacDonald & Preiss, 1985).

Both groups (soluble and granule bound starch synthases), can further be divided into two other groups, based on their elution from a sepharose column (MacDonald & Preiss, 1985). These are probably the isoforms of GBSS and SSS, which have now been found in several species including pea, potato and wheat (Dry et al., 1992; Denyer et al., 1995; Edwards et al., 1995).

Although GBSSI of maize can extend amylopectin *in vitro* (MacDonald and Preiss, 1985), it is *in vivo* predominantly involved in the synthesis of amylose. This is shown by the absence of amylose in mutants of different species lacking the GBSSI gene (Shannon and Garwood, 1984). Moreover, Visser et al. (1991) and Salehuzzaman et al. (1993) demonstrated that by using an antisense GBSSI gene from potato and cassava respectively, there can be a reduction of the GBSS activity. This led to reduced amylose levels in potato tuber starch down to about 0%.

SSS is thought to be predominantly involved in the synthesis of amylopectin. *Chlamydomonas st-3* mutants, defective for one (SSSII) of two isoforms of SSS, showed an increased amylose content, whereas the length of the amylopectin chains shifted from intermediate size (8-50 units) to short chains (2-7 units) (Fontaine et al., 1993).

Now that the GBSSII isoform in wheat (Edwards et al., 1995) and potato (Denyer et al., 1995) has been found in the granule bound fraction and the soluble fraction, the distinction between the granule bound and soluble starch synthases has become less profound. In potato, GBSSII is present throughout the development of the tuber, but only accounts for a maximum of 15% of the total starch synthase activity in this tissue. Northern analysis indicated a prominent role for GBSSII in the early stages of pea development (Edwards et al., 1995).

Branching Enzyme

Branching enzyme is present in multiple isoforms in several plant species like spinach, sorghum and maize (Hawker et al., 1974; Boyer, 1985; Fisher et al., 1993). In

cassava Salehuzzaman et al. (1992) were able to clone only one type of BE cDNA from a cassava tuber specific library. This cDNA has 70-75% similarity with BEI from other plant species but less with BEII. The open reading frame encodes a protein of 852 amino acids of which 74 amino acids form a transit peptide whose cleavage site motif was identified as ISA/A. The mature protein of 778 amino acids has a calculated MW of 88.7 kD and is highly expressed in cassava tubers. The enzyme also shows differences in expression between various cassava genotypes. It remains unclear as to whether or not the different protein bands which react with the BE antibody, in cassava, represent different isoforms of BE (Salehuzzaman et al., 1992).

The activity of BE is important for starch quality and quantity. The wrinkled seeded pea and the amylose extender mutant of maize are due to the lack of activity of one of the isoforms of BE. Consequently the plants are characterised by less branched amylopectin, a high ratio of amylose over amylopectin, reduced starch levels, increased amounts of sugars, as well as deeply fissured starch grains (Bhattacharyya et al., 1990, Shannon and Garwood, 1984)

Production of new cassava varieties

The availability of cloned and characterised cassava starch genes has opened new avenues for altering cassava starch composition and structure. The development of new cassava cultivars with starch having a range of physico-chemical properties and uses is dependent on the availability of a reproducible transformation and regeneration method. For cassava such a protocol has recently become available (Sofiari, 1996). With this procedure cassava embryogenic calli are bombarded with gold/tungsten particles coated with DNA of the appropriate gene constructs. The transformed embryogenic calli are then selected using a selection marker such as kanamycin or luciferase. The calli are allowed to regenerate into plantlets using the technique of somatic embryogenesis (Raemakers et al., 1992). Transgenic cassava plantlets can be easily screened in vitro without the need to wait for at least six months before tuber formation in the field. This can be done using the starch filled thickened stems obtained when *in vitro* plantlets are grown on high sucrose media (Salehuzzaman et al., 1994).

The content and composition of cassava starch can be altered by down regulation or increase of the expression of the committed enzymes in the pathway of starch biosynthesis. In general down regulation of any gene product can be achieved by the antisense technique.

Antisense inhibition of gene expression leads to a reduction in the steady state mRNA levels, a reduction in the amount of protein, and consequently reduced enzyme activity. Before the advent of a reproducible transformation protocol in cassava, Salehuzzaman et al. (1993) were able to demonstrate the antisense effect of cassava GBSSI in potato. The use of antisense technology for down regulation of genes has now become common. It has been used in suppression of chalcon synthase (CHS) gene expression in *Petunia* (van der Krol et al., 1988), potato for example for AGPase B (Müller-Röber et al., 1992), GBSS (Visser et al., 1991) and UDP glucose pyrophosphorylase (Zrenner et al., 1993).

Use of the antisense technique to produce new cassava cultivars has a distinct advantage over the use of naturally occurring or chemically induced mutants. Most of these mutations are recessive in nature whilst an antisense gene acts as a dominant suppressor gene. It can thus be used directly in allotetraploid cassava thereby reducing the time required to breed a new variety for this trait.

Modifying cassava starch content

Reduction of starch content in cassava could be achieved by using antisense AGPase gene constructs. Inhibition of AGPase would in principle reduce the amount of ADP-glucose available for amylose and amylopectin formation and reduce starch levels in the cassava tubers. Such experiments have been carried out using antisense AGPase S gene constructs by Müller-Röber et al. (1992) in potato. The transgenic plants that showed the highest inhibition had AGPase activity that was less than 2% of the wild type. Additionally the dry weight was reduced by 40% compared to the wild type potato. The tubers stored more sugars (sucrose up to 30% and glucose 8%) and less storage protein.

Reduction in branching enzyme activity in cassava with antisense constructs may also decrease starch content as for the *rugosus* (*r*) mutation in pea. Pea plants with this mutation, in addition to increased sucrose levels, also exhibited higher than normal lipid levels (Bhattacharyya et al., 1990). Starch altered in this way is commercially important, due to its greater palatability it may be desirable for use in food products.

Storage organs devoid of starch can also be modified genetically to become storage sites for other compounds. Such compounds could be sucrose derived ones such as fructans and cyclodextrins as has been shown in potato (Oakes et al., 1991, van der Meer et al., 1994). In the field, plants with high sucrose levels may become susceptible to pathogen attack during germination or sprouting. However, the availability of organ specific,

inducible promoters is expected to make it possible to express the gene constructs in specific organs and at the appropriate time.

Increasing starch content in plants could have a very dramatic effect on the economics of plant production. It would enable the cost of tuber harvesting and transport to be offset by the profit from an increase in the dry weight to fresh weight ratio. In cassava, starch content could be increased by over-expression of the AGPase enzyme or by the use of the mutant bacterial AGPase gene (*glg*C) as was achieved in potato (Stark et al., 1992)

Modifying cassava starch structure

In cassava, an altered starch structure can be introduced by reducing the activities of the "committed" enzymes especially BE and GBSS. This will considerably alter the ratios of amylose to amylopectin as well as the degree of branching in the starch. A range of cassava cultivars with altered granule size, as well starch composition can thus be theoretically produced.

Genetic alterations in starch structure have been reported in other starch storing plants. The cassava GBSSI gene, when introduced in antisense in wild type potato, led to the production of starch with reduced levels of amylose and in some cases to amylose free potato starch (Salehuzzaman et al., 1993). A similar phenomenon was observed for potato GBSSI in potato (Visser et al., 1991)

An amylose free variety or mutant is currently not available for cassava. Hence the availability of GBSSI as well as GBSSII would be ideal for the production of such plants in cassava based on the antisense suppression of the endogenous gene (s). Plants with a range of amylose were produced in potato by using the antisense approach (Kuipers et al., 1994). Some of these genetic modifications may result in reduced synthesis and yield of starch. This is the case with most naturally occurring mutants (Visser & Jacobsen, 1993). This may be overcome by incorporating genetic strategies that increase yield like over-expression of AGPase. In some cases the type of starch produced such as the amylose free type is of such high value that the profits offset possible losses due to yield (Visser & Jacobsen, 1993).

Outline of the thesis

In this study the cloning and characterisation of cassava cDNAs encoding enzymes involved in cassava starch biosynthesis was undertaken. Chapter 2 describes the cloning and developmental Northern analyis of a granule bound starch synthase (GBSSII) of cassava. The possible role of GBSSII *vis a vis* GBSSI in amylose synthesis is discussed. Chapter 3 examines the critical role of the enzyme ADP glucose pyrophosphorylase (AGPase) in starch biosynthesis. In this chapter the isolation of two cDNAs encoding the small and large subunits of AGPase is described. In addition to sequence and developmental Northern analyses, investigations into AGPase activity in various cassava tissues are described. Chapter 4 is devoted to the transformation and analysis of transgenic potato plants carrying a cassava antisense AGPase B gene construct. In Chapter 5 the use of particle bombardment to successfully transform cassava friable embryogenic calli with the cassava AGPase B antisense cDNA is described. Investigations of possible changes in starch biosynthesis in transgenic cassava plants are described and discussed. In this chapter investigations on the improvement of the transformation and regeneration procedures for cassava were also performed. A general discussion focused on the impact of genetic modification of cassava starch, the potential benefits to resource poor farmers in southern countries of introducing transgenic cassava as well as the possible risk assessment strategies is provided in Chapter 6.

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Isolation, characterisation and developmental regulation of granule bound starch synthase II from cassava (*Manihot esculenta* Crantz)

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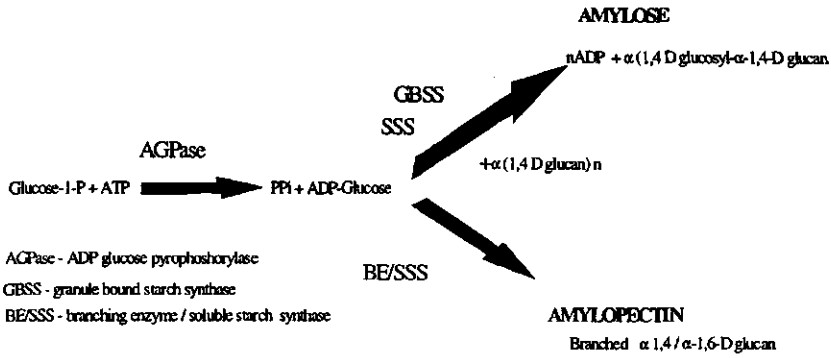
Isolation, characterisation and developmental regulation of granule bound starch synthase II from cassava (*Manihot esculenta* Crantz)

Abstract

A 2708 bp cDNA clone coding for granule bound starch synthase II (GBSSII) was isolated from a cassava tuber specific cDNA library. GBSSII encodes a 751 amino acid polypeptide that showed homology to potato (61%) and pea (59%) granule bound starch synthase II (GBSSII). The derived amino acid sequence of this cassava GBSSII exhibited low sequence homology to cassava GBSSI (35% identity). There was an N-terminal domain of 203 amino acids in cassava GBSSII which was similar in size to that found in pea and potato and thus was characteristic of GBSSII polypeptides. Southern analysis of cassava genomic DNA as well as segregation analysis of a cross between the cassava cultivars TMS 30572 and CM 2177-2 revealed that GBSSII was a single copy gene that was localised on linkage group T of the male derived cassava genetic map. Cassava GBSSII was highly expressed in young leaves, and exhibited much lower expression in developing tubers. These results are indicative of the differing and complementary role to GBSSI that GBSSII plays in leaf and tuber starch production.

Introduction

In general plant starch consists of 20% α 1,4 linked glucan chains termed amylose and of 80% amylopectin, a polymer composed of α 1,4 and α 1,6 linked glucan chains. The generally accepted pathway for starch biosynthesis in higher plants and algae involves the following reactions and enzymes.



The critical role of AGPase and the correlation between AGPase activity, ADP-glucose formation and ultimately starch synthesis has been established for several plant species (Preiss et al., 1982). This lends credence to the above pathway being the predominant route by which starch is synthesised in higher organisms.

Starch synthases are involved in extending glucan chains. Maize, rice, sorghum *waxy* mutants and the amylose free mutant (*amf*) of potato have reduced starch synthase activity accompanied by appreciably reduced amylose levels in their starch (Echt and Schwartz, 1981; Sano, 1984; Hseih, 1988; Jacobsen et al., 1989). These mutants lack a protein of approximately 60 kDa which is present in the wildtype or *Waxy* plants. This starch synthase was shown to be largely bound to the starch granule and was termed granule bound starch synthase (GBSS) in contrast to soluble starch synthases which are present in the stroma of the amylopast. Little or no change in total starch content in *waxy* compared to *Waxy* grains was observed thus affixing the role of GBSS to amylose formation and less to amylopectin synthesis (Shannon and Garwood, 1984). Using this protein, cDNA clones have been isolated from several plant species including potato and cassava (Salehuzzaman et al., 1993). The primary sequences of GBSS are highly conserved in all species examined thus far (Salehuzzaman et al., 1993). The role of GBSS in amylose synthesis has been demonstrated further by transforming potato with an

antisense GBSS gene from potato and cassava. Some of the transgenic plants produced had no starch synthase activity and the tubers contained amylose free starch. (Visser et al., 1991, Salehuzzaman et al., 1993).

Studies carried out on *Chlamydomonas* GBSS mutants showed that in addition to lacking amylose they lack a particular amylopectin fraction thereby indicating that GBSS may also be directly or indirectly involved in amylopectin synthesis (Delrue et al., 1992). It was further established that whilst GBSS was important in certain tissues such as endosperm, pollen, embryo sac etc, the waxy mutation did not reduce amylose levels in other parts of the maize plant or at different developmental stages (Echt and Schwartz, 1981). The existence of several isoforms of granule bound starch synthase was thus postulated. These starch synthase isoforms have been found in maize; with 4 GBSS and 2 SS polypeptides (Macdonald and Preiss, 1985), potato and developing pea embryos with a 60 kDa (GBSSI) and 77 kDa GBSSII polypeptide (Dry et al., 1992). The 77 kDa GBSS polypeptide, termed GBSSII, is highly active in developing pea embryos and has been shown also to be active in both the soluble phase and granule bound in pea and potato. The GBSSII of potato plays a relatively minor role in starch synthesis and is thought to contribute only 15% of the total amylose content (Edwards et al., 1995) but to 60-70% of the synthase activity of pea embryos (Denyer and Smith, 1992).

Now that the GBSSII isoform of pea and potato has been shown to be present in the granule bound and soluble phases, the distinction between the two categories of starch synthases (SS) has become less profound. It has been suggested that the soluble starch synthase (SSS) may actually become bound to starch granules due to entrapment within the growing starch granule (Mu et al., 1994). Within the starch granule SS would then make predominantly amylose whilst in the soluble phase it predominantly makes glucan chains which are more readily acted upon by branching enzyme (BE). However the evidence for this view is largely circumstantial. What remains clear is that there are several isoforms of SS within a plant. These are active in both the granule bound and soluble phases. The extent to which each SS isoform participates in starch synthesis is largely dependent on the tissue and stage of development of the plant.

This study was carried out to determine if there were any GBSS isoforms in the tropical crop cassava (*Manihot esculenta* Crantz). Analysis of the primary sequence of a putative GBSSII of cassava as well characterisation of the expression of the gene during development was carried out.

Materials and Methods

Screening of a cassava cDNA library

Approximately $3{,}0x10^5$ plaque forming units of a lambda gt11 cassava tuber specific cDNA library were screened with a 1.5 kb fragment of the potato GBSSII cDNA (kindly provided by Dr. C. Martin, John Innes Institute, Norwich U.K.). The probe was labelled with [32P] dCTP (2'-deoxycytidine 5'triphosphate) by the random primer labelling technique (Feinberg and Vogelstein, 1983). The blots were hybridised overnight at 60°C and washed three times with 2xSSPE (3M NaCl, 0.2 M Sodium phosphate) and 0.1% Sodium dodecyl sulphate (SDS) at 60°C for 30 minutes each time. Autoradiography was carried out at -80°C within intensifying screens. The positive cDNA was isolated and cloned into the KpnI site of pUC18.

Sequencing and Sequence analysis

The clones and subclones of the putative GBSSII cassava cDNAs (in pUC18) were sequenced using the dideoxy method of Sanger et al. (1977). The analysis and manipulation of the sequences was carried out using the PC-Gene programme (Intelligenetics, Mountain View, CA, U.S.A). Homology searches to sequences in data banks was carried out using the blast programme (Altschul et al., 1990) on the WWW site of the NCBI (http://www.ncbi.nlm.nih.gov).

Plants and bacteria

The Latin American cassava genotype M.col 22 was grown in the greenhouse at 25-35°C. Leaves and tubers at various developmental stages were harvested, frozen in liquid nitrogen and stored at -80 °C for later use. The *E.coli* strain DH5α (Bethesda Research Laboratories) was cultured according to standard protocols (Sambrook et al., 1989)

Southern Hybridisation

Genomic DNA isolation from cassava was carried out using the method of Dellaporta et al. (1983). The DNA was digested with various enzymes, electrophoresed on 0.8% agarose gels and blotted on to nitrocellulose. Hybridisation with the isolated cassava

cDNA labelled with [32P] dCTP was carried out at 60°C for 16 hours. The filters were washed twice with 2X SSPE +0.1% SDS at 65°C. The blots were exposed to Kodak XOMAT-AR films between intensifying screens at -70°C for 2 days.

Localisation of the putative GBSSII on to the cassava genetic map

The cassava mapping population was described by Fregene et al. (1997). It consisted of 150 F_1 plants from an interspecific cross between TMS 30572 (the female parent), an elite cultivar tolerant to african cassava mosaic disease, and CM 2177-2 (the male parent) which had tolerance to bacterial blight and good cooking qualities. Localisation of the GBSSII gene on to the cassava genetic map was carried out using the computer package MAPMAKER (Lander et al., 1987) and linkage positions were assigned as described by Fregene et al. (1997).

Northern Hybridisation

RNA preparation and northern blot analysis was performed according to the protocol of Visser et al. (1989). Some 50µg of total RNA were used per lane. The RNA was then transferred to Hybond-N (RPN203N, Amersham, U.K.). Hybridisation and washing of filters was performed in the same way as described for southern blot analysis.

Starch isolation

Starch was isolated from greenhouse grown cassava tubers as described by Kuipers et al., (1994). Cassava tubers were washed, homogenised in extraction buffer (50 mM Tris pH 7.5, 10 mM EDTA, 2 mM $Na_2S_2O_5$, 1 mM DTT), and then filtered through synthetic cloth. The solution was allowed to stand at room temperature to allow the starch granules to settle. The starch was washed twice with extraction buffer and acetone, collected and dried at 4°C.

SDS PAGE and Immunoblotting

An amount of 20 mg finely ground cassava leaf or starch was boiled in 200µl of sample buffer (20mM Tris pH 8.0, 2 mM EDTA, 20% glycerol, 2% SDS and 10% 2-mercaptoethanol). The solubilised protein was electrophoresed on a 10% SDS polyacrylamide gel, and then blotted on to a nitrocellulose membrane. The blot was then analysed immunochemically as described by Hovenkamp-Hermelink et al. (1987) using

antisera raised against potato GBSSI and GBSSII (Vos Scheperkeuter et al., 1986; Edwards et al., 1995).

Results and Discussion

Sequence of GBSSII

Screening of a cassava tuber specific cDNA library, using a potato GBSSII cDNA, resulted in one positive clone being isolated, after three rounds of screening and amplification. The cDNA hybridised strongly with the potato GBSSII cDNA under stringent conditions. It was cloned into the KpnI site of pUC18, and characterised by restriction analysis (Fig.1a). The entire cassava GBSSII cDNA was sequenced.

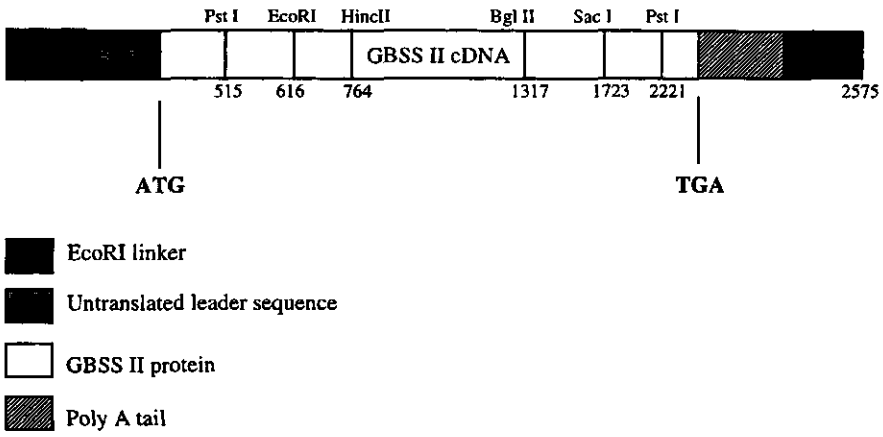


Fig 1a. Restriction map of the cassava GBSSII cDNA

This clone of about 2.7 kb proved to be full length: there is an ATG start codon at position 150 and a TGA stop codon at position 2303 on the nucleotide sequence of cassava GBSSII. Several putative polyadenylation signals AATAAA (Joshi, 1987) are present at positions 2264 and 2348 upstream of the poly A tail at the 3'end (Fig. 1b). Two putative hairpin loops centred at position 1318 and 1092 are predicted. In addition there are 9 inverted repeats of minimum length 10 bp. Two of the repeats have palindromic sequences and one spans the cDNA being set at position 80 (ATCCAAACCA) and also at position 2502 (TGGTTTGGA) [Fig. 1b]

```
   1 GTTTGATGAA CCCCATCTCA AGCTGTTGGG GCTGTCGCAC TTGTGAGAAA TGGCATTTAT
  61 AGGATCACTT CCTTTTATTA TCCAAACCAA AGCAGAAAGT TCTGTCCTTC TCCATGACAA
 121 AAACCTACAG CGATCCAGAT TCTCCGTTTT CCCATGTAGA TCACAAAACT CTTTTAATTT
 181 AGCCGTTTCG TTATCTTTGA GTTTTAAGCC TGTAAGAGCT ACAGGTAAGG AAGGCGTTAG
 241 TGGTGATGGG TCAGAGGATA CACTTCAAGC CACCATCGAG AAAAGCAAGA AAGTTCTCGC
 301 CTTGCAAAGG GACCTACTTC AGAAGATTGC TGAAAGAAGG AAATTGGTTT CTTCTATACA
 361 AAGTAGTGTT GGTGACCACG ACACAAACAA AACTTCTCAT GAACAGAGGG AAAACTCTTT
 421 GGCAAATTCA GATAATACTT CAACTAGTGA TGTGAATATG CACCAACAGC AAAATGGCCC
 481 AGTTCTTCCG AGTAGCTATG TCCATTCAAC TGCAGATGAG GTATCAGAAA CTGCATCTTC
 541 AGCTATTAAT AGAGGTCATG CTAAAGATGA TAAGGAACTT GAACAACATG CATCTCCTAG
 601 AACAGCCTTT GTTAAGAATT CTACCAAACA GTTTAAAGAG ATGGATTCTG AGAAACTACA
 661 GACAGATGAG ATACCATCTT TTCTTTCAAA CACCACAGAT ATTTCCACTA TAAATGAAGA
 721 AAATAGTGAA CATTCAAATG AATCAACCTC ACCTATGGTC GACATTTTTG AAAGTGATTC
 781 TATGACTGAA GACATGAAGC CACCTCCTTT GGCTGGGGAC AATGTCATGA ATGTTATTTT
 841 GGTAGCTGCA GAATGTGCTC CATGGTCCAA AACAGGTGGC CTTGGTGATG TCGCTGGATC
 901 TTTACCAAAG GCTTTGGCTC GGCGTGGACA TCGGGTTATG GTTGTGGCAC CGCGATATGG
 961 CAACTATGTT GAACCTCAGG ATACTGGAGT CCGAAAGAGG TATAAGGTGG ATGGTCAGGA
1021 TTTTGAAGTA TCATACTTCC AAGCCTTCAT TGATGGGGTT GATTTTGTAT TCATTGACAG
1081 TCCTATGTTT CGCCACATAG GGAATGATAT ATATGGAGGA AACAGAATGG ATATATTAAA
1141 GAGGATGGTA TTATTTTGCA AAGCTGCTGT TGAGGTTCCT TGGCATGTCC CATGTGGTGG
1201 AGTCTGCTAT GGGGATGGAA ATTTGGCTTT CATTGCAAAT GATTGGCATA CAGCATTGTT
1261 GCCAGTGTAT CTGAAGGCAT ATTATCGGGA TAATGGTTTA ATGCAATATA CAAGATCTGT
1321 TCTTGTAATT CATAACATAG CTCACCAGGG TCGGGGTCCC AGTGGAGATT TCTCTTACGT
1381 GGGTCTACCA GAACATTACA TTGATCTCTT CAAACTGCAT GATCCGATTG GTGGTGACCA
1441 CTTCAATATC TTTGCACCTG GTCTTAAGGT GGCAGATCGT GTGGTTACTG TTAGTCATGG
1501 ATACGCCTGG GAGCTTAAAA CATCTGAAGG TGGTTGGGGT CTTCACAATA TCATAAATGA
1561 GAACCACTGG AAATTGCAGG GCATTGTTAA TGGGATTGAT GCCAAAGAAT GGAATCCACA
1621 GTTTGATATT CAACTGACAT CAGATGGTTA TACTAACTAT TCCCTGGAAA CACTTGATAC
1681 TGGCAAGCCT CAGTGCAAGA CAGCCTTACA GAACGAGCTC CGGTTTGCCA TCCCCCCAGA
1741 TGTCCCTGTT ATTGGGTTCA TTGGAAGGTT GGATTATCAG AAAGGTGTCG ATCTCATAGC
1801 TGAGGCAATT CCCTGGATGG TGGGTCAGGA TGTGCAACTA GTAATGTTGG GTACTGGCAG
1861 ACAAGACTTG GAAGAGATGC TTAGACAATT TGAAAACCAA CATAGAGATA AAGTGAGGGG
1921 ATGGGTTGGT TTTTCTGTGA AGACAGCTCA CAGGATAACT GCTGGTGCAG ATATTTTGCT
1981 CATGCCATCA AGATTTGAAC CATGTGGGCT AAACCAGTTA TATGCTATGA TGTACGGGAC
2041 GATTCCTGTA GTACACGCTG TGGGTGGACT AAGGGACACG GTGCAACCTT TCGATCCATT
2101 TAATGAGTCG GGGCTTGGGT GGACATTTGA TAGCGCTGAA TCACATAAAC TGATACATGC
2161 ATTAGGCAAT TGCTTGCTCA CTTACCGAGA GTACAAGAAG AGCTGGGAAG GCCTGCAGAG
2221 AAGAGGGATG ACTCCAAACC TCAGCTGGGA CCATGCTGCT GAGAAATATG AGGAGACTCT
2281 TGTTGCAGCC AAGTACCAGT GGTGAGCAAT AGTGCTTTTC CTTAAATTTT GACTTTTTTT
2341 TTTCTGTTAA ATAATGCTTC CAAGAGGTTG CCCTCTGATG CTAGTAAGGG GGCCAATAGA
2401 TGGCCATGTA TGTTCTGCTA TATAGCTCAA TGTGTAATCA GCTTAGAGTT ATGATTCAGG
2461 AGTTGTAATC CTTTCTGGTT TTATCACACA TTAGCAGAGG ATGGTTTGGA TGTGATGAGT
2521 GGTGACCCAT GTTTTATCCT TGTGCTTATT CTGTTATCTG AAAAAAAAAA AAAAA

DNA sequence composition:    739 A;   477 C;   614 G;   745 T;
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Fig. 1b. The complete nucleotide sequence of the cassava GBSSII cDNA. The translational start codon (ATG) and stop codon (TGA) are shown in bold. The putative polyadenylational signals AATAAA are underlined. One inverted repeat (situated at position 80 and 2502) which is also palindromic is highlighted in bold and italics.

Properties of the cassava GBSSII protein

The cassava GBSSII cDNA has an open reading frame of 751 amino acids. The protein has a calculated molar mass of 83.7 kDa. It has a net negative charge at pH 7.0 and is hydrophobic. Cassava GBSSII has a largely hydrophilic and serine rich N-terminal region. A transit peptide (TP) which is thought to target the enzyme to the amyloplast is predicted in position 1-57 of cassava GBSSII according to the rules of Gavel and von Heijne (1990). This is slightly smaller than the 78 amino acid GBSSI TP which has a cleavage site predicted as IVC/G (Salehuzzaman et al., 1993) whereas that of GBSSII is predicted as being PVR/A. The mature protein is thus predicted to be 694 amino acids long with a calculated molar mass of 77.5 kDa.

There is a 203 amino acid N-terminal region of GBSSII which forms part of the mature protein. This domain is effectively the reason why GBSSII is much larger than GBSSI of cassava. The N-terminal domain of cassava GBSSII shares only 10% homology with that of the GBSSI N-terminal region. Significantly similar N-terminal extensions have been found in pea and potato GBSSII (Dry et al., 1992; Edwards et al., 1995) marking this out as a feature distinguishing these GBSS isoforms. The N-terminal region of cassava GBSSII exhibits low homology to the N-terminal domain of potato GBSSII (28.5%) and of pea GBSSII (22.6%) [Fig. 2]. This homology is largely due to the high proportion of the serine amino acids which makes this domain highly flexible. Within the cassava GBSSII N-terminal domain there is a 210 amino acid ORF that is in a different frame from the mature protein. Its significance is unclear at present.

At the C-terminal of this domain there is a region of three consecutive prolines (PPP) which Martin and Smith (1995) have termed the turn in the "flexible arm" of GBSSII. Expression of pea GBSSII in *E. coli* without the novel N-terminal domain resulted in a fully active enzyme indicating that the flexible arm is not involved in the catalytic mechanism of the enzyme (Martin and Smith, 1995). Whilst the role of this novel N-terminal domain is still unclear some workers have suggested that it may be involved in physical association of GBSSII with starch branching enzyme. It has been also suggested that this region may be important in the partitioning of the enzyme between the soluble and granule bound phases. However, analysis of the primary sequence of the N-terminal region of GBSSII does not readily reveal these functions. More research is required to determine the significance of the N-terminal domain.

```
CASGB2T     ATGK---EGVSGDGSEDTL-QATIEKSKKVLALQRDLLQKIAERRKLVSS    46
POTGB2T     ATGENSGEAASADESNDAL-QVTIEKSKKVLAMQQDLLQQIAERRKVVSS    49
PEAGB2T     AVGKSFGADENGDGSEDDVVNATIEKSKRFLLCKGNLFNRLLKERNLVSS    50
            *.*.   .. ..*.*.*.. ..****** .  *   . .*.... . *..***

CASGB2T     IQS-SVGDHDTNKTSHEQRENSLANSD-------------NTSTSDVNMH    82
POTGB2T     IKS-SLAN---AKGTYDGGSGSLSDVDIPDVDKDYNVTVPSTAATGITDV    95
PEAGB2T     IDSDSIPGLEGNGVSYESSEKSLSRDSNPQ-----------KGLPAAAVL    89
            *.* *. .   .  ...  . **.  .                .. .. .

CASGB2T     QQQNGPVLPSSYVHSTAD---EVSETASSAINRGHAKDDKELEQHASPRT   129
POTGB2T     DKNTPPAISHDFVESKREIKRDLADERAPPLSRSSITASSQISSTVSSKR   145
PEAGB2T     LKPNGGTVSFNYVRSKETETWAVS---SVGINQGFDEIEKK---------   127
             . .  ... ..* *. .   ...   .  ....  . ...

CASGB2T     AF------VKNSTKQFKEMDSEK----LQTDEIPSFLSN-TTDISTINEE   168
POTGB2T     TLNVPPETPKSSQETLLDVNSRKSLVDVPGKKIQSYMPSLRKESSASHVE   195
PEAGB2T     -----NDAVKASSKLHFNEQIKNKLYERPDTKDIS--SSIRT--SSLKFE   168
                     *.* .   . . .    ....  *  ..  .  *. . *

CASGB2T     NSEHSNESTSPMVDIFESDSMTEDMKPPPLAGDNV-   203
POTGB2T     QRNENLEGSSAEANEETEDPVNIDEKPPPLAGTNV-   230
PEAGB2T     NFEGANEPSSKEVANEAENFESGGEKPPPLAGTNV-   203
            . . . * .*  ..  ...  . . *******.**
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Fig. 2. Alignment of the N-terminal domains of the GBSSII isoforms of cassava (CASGB2T), potato (POTGB2T) and pea (PEAGB2T). Amino acids perfectly conserved in all three sequences are indicated by '*' whilst those which are well conserved in two of the sequences are indicated by '.' The prolines PPP which form the hook of the flexible region are highlighted in bold. Sequence data are from: CASGBT2 (present work); POTGB2T (Edwards et al., 1995); PEAGB2 (Dry et al., 1992).

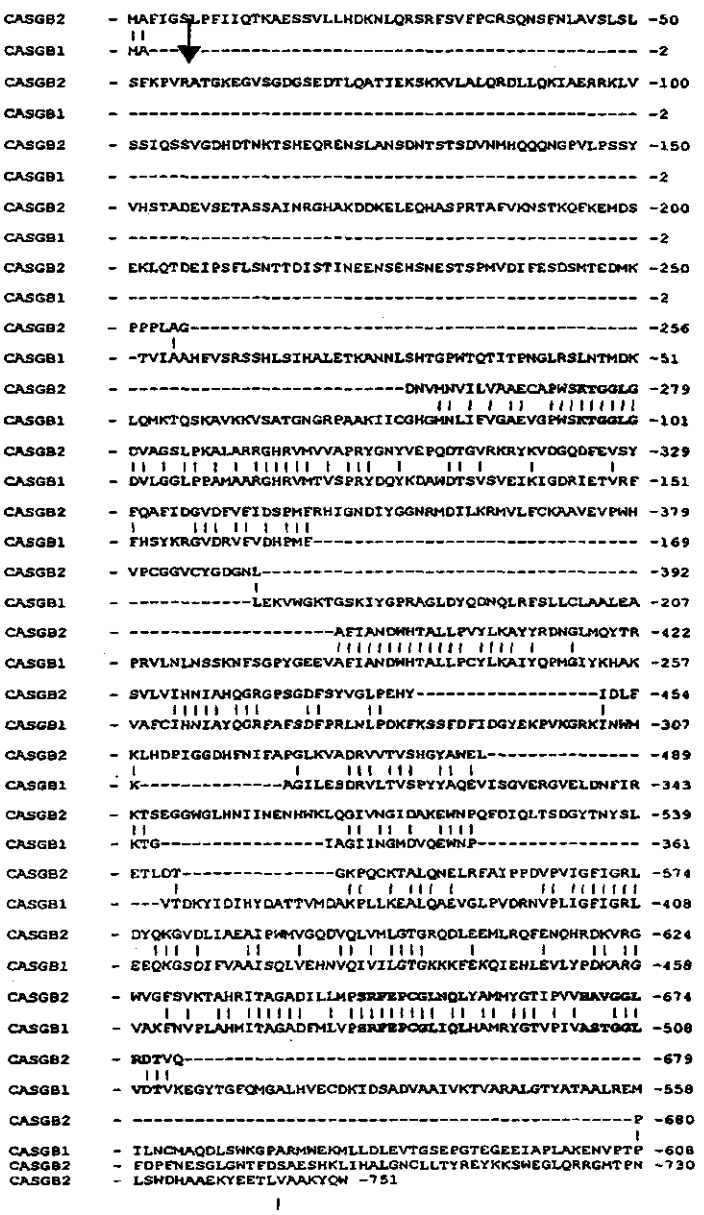

Fig 3: Amino acid sequence alignment of cassava granule bound starch synthase II of cassava (CASGB2) with cassava GBSSI (CASGB1). The cleavage site motif (PVRA) for cassava GBSSII is shown and the splice site is indicated by an arrow. The conserved regions encompassing the KTGGL ADP-glucose binding site motif (Furukawa et al., 1990) BOX I; conserved domains SRFEPCGLXQL (BOX II) and XXGGLXDT (BOX III) are shown in bold. Source of CASGB1 (Salehuzzaman et al., 1993).

```
CASGB2            MAFIGSLPFIIQTKAESSVL-LHDKNL-QRSRFSVFPCRSQNSFNLAVSL    48
POTGB2            M---------------ENSIL-LHSGNQ-FHPNLPLLALRPKK-LSLIHGS    33
PEAGB2            M--------MLSLGSDATVLPFHAKNLKFTPKLSTL--NGDLAFSKGLGV    40
                  *               ....* .*. *     .... .  . .  ..   .
                                    ↓

CASGB2            S----------LSFKPVRATGK--EGVSGDGSEDTL-QATIEKSKKVLAL   85
POTGB2            SR-----EQMWRNQRVKATGENSGEAASADESNDAL-QVTIEKSKKVLAM    77
PEAGB2            GRLNCGSVRLNHHKQHVRAVGKSFGADENGDGSEDDVVNATIEKSKRFLLC    90
                  .             ..*.*.*.    .. ..*.*.*.. ..******. *
CASGB2            QRDLLQKIAERRKLVSSIQS-SVGDHDTNKTSHEQRENSLANSD------  128
POTGB2            QQDLLQQIAERRKVVSSIKS-SLAN---AKGTYDGGSGSLSDVDIPDVDK  123
PEAGB2            KGNLFNRLLKERNLVSSIDSDSIPGLEGNGVSYESSEKSLSRDSNPQ---  137
                  . .*.... . *..****.* *. .   .  ...  . **.   .
CASGB2            --------NTSTSDVNMHQQQNGPVLPSSYVHSTAD---EVSETASSAINR  168
POTGB2            DYNVTVPSTAATGITDVDKNTPPAISHDFVESKREIKRDLADERAPPLSR  173
PEAGB2            ---------KGLPAAAVLLKPNGGTVSFNYVRSKETETWAVS---SVGINQ  176
                            .. .. .   .  . ... ..* *. .   ...   .  ...
CASGB2            GHAKDDKELEQHASPRTAF------VKNSTKQFKEMDSEK----LQTDEI  208
POTGB2            SSITASSQISSTVSSKRTLNVPPETPKSSQETLLDVNSRKSLVDVPGKKI  223
PEAGB2            GFDEIEKK---------------NDAVKASSKLHFNEQIKNKLYERPDTKD  212
                                          *.*  .   .  .  .      ....
                  .  .  ...
CASGB2            PSFLSN-TTDISTINEENSEHSNESTSPMVDIFESDSMTEDMKPPPLAGD  257
POTGB2            QSYMPSLRKESSASHVEQRNENLEGSSAEANEETEDPVNIDEKPPPLAGT  273
PEAGB2            IS--SSIRT--SSLKFENFEGANEPSSKEVANEAENFESGGEKPPPLAGT  258
                   *   ..  .  *. . *.  . .  * .*   ..  ...  . . *******.
                                   BOX I
CASGB2            NVMNVILVAAECAPWSKTGGLGDVAGSLPKALARRGHRVMVVAPRYGNYV  307
POTGB2            NVMNIILVASECAPWSKTGGLGDVAGALPKALARRGHRVMVVAPRYDNYP  323
PEAGB2            NVNMIILVSAECAPWSKTGGLGDVAGSLPKALARRGHRVMIVAPHYGNYA  308
                  **   .***..*****************.*************.***.*.**
CASGB2            EPQDTGVRKRYKVDGQDFEVSYFQAFIDGVDFVFIDSPMFRHIGNDIYGG  357
POTGB2            EPQDSGVRKIYKVDGQDVDVTYFQALLMDCDFVFIHSHMFRHIGNNIYGG  373
PEAGB2            EAHDIGVRKRYKVAGQDMEVTYFHTYIDGVDIVFIDSPIFRNLESNIYGG  358
                  *..* **** ***.*** .*.**.. . .  *.***.*..**.....****
CASGB2            NRMDILKRMVLFCKAAVEVPWHVPCGGVCYGDGNLAFIANDWHTALLPVY  407
POTGB2            NRVDILKRMVLFCKAAIEVPWHVPCGGVCYGDGNLVFIANDWHTALLPAY  423
PEAGB2            NRLDILRRMVLFCKAAVEVPWHVPCGGICYGDGNLVFIANDWHTALLPVY  408
                  **.***.*********.**********.*******.************.*
CASGB2            LKAYYRDNGLMQYTRSVLVIHNIAHQGRGPSGDFSYVGLPEHYIDLFKLH  457
POTGB2            LKAYYRDNGIMNYTRSVLVIHNIAHQGRGPLEDFSYVDLPPHYMDPFKLY  473
PEAGB2            LKAYYRDHGLMNYTRSVLVIHNIAHQGRGPVEDFNTVDLSGNYLDLFKMY  458
                  *******.*.*.*****************  .**. *.*.  .*.* **..
CASGB2            DPIGGDHFNIFAPGLKVADRVVTVSHGYAWELKTSEGGWGLHNIINENHW  507
POTGB2            DPVGGEHFNIFAAGLKTADRVVTVSHGYSWELKTSQGGWGLHQIINENDW  523
PEAGB2            DPVGGEHFNIFAAGLKTADRIVTVSHGYAWELKTSEGGWGLHNIINESDW  508
                  **.**.******.***.***.*******.******.******.****..*
CASGB2            KLQGIVNGIDAKEWNPQFDIQLT-SDGYTNYSLETLDTGKPQCKTALQNE  556
POTGB2            KLQGIVNGIDTKEWNPELDVHLPRSDGYMNYSLDTLQTGKPQCKAALQKE  573
PEAGB2            KFRGIVNGVDTKDWNPQFDAYLT-SDGYTNYNLKTLQTGKRQCKAALQRE  557
                  *..*****.*.*.***..*  *. **** **.*.**.***.***.***.*
CASGB2            LRFAIPPDVPVIGFIGRLDYQKGVDLIAEAIPWMVGQDVQLVMLGTGRQD  606
POTGB2            LGLPVRDDVPLIGFIGRLDPQKGVDLIAEAVPWMMGQDVQLVMLGTGRRD  623
PEAGB2            LGLPVREDVPIISFIGRLDHQKGVDLIAEAIPWMMSHDVQLVMLGTGRED  607
                  *  .... ***.*.****** **********.***...*********** *
                                                           BOX II
CASGB2            LEEMLRQFENQHRDKVRGWVGFSVKTAHRITAGADILLMPSRFEPCGLNQ  656
POTGB2            LEQMLRQFECQHNDKIRGWVGFSVKTSHRITAGADILLMPSRFEPCALNQ  673
PEAGB2            LEQMLKEFEAQHCDKIRSWVGFSVKMAHRITAGSDILLMPSRFEPCGLNQ  657
                  **.**..** ** **.*.*******  .******.************.***
                                 BOX II1
CASGB2            LYAMMYGTIPVVHAVGGLRDTVQPFDPFNESGLGWTFDSAESHKLIHALG  706
POTGB2            LYAMKYGTIPVVHAVGGLRDTVQPFDPLMSQDWGGPSDRAEASQLIPRIR  723
PEAGB2            LYAMSYGTVPVVHGVGGLRDTVQPFNPFDESGVGWTFDRAEANKLMAALW  707
                  **** ***.****.************.*. . . *  . *.**. .*.   .
CASGB2            NCLLTYREYKKSWEGLQRRGMTPNLSWDHAAEKYEETLVAAKYQW  751
POTGB2            NCLLTYREYKKSWEGIQTRCMTQDLSWDNAAQNYEEVLIAAKYQW  768
PEAGB2            NCLLTYKDYKKSWEGIQERGMSQDLSWDNAAQQYEEVLVAAKYQW  752
                  ******..*******.* * *...****.**..***.*.******
```

Cassava GBSSII is more similar to other GBSSII sequences than to GBSSI of cassava

Comparison of the derived amino acid sequence of cassava GBSSII to that of cassava GBSSI revealed an overall 35% amino acid identity (Fig. 3). This is low compared with the identity of cassava GBSSI to potato GBSSI of 74% (Salehuzzaman et al., 1993). At the same time cassava GBSSII, minus the N-terminal region, exhibits increased amino acid sequence homology with other cloned GBSSII cDNAs from pea (79% identity) and potato (80% identity) [Fig. 4].

A dendrogram was produced after comparison of cassava GBSSII with isoforms of GBSS in potato, pea, cassava, and *E.coli*. The GBSSII sequences fall, because of their high similarity, into a distinct group separate from that formed by the GBSSI type and also one which encompasses the prokaryotic glycogen synthase genes as illustrated by *Escherichia coli* glg A (Fig. 5). It is also noteworthy to mention that potato soluble starch synthase 3 (POTSSS3) exhibits greater homology to GBSSII sequences when only the region from position 780 to 1230 of the polypetide is used in alignments. This may indicate that one of the ways starch synthases may have evolved is by introgression of additional sequences within the N-terminal region whilst maintaining the basic sequences required for starch synthase activity.

Thus despite the rather low homology between GBSSII and GBSSI certain domains which may play a critical role in the catalytic and allosteric activity of the enzyme are conserved. These include the glycine rich Box I encompassing the **KTGGL** ADP-glucose binding site (Furukawa et al., 1990). In addition other highly conserved regions denoted as BOX II (SRFEPCGLXQL) in which there is 90% identity and Box III (XXGGLXDT) with 75% homology, were identified (Fig. 3). Such regions and other

Fig. 4 Alignment of the granule bound starch synthase sequences from pea (PEAGB2), potato (POTGB2) and cassava (CASGB2). Conserved regions (BOXI, BOX II and BOX III) are indicated as for Fig. 3. The putative splice site of the transit peptide of cassava GBSSII is indicated by an arrow.

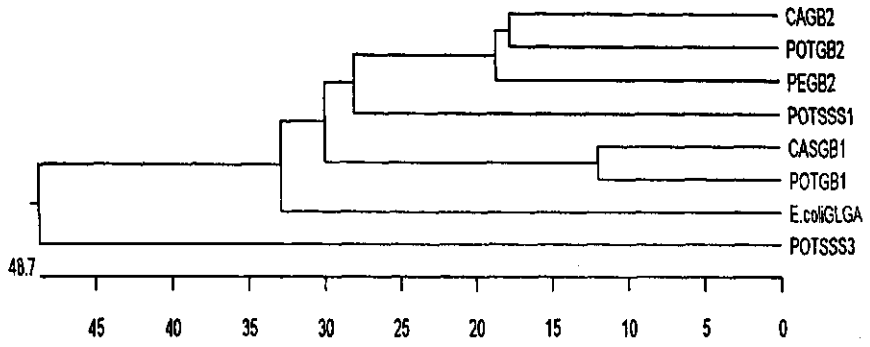

Fig. 5 Phylogenetic tree derived from alignment of starch synthases from cassava (CA), potato (POT), pea (PE) and *Escherichia coli* (*glg A*). The isoforms are denoted as GB1 (GBSSI) and GB2 (GBSS2). For potato soluble starch synthase the isoforms are SSSI and SSS3. Sequence data are from the following sources CAGB2, present work; CASGB1, Salehuzzaman et al., 1993; POTGB1, Visser et al., 1989; POTGB2, Edwards et al., 1995; *E.coli* GLGA, Kumar et al., 1989; POTSS3 and POTSSS1, Abel, 1995; PEGB2, Dry et al., 1992).

conserved single amino acid positions indicate their importance to the overall structure of the starch synthase protein in relation to its function in starch polymerisation.

Southern analysis and localisation of GBSSII on to the genetic map of cassava

Southern analysis of allotetraploid cassava revealed that GBSSII is a low copy number gene (Fig. 6a). This is a situation which is common amongst the known starch genes in cassava (Salehuzzaman et al., 1993; Munyikwa et al., 1997). Linkage analysis of a segregating population of 150 F_1 cassava plants enabled the GBSSII gene to be localised on to the male derived linkage group T (Fig. 7a and Fig 7b). Although the genetic map of cassava is still under construction, currently consisting of 20 non-overlapping male and female linkage groups instead of the 9 linkage groups expected (Fregene et al., 1997), the map position of cassava GBSSII indicates that this gene is localised on one linkage group and not dispersed throughout the genome. This is in contrast to the small subunit genes of ADP-glucose pyrophosphorylase which appear to be localised on at least two positions

within the cassava genetic map (Fregene personal communication). Thus on the basis of

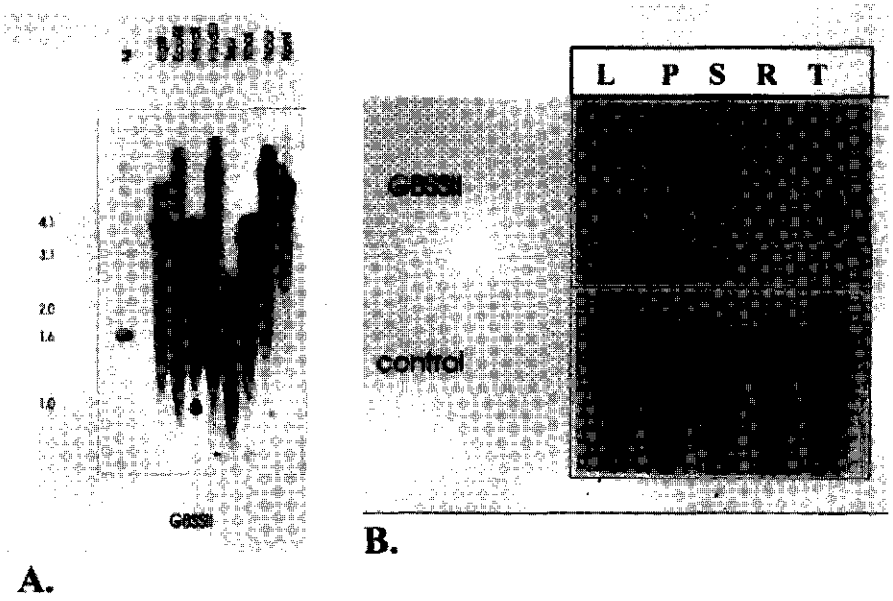

Fig 6A: Southern blot analysis of cassava DNA. 10 μg per of cassava DNA per lane was digested with the enzyme indicated, electrophoresed and probed with the 2.5 kb putative cassava GBSSII cDNA. Molecular weight markers are indicated in kb on the left.

Fig 6B: Organ specific expression pattern of cassava GBSSII. The lanes are: L, leaves; P, petioles; S, stems; R, roots; T, tubers. For each tissue type 40mg of RNA was electrophoresed and probed with the putative cassava GBSSII cDNA labelled with [32P] dCTP.

the simple pattern of restriction fragments and the simple segregating pattern in the offspring it can be concluded that GBSSII is a single copy gene on the male linkage group T.

Localisation of GBSSII and other starch genes will aid in the construction of a genetic map for cassava and enable breeders to be able to monitor transfer of traits associated with starch quality and quantity.

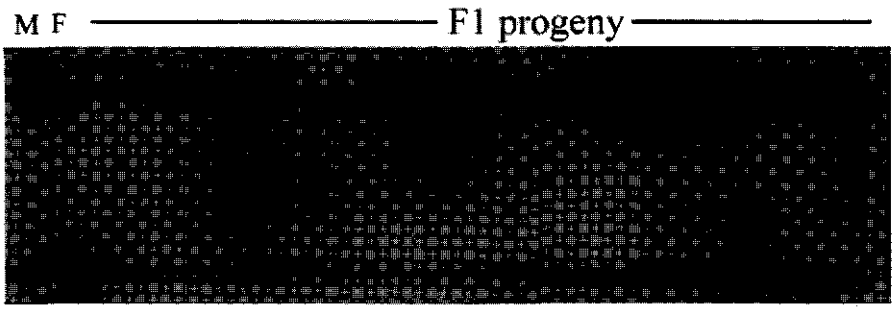

Fig 7a

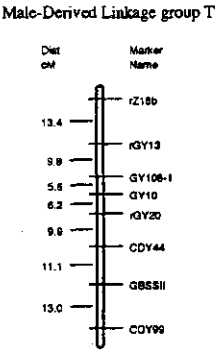

Fig. 7b

Fig. 7a Southern hybridisation of Hind III digests of parental lines and mapping population probed with cassava GBSSII. F, female parent (TMS 30572), M, male parent (CM 2177-2). An amount 10μg of genomic DNA was used per lane. The probe used was the 2.5 kb cassava GBSII cDNA. Only 28 out of a total 150 F1plants probed are shown in this panel.

Fig. 7b Localisation of cassava GBSSII on the male derived genetic map of cassava. The other markers shown are RFLP markers (CDY; cDNA, GY genomic, and the RAPD marker denoted as rZ18b).

Tissue specific expression of GBSSII

GBSSII is highly expressed in leaf tissue while expression in other tissues is low (Fig. 6b). This is different from the expression of cassava GBSSI which is most prominent in tubers and low in leaves (Salehuzzaman et al., 1993).

Developmental Expression of Cassava Granule Bound Starch Synthases

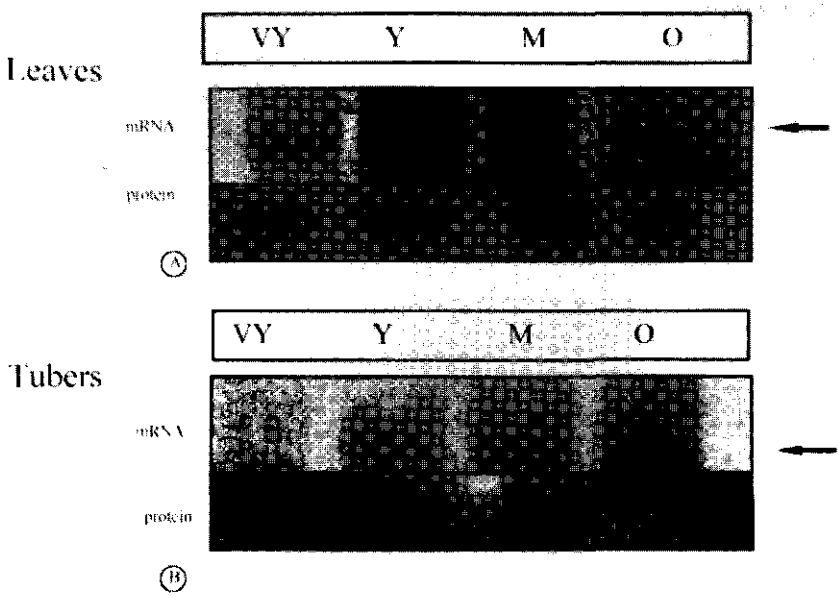

Fig. 8: Expression of cassava GBSSII in tissues at different developmental stages. Arrows indicate the position of the GBSSII mRNA. VY = very young : leaves (1cm) and tubers (1 cm diameter) Y = young: leaves (1 to 6 cm long) and tubers (1-3.0 cm diameter); M = middle : leaves (7 to 12 cm long) and tubers (3.5 to 5.0 cm diameter); O = old leaves (> 12 cm) and tubers (>5.0 cm in diameter)

Expression in leaves

Developmental northern analysis of total cassava leaf RNA revealed a GBSSII transcript of about 3.0 kb. Cassava GBSSII exhibits high expression in leaves with the highest level of expression being in source leaves and lowest in sink leaves (Fig. 8a). However, the pattern of expression was not closely matched by the polypeptide level as the amount of protein was approximately the same at all stages of leaf development.

While there was no discernible RNA transcript in leaves for GBSSI, a clear antigenic reaction was observed on western blots probed with GBSSI antibody (results not shown). This may indicate very low GBSSI expression in leaves that would require a more sensitive technique than total RNA northern blotting to detect it.

Expression in tubers

GBSSII mRNA starts to accumulate in the very young tubers and is expressed to a similar level in sink tubers. Similarly the pattern of polypeptide accumulation mimicked the transcript levels (Fig. 8b). GBSSI exhibits high constant expression in tubers at different developmental stages. At the polypeptide level the amount of protein produced in these different categories is approximately the same (Salehuzzaman et al., 1993).

Based on the results above it would seem that there are differing roles for cassava GBSSII and cassava GBSSI in tuber and leaf amylose production. The higher expression of GBSSII in source leaves would indicate a prominent role for the enzyme in this tissue, whereas GBSSI would seem to be more important in sink tubers where it is highly expressed with high enzyme activity (Salehuzzaman et al., 1993). In pea it was observed that GBSSII is expressed in all organs, but most abundantly early in embryo development and in roots (Dry et al., 1992). Whereas potato GBSSII is present throughout the development of the tuber, it only accounts for a maximum of 15% of the total starch synthase activity in this tissue (Edwards et al., 1995). Thus for cassava in addition to being expressed early on during development in leaves, GBSSII would seem to have a complementary role to GBSSI the predominant amylose producer in tubers.

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Isolation and characterisation of cDNAs encoding the large and small subunits of ADP-glucose pyrophosphorylase from cassava (*Manihot esculenta* Crantz)

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Isolation and characterisation of cDNAs encoding the large and small subunits of ADP-glucose pyrophosphorylase from cassava (*Manihot esculenta* Crantz)

Abstract

Screening of a tuber specific cassava cDNA library resulted in the isolation of full length cDNA clones designated AGPase B and AGPase S. The two clones exhibited 35% and 54% homology with each other at nucleotide and amino acid sequence levels, respectively. AGPase B has high homology (74-84%) with previously cloned small AGPase subunit polypeptides. AGPase S also exhibits high amino acid sequence homology to polypeptides from genes encoding the large subunit of AGPase enzymes in other plant species. Analysis of the genomic DNA of allotetraploid cassava revealed that AGPase B and AGPase S are low copy genes and originate from different loci in the cassava genome. Segregation analysis of a cross between the cassava cultivars TMS 30572 and CM 2177-2 revealed that AGPase S is a single copy gene that is localised on the female derived linkage group E of the cassava genetic map. The two genes are expressed in all cassava tissues but AGPase B exhibits a higher steady state mRNA level than AGPase S and is highly expressed in leaf and tuber tissue. Post-transcriptional control of small subunit polypeptide levels could be inferred from the discrepancy between AGPase B mRNA and polypeptide levels. The AGPase enzyme activity was much higher in young cassava leaves as compared to older leaves and tubers. Cassava leaf AGPase activity was increased 3 fold by the addition of 3-PGA and inhibited by up to 90% in the presence of inorganic phosphate (Pi). The tuber enzyme was relatively unaffected by 3PGA but was highly inhibited by Pi.

Introduction

Glycogen and starch are important storage compounds in bacteria and plants, respectively. These polysaccharides share important similarities in the way in which they are synthesised and in their structure. Basically, they are α-1,4 glucan compounds with varying degrees of branching, and have highly analogous enzymes involved in their synthesis namely ADP glucose pyrophosphorylase (AGPase), glycogen or starch synthases and branching enzyme (Okita, 1993).

AGPase (AGPase; EC 2.7.7.27) catalyses the synthesis of ADP-glucose and inorganic phosphate from ATP and glucose-1-phosphate. This reaction is a critical step in the synthesis of bacterial glycogen and plant starch as ADP glucose acts as the main glucosyl donor for α-glucan synthesis by branching enzyme and various starch synthases (Preiss, 1991)

The bacterial AGPase enzyme is homotetrameric in structure and is encoded by a single gene locus the *glg C* gene in *Escherichia coli*. The enzyme has a molecular weight of approximately 200kDa with each subunit being 48kDa (Preiss, 1991). In contrast the plant enzyme has a much more complex heterotetrameric structure. It is about 210-240 kDa in size and is based on 2 subunit types (Copeland and Preiss, 1981), a large subunit (between 54 and 60 kDa) and a small subunit (between 50 and 55 kDa). The two subunits are encoded by different genes in various plant species (Bhave et al., 1990, Bae et al., 1990, Müller-Röber et al., 1990). Both subunits are required for full activity of AGPase. Mutants lacking either of the two subunits in maize (Hannah et al., 1980), Arabidopsis (Lin, 1988), pea (Smith et al., 1989) and transgenic potato plants expressing an AGPase antisense cDNA (Müller-Röber et al., 1990), exhibit significant reductions in AGPase activity and consequently reductions in starch levels. This is exemplified by the Arabidopsis mutant *adg2* which has 5% ADP-glucose pyrophosphorylase activity and only 40% of the wild type starch due to lack of the large subunit of AGPase (Lin, 1988).

The bacterial and plant AGPases differ further in the way in which they are regulated. In *E.coli* AGPase is activated by fructose 1,6 diphosphate and inhibited by adenine monophosphate (AMP) or adenosine diphosphate. The plant AGPases in photosynthetic tissue are closely regulated by fluctuations in the ratios of the photosynthetic metabolites 3-phosphoglyceric acid (3-PGA) and orthophosphate (Pi). Studies on AGPase from non-photosynthetic tissue have shown that the enzyme shows similar responses to allosteric activation and inactivation by 3-PGA and Pi respectively, like the enzymes from photosynthetic tissue. Generally plant as well as algal and

cyanobacterial AGPases are activated by 3-PGA and inhibited by orthophosphate (Preiss, 1991). An exception is the barley AGPase which is relatively insensitive to these metabolites and actually shows high activity in the absence of 3-PGA (Kleczkowski, 1993).

CDNA as well as some genomic clones encoding plant AGPases have been cloned from several species such as maize (Bhave et al., 1990), sugar beet (Müller-Röber et al., 1995), rice (Anderson et al., 1989) potato (Müller-Röber et al., 1990), spinach (Smith-White and Preiss, 1992), and sweet potato (Bae and Liu, 1997).

It has been shown that multiple genes encode the large or S subunits in plants such as potato and *Arabidopsis* where from each 3 genes encoding AGPase S isoforms have been cloned (La Cognita et al., 1995; Villand et al., 1993). These genes are strongly tissue specific (AGPase S3 of potato is only expressed in tubers, La Cognata et al., 1995) and are induced under specific conditions such as increased sucrose levels as is the case with AGPase S2 of potato whose expression was induced 2 to 3 fold by exogenous sucrose (La Cognata et al., 1995). Investigations into the small or B-subunit have also revealed the existence of various small subunit isoforms which also exhibit tissue specificity in bean, maize and sweet potato (Weber et al., 1995; Prioul et al., 1994; Bae and Liu, 1997). Although the cDNAs encoding the small subunit isoforms in sweet potato did not exhibit differences in tissue specificity, one of them was sucrose inducible as well as light responsive (Bae and Liu, 1997).

The molecular identification and characterisation of genes involved in starch biosynthesis in the tropical root crop cassava (*Manihot esculenta* Crantz) is our major goal. This will provide us with invaluable tools not only for analysing the process of starch biosynthesis, but also for modifying the composition and quantity of starch produced by cassava plants. This paper describes the cloning and characterisation of two cDNAs encoding the small and large subunit of AGPase from cassava respectively.

Materials and Methods

Plant Material and Bacterial Strains

Cassava genotype M.Col22 was used in this study. The plants were sprouted from stem cuttings and grown in the greenhouse under a 16 hour light/ 8 hour dark regime. The day and night temperatures were maintained at 28°C and 16°C respectively. Leaves, tubers, roots, petioles and stem were harvested from plants which had been growing for 6 months. The tissue was frozen in liquid nitrogen and stored at -80°C for later extraction of DNA and RNA or homogenised immediately for antigen and enzyme activity determination. *Escherichia coli* strains DH5 alpha and Y1090 were cultured and transformed using standard techniques (Sambrook et al., 1992).

Screening of cDNA library

A cassava M.Col 22 tuber specific cDNA library (preparation described in Salehuzzaman et al., 1993) in lambda gt11 was screened by plaque hybridisation for the genes encoding ADP-glucose pyrophosphorylase B and S subunits. The cloned genes from potato labelled with [^{32}P] dCTP by the random primer labelling technique (Feinberg and Vogelstein, 1983) were used as probes. Approximately 2 x10^5 pfu were screened for each probe at the first round of screening. The blots were hybridised overnight at 60°C in 5 X SSPE, and 0.3% SDS with 100mg/ml denatured herring sperm DNA added. Washing was carried out three times with 2xSSPE + 0.1% SDS at 60°C for 30 minutes each time followed by autoradiography, at -80°C, within intensifying screens. The positive cDNAs obtained were cloned into the EcoRI site of pUC19.

Isolation of DNA

Minipreps of DNA from recombinant lambda gt11 were prepared from cultures of *E.coli* (Y1090) infected with individual plaques. These were grown at 43°C for 6 hours on L agar with 50ug/ml ampicillin and 10mM $MgSO_4$. Minipreps and large-scale preparations of plasmid DNA were carried out according to Sambrook et al. (1992). Genomic DNA isolation from cassava was carried out using the method of Dellaporta et al. (1983).

Sequencing

The dideoxy chain termination method (Sanger et al., 1977) was used for sequencing the clones and subclones of isolated cassava cDNAs. Sequences were analysed for homology to data bases using the blast programmes (Altschul et al., 1990) located at the WWW site of NCBI (http://www.ncbi.nlm.nih.gov). All other sequence manipulations and analyses were carried out using the PC-Gene programme (Intelligenetics, Mountain View, CA, USA).

Southern and Northern Hybridisation

Cassava DNA, digested with various enzymes, was electrophoresed on 0.8% agarose gels and blotted on to nitrocellulose. Total RNA from plant organs was denatured using formaldehyde and formamide. This was then size fractionated in denaturing 1.4% (w/v) agarose gels. The RNA was then transferred to Hybond-N (RPN203N, Amersham, UK). Hybridisation with the isolated cassava cDNAs was carried out at 60°C as described by Salehuzzamman et al., (1992).

Localisation of the putative AGPase cDNAs on to the cassava genetic map

The cassava cDNAs were mapped using 150 F_1 plants from an interspecific cross between TMS 30572 (the female parent) and CM 2177-2 (the male parent) described in Fregene et al, (1997). Localisation of the AGPase B and AGPase S cDNAs on to the cassava genetic map was carried out using the computer package MAPMAKER (Lander et al, 1987) and linkage positions were assigned as described by Fregene et al, 1997.

Cloning of the AGPase B insert in the expression vector

From the results of sequencing AGPase B (see results section) primers with a BamHI restriction site, were synthesised for PCR amplification of the coding sequence of AGPase B. The PCR product was cloned directly into the pGEMT vector (Promega, Madison, WI, USA). This was followed by restriction with BamHI and Hind III resulting in a 1 kb Bam/Hind AGPase B insert containing an in frame ATG start codon. The expression vector pQE-32 (Qiagen GmbH, Dusseldorf, Germany), carrying a 6 X Histidine affinity tag at the 5' site of the expression box, cut with BamHI and HindIII was then ligated with the 1 kb Bam/Hind AGPase insert to give pQEB and its sequence was verified.

Expression of AGPase in E.coli

pQEB was transformed into *E.coli* M15. This *E.coli* strain contains the plasmid pREP4. This plasmid carries the gene for neomycin phosphotransferase as well as the IPTG induceable lac I gene which encodes the lac I repressor. Over-expression of the AGPase B protein as well as its purification using an NI-NTA resin column was carried out according to the manufacturer's recommendations (Qiagen).

Polyclonal antibodies were raised in a rabbit against the cassava AGPase B protein and obtained using standard techniques (Sambrook et al., 1992). The final serum, giving a high antibody titer, termed "ANTI B" serum, was collected and stored at -20°C.

SDS-PAGE and Western Blot Analysis

Cassava tuber and leaf tissue were ground in extraction buffer (50 mM Tris-HCl pH 7.5, 10mM EDTA, 2 mM $Na_2S_2O_5$, and 2.5 mM Dithriothreiol). The homogenate was filtered and then centrifuged at 13000rpm for 5 mins. The supernatant was concentrated to 2 mg/ml protein, mixed 1:1 with sample buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 25% glycerol, 2% SDS, 10% 2-mercaptoethanol) and then boiled for 5 mins followed by electrophoresis in a 10% SDS polyacrylamide gel. The separated proteins were then transferred onto nitrocellulose membranes. The blots were blocked with 3% BSA, probed with "ANTI B" serum diluted at 1:500. Goat anti-rabbit IgG phosphatase conjugate (diluted 200 times) was used as the secondary antibody. The immuno reactive bands were detected by incubating the membranes in the dark in 100ul AF buffer (100 mM Tris-HCl pH 9.5, 100mM NaCl, 5mM MgCl2) containing 200ul NBT (4 nitro-blue tetrazoliumchloride; 75 mg/ml in dimethylformamide) and 200ul BCPIP (5-bromo-4-chloro-3-indoyl phosphate; 50 mg/ml in H2O).

AGPase Enzyme activity

The formation of NADH measured at 340nm and 25°C, using a continuous spectrophotometric method was used to determine AGPase activity (in the reaction leading to Glucose-1-P synthesis) in various cassava tissues. Enzyme extraction and reaction was carried out as described by Weber et al., 1994. NADH synthesis was linear for at least 15 minutes. The allosteric regulators 3-PGA and Pi were added to the reaction at between 0.5 to 5mM.

Results

cDNA library screening

About 2.0 x 10^5 plaque forming units of a cassava tuber specific cDNA library were screened separately with: (a) a 1.2 kb EcoRI fragment of the potato AGPase B cDNA, (b) a 1.5 kb EcoRI cDNA fragment of the potato AGPase S2 cDNA and (c) a 1.5 kb EcoRI fragment of the potato AGPase S3 cDNA. Hybridisation with the 1.2 kb EcoRi fragment of AGPase B resulted in the isolation of 6 cDNA clones, ranging in size from 1.5 to 2.0 kb. Digestion of the putative cassava AGPase B clones revealed identical restriction sites. The largest clone B45-1 (2.0 kb), hence forth referred to as AGPase B, was completely sequenced.

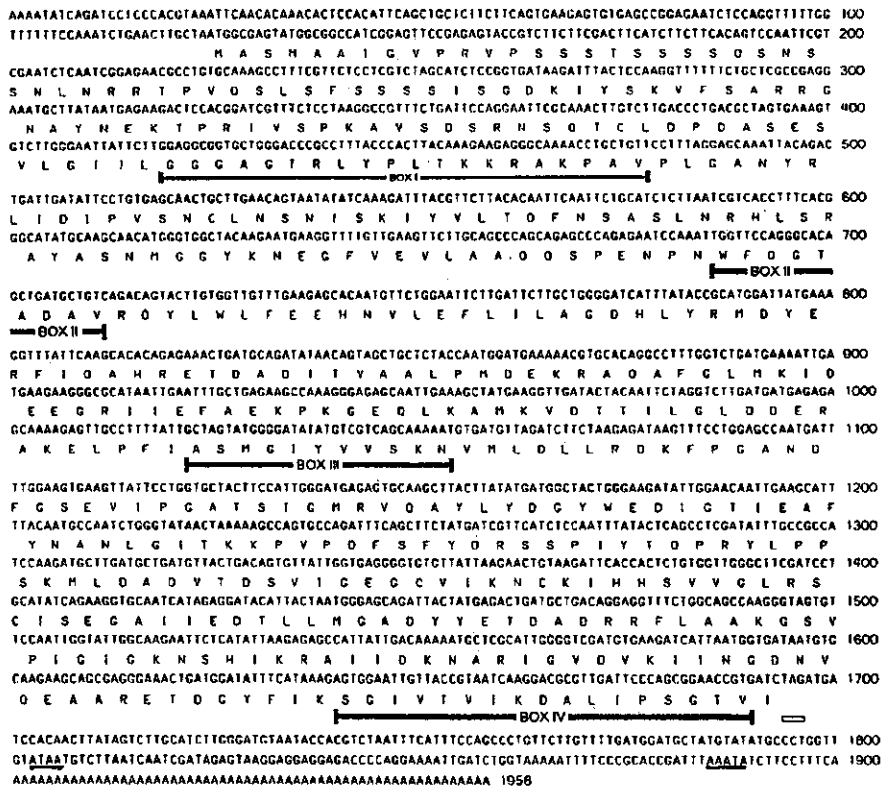

Fig. 1 The complete nucleotide and derived amino acid sequence of the cassava AGPase B cDNA. Regions which have been shown to be functionally important and are conserved in bacterial and plant AGPases are highlighted as: Box I ([AG]GGXG[ST]XLX(2)L[TA]X(3)AXPAV) and Box II (W[FY]XGTA[DN][AS][LIVMFYW]) have been proposed to be part of the allosteric and or substrate binding site in the *E.coli* enzyme glgC (Nakata *et al*, 1991). Box III (ASMG[LIVM]Y[IV][LIVMFY]X(2)[DENPH]), corresponds to a conserved region in the central part of the enzyme. Box IV (SGIVTVIKDALIPSGTV) has been proposed to be the binding site for the activator 3-PGA in higher plant enzymes (Smith-White and Preiss, 1992). The putative polyadenylation signals AATA are underlined.

Ten clones of 2.5 kb in size, were isolated with the potato AGPase S2 probe and two clones of 2.5 kb each were isolated using the potato AGPase S3 probe. Restriction analysis as well as hybridisation between the S2 and S3 cassava clones under stringent conditions (65°C) suggested that the two sets of clones were identical. This was confirmed by sequencing the 2.5 kb S2 -24 and S3-312 cDNAs which will now be referred to as the AGPase S cDNA.

Structure of cassava AGPase B and AGPase S cDNAs

The cassava AGPase B cDNA is 1958bp long (Fig. 1). It has an open reading frame (ORF) of 1572 bp. There is a 124 bp 5' untranslated leader sequence, and a 262 bp 3' untranslated sequence terminated by a poly A stretch of 59 nucleotides. There are putative polyadenylation signals ATAA (Joshi, 1987) at position 1803 and 1884 on the AGPase B sequence.

The AGPase S cDNA is 2385 bp long (Fig. 2). It has an ORF which at 1593 bp is only 21 nucleotides larger than that of AGPase B. There is a long 5' untranslated leader sequence and 3' untranslated sequence of 419 bp and 373 bp respectively in the AGPase S cDNA. AGPase S has a poly A stretch of 6 nucleotides and there is a putative polyadenylation site located 215 bp from the poly A tail.

In frame stop codons (TAA) were located upstream of the ATG start codons for both ORFs indicating that the cDNAs are full length. The sequences flanking the start codon for AGPase B (AGT ATG GC) and AGPase S (GGA ATG GA) differ from the consensus sequences proposed for eucaryotes of, ACC ATG GC , by Kozak (1984) and ACA ATG GC, Lutcke et al., (1987).

The AGPase B cDNA shares a similarity between 82.2 % and 88.8% with other cloned small subunit cDNAs but only 40.0 % similarity at nucleotide level to the AGPase S cDNA . On the other hand the AGPase S cDNA has nucleotide identity of between 54% and 74% with cloned large subunit AGPase cDNAs.

The cassava AGPase S and B proteins

Translation of the 1572 bp AGPase B ORF resulted in a polypeptide which is 524 amino acids long, overall negatively charged at pH 7.0 and has a MW size of 57.3 kDa. The 1593 nucleotides of the AGPase S ORF translate into a protein of 531 amino acids. The polypeptide has a calculated MW of 58.7 kDa and is overall positively charged at pH

7.0.

Comparison of the AGPase B and AGPase S polypeptides shows that they share 45% homology at the amino acid level (Fig. 3). There is larger divergence in homology between the two proteins in the N-terminal region.

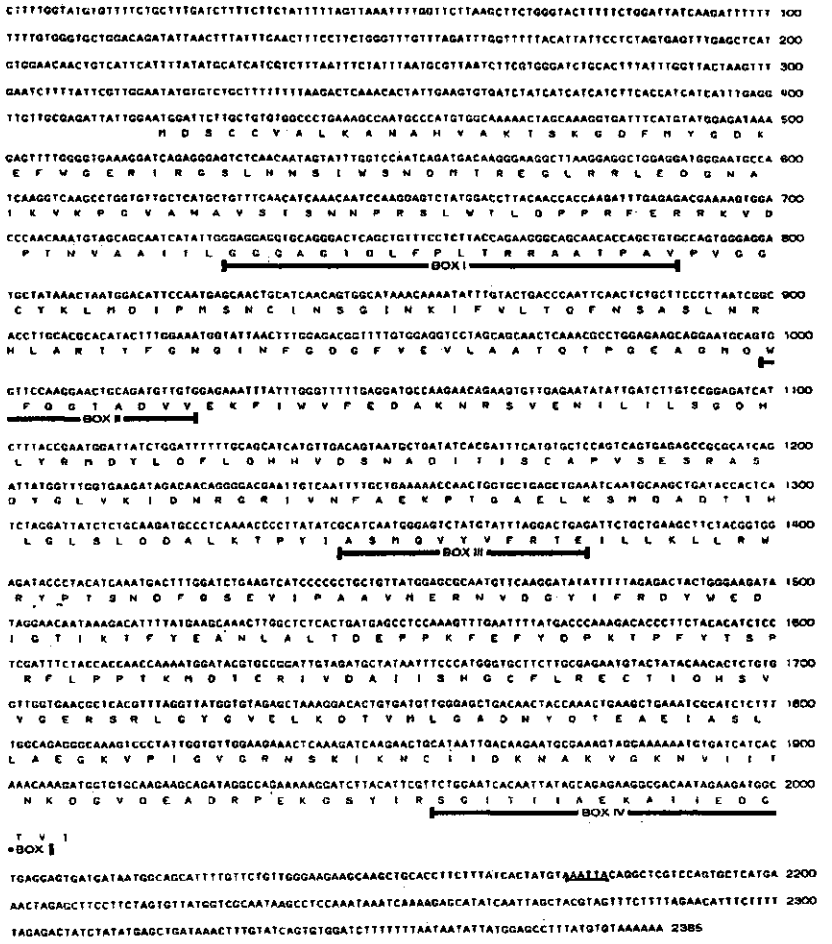

Fig. 2 Nucleotide and derived amino acid sequence of the cassava AGPase S cDNA. Functionally important and regions conserved in other bacterial and plant AGPases are highlighted as Box I to Box IV. They are illustrated as for Fig 1.

Transit peptides (TPs)

A transit peptide is predicted in the N terminal region of the amino acid sequence of both AGPase B and AGPase S based on features common to chloroplast TP as suggested by Gavel and von Heijne (1990). These features include the high proportion of hydroxylated S and T amino acids, and the hydrophobic A amino acid (totalling 40% in AGPase B). No cleavage site conforming to the IXA!A consensus motif for chloroplast TP could be identified for either AGPase transit peptide. However, based on the predicted start of the mature protein for the *spinach* leaf AGPase (Morell et al., 1987) the AGPase S TP is approximately 69 amino acids long (7.5 kDa) and that of AGPase B is 74 amino acids

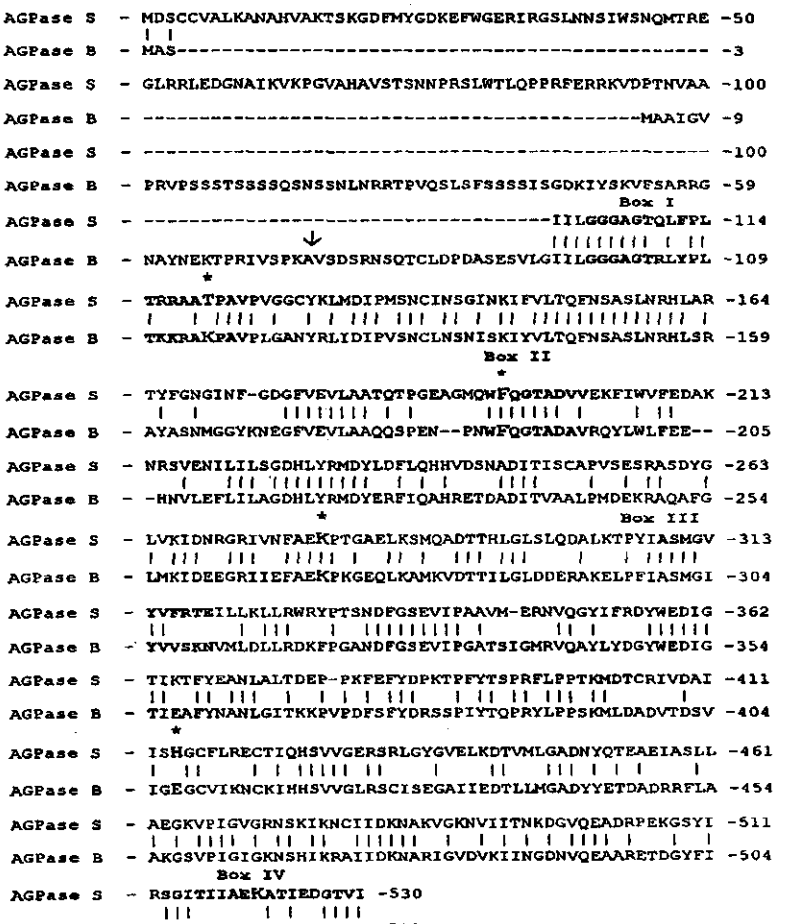

Fig. 3 Alignment of the deduced amino acid sequences of cassava AGPase B and AGPase S. The conserved regions Boxes I to IV are highlighted and explained as for Fig. 1.

long with a calculated MW of 8.0 kDa. This results in a mature protein of 50 kDa and 48.4 kDa for AGPase S and AGPase B respectively. Comparison of the TP regions of the two cassava AGPase polypeptides reveals a low amino acid homology of only 12% (Fig. 3).

The cassava AGPase proteins are very similar to other AGPase subunits.

The deduced amino acid sequences of the cassava AGPase S and AGPase B showed significantly high similarities to already sequenced AGPase amino acid sequences from other plant and bacterial species. AGPase B is highly homologous to the small subunit

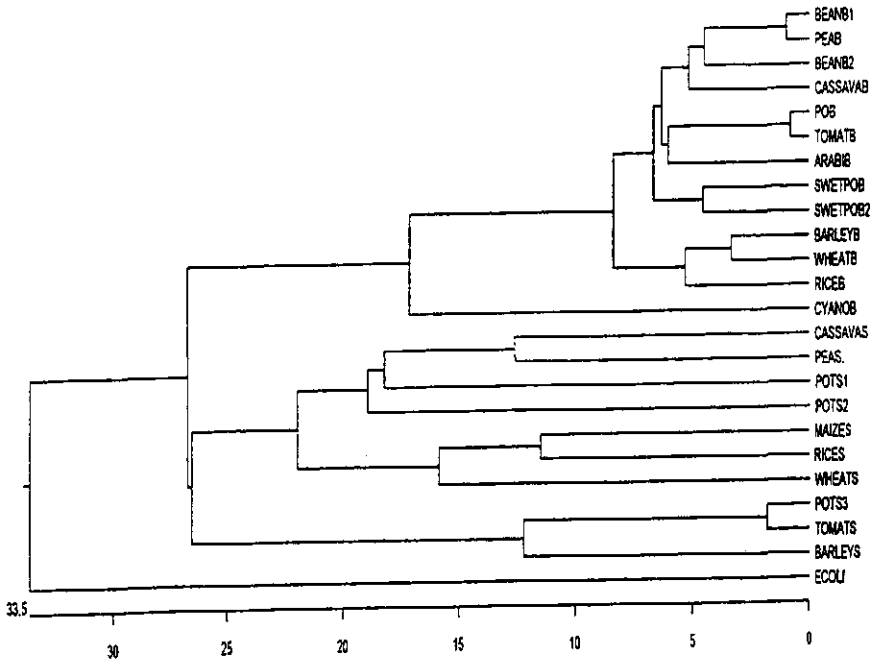

Fig. 4. Dendogram of the evolutionary relationship amongst the various plant and bacterial AGPases. This was constructed using the unweighted pair group maximum averages(UPGMA) method of Weir (1990). S=large subunit and B = small subunit of ADP- glucose pyrophosphorylase. Sequence data are from the following sources with Genbank accession numbers given for unpublished sequences. Arabidopsis - ARABIB(Choi and Okita, unpublished accession # U70616); Barley B (Thorsjornsen et al, 1996); Barley S (Eimert et al., 1992); Bean B and Bean B2 (Weber et al., 1995), Cassava B and S (present work); Cyanobacteria - CyanoB (Charng et al., 1992); *E.coli*- ECOLI (Baecker et al., 1993); Maize S (Bhave et al., 1990); PEA B and S (Burgess et al., 1997);Potato B - POB (Okita et al, 1990); Potato S1- POTS1 (Okita and Preiss, 1991); POTS2 and POTS3 (La Cognita et al., 1995); Rice B (Anderson et al., 1989); Rice S (Kavakli et al., 1996); Sweetpotato B and B2 - SWETPOB and SWETPOB2 (Bae and Liu, 1997); Tomato B - TOMATB (Chen and Janes, unpublished accession # L411260); TOMATS - (Parks and Chung, unpublished accession # U85497); Wheat B (Ainsworth et al., 1993); Wheat S (Park and Chung, unpublished accession # U85497).

cDNAs, in particular to pea AGPase B with which it shares 88.8% similarity. On the other hand AGPase S exhibits greater identity to genes encoding the large subunit - being 73.7 % identical to the pea AGPase large subunit gene. Based on percentage similarity the small subunit sequences can further be grouped into monocots and dicots (Fig. 4). Three regions, which are conserved in all AGPases were identified in both AGPaseS and AGPase B (Fig. 3). The first two regions, Box I ([AG]GGXG[ST]XLX(2)L[TA]X(3)AXPAV) and Box II (W[FY]XGTA[DN][AS][LIVMFYW]) have been proposed to be part of the allosteric and or substrate binding site in the *E.coli* enzyme *glg*C (Nakata et al., 1991). The third region, Box III (ASMG[LIVM]Y[IV][LIVMFY]X(2)[DENPH]), corresponds to a conserved region in the central part of the enzyme. The fourth conserved domain of 17 amino acids found within the C-terminal region, Box IV (SGIVTVIKDALIPSGTV) has been proposed to be the binding site for the activator 3-PGA in higher plant enzymes (Smith-White and Preiss, 1992). Within these conserved domains several functionally important amino acids were also found to be highly conserved (Fig.3). These include Lys 39 (numbered as per the bacterial *glg* C gene) which has been suggested as being located in the allosteric or substrate binding site in E.coli and may contribute to the regulatory or catalytic activities of the plant enzyme (Baecker et al, 1983; Okita et al., 1993). In the AGPase B sequence Lys 39 is replaced, as in most small subunit polypeptides, by arginine (Fig. 3) . In the AGPase S sequence the corresponding amino acid is threonine. Another important amino acid that is also involved in substrate binding in E.coli is Tyr 114. This is present as phenylalanine in both cassava AGPase sequences. This amino acid is thought to co-ordinate the adenine rings of ATP or ADP glucose via hydrophobic interactions (Olive et al., 1989). The dendrogram (Fig. 4) shows how closely related the various cloned AGPase sequences are. There is greater similarity amongst the small subunit genes and more heterogeneity amongst the large subunit genes.

Southern analysis and localisation of the AGPase genes on to the genetic map of cassava

Characterisation of B45 and S312 by hybridisation to genomic DNA of cassava M. Col 22 digested with various enzymes resulted in several bands being detected in each lane for both cDNAs (Fig. 5a). The different banding patterns obtained with the AGPase B and AGPase S cDNAs are a further indicator that these are indeed distinct genes. Linkage analysis of a segregating population of cassava plants enabled the AGPase S gene to be

localised on to the female derived linkage group E (Fig. 5b). The AGPase S gene is a single copy gene and exhibits a simple segregating pattern in the offspring (Fig. 5b). This is in contrast to the small subunit genes of ADP-glucose pyrophosphorylase that appeared to be localised on at least two positions within the cassava genetic map (Fregene personal communication).

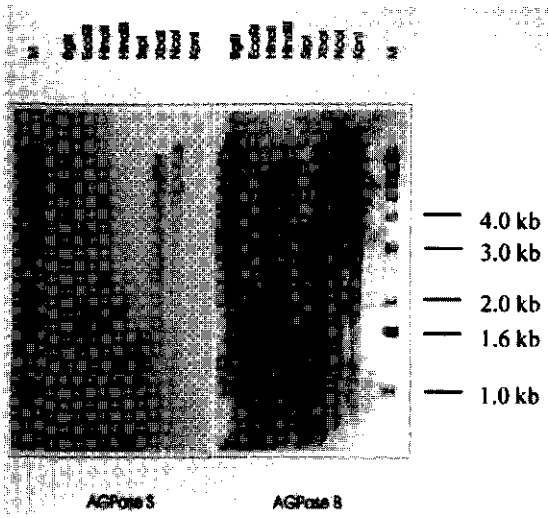

Fig. 5a

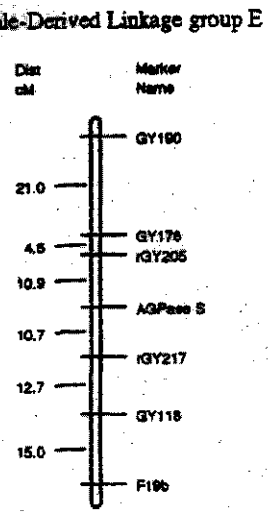

Fig. 5b

Fig. 5a. Southern blot of cassava DNA, probed with ^{32}P labelled AGPase B and AGPase S cDNA clones. An amount of 10µg of digested DNA was loaded per lane. Molecular weight markers and the restriction enzymes used are indicated.

Fig. 5b Position of AGPase S on the female derived linkage group E of the genetic map of cassava

AGPase B is highly expressed in leaf tissue and AGPase S is expressed in all starch synthesising tissues

The expression patterns of the cassava B45 and S312 cDNAs were determined by Northern hybridisation using RNA from various cassava tissues of greenhouse grown plants. The AGPase B cDNA hybridised to a mRNA transcript of 2.0 kb in size. The gene was highly expressed in leaf tissue while the expression in other tissues, including the tubers, was very low (Fig. 6). The AGPase B transcript was of the same size in all tissues examined. AGPase S, hybridises to a 2.3 kb transcript, and is expressed to the same extent in leaves, petioles, roots and tubers (Fig. 6) i.e. in all cassava tissues in which starch is synthesised. However, AGPase B exhibits considerably higher steady state mRNA levels than AGPase S in all the tissues examined.

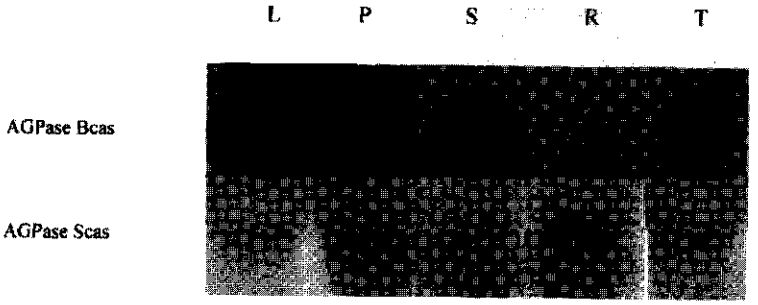

Fig. 6. Expression of AGPase B and AGPase S in different tissues of greenhouse grown cassava

MCol 22 plants. An amount of 40 µg of total RNA was run in each lane and probed with ^{32}P labelled AGPase B and AGPase S cDNAs. L, leaf; P, petiole; S, stem, R, root; T, tuber. In order to ensure to ensure equal loading of RNA per lane the same blot was deprobed and rehybridised with a 28S rRNA probe (results not shown).

Expression of AGPase B in *E.coli*

In order to study the distribution of the small subunit gene product in cassava, a 1 kb BamHI/Hind III fragment containing the transit peptide and three conserved AGPase regions was cloned and expressed in *E.coli*. The expressed protein has a size of about 40 KDa (Fig. 7, lane 1). This lies in the same range as the calculated size of 38 kDa for a protein expressed from a 1 kb DNA fragment. Polyclonal antibodies specific to the AGPase small subunit were produced (as described in material and methods) and used in

western blot analysis of cassava tissue.

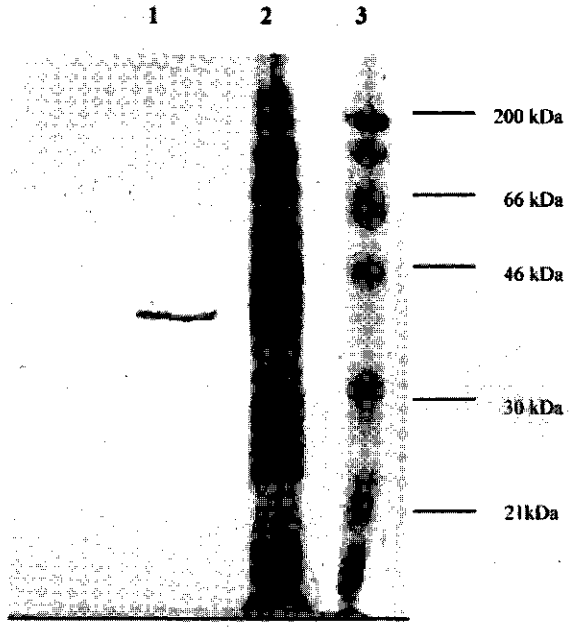

Fig. 7. Profile of cassava AGPase B protein purified from *E.coli* on SDS page. 1 = purified protein; 2 = induced total protein, 3 = marker lane. Molecular weight markers are indicated on the right in kD.

Western analysis of cassava AGPase

Crude protein extracted from cassava leaves, petioles, stems, roots and tubers was electrophoresed in a 10% SDS polyacrylamide gel. Polyclonal antibodies raised against the cassava small subunit cDNA detected a 50 kDa band (Fig. 8 lanes 1 and 2). This band possessed the same size in all tissues examined. Degradation of this band was observed especially in tuber tissue at 4 °C even when the extraction was carried out in the presence of reducing agents such as SDS or TCA (Fig. 8 lane 4).

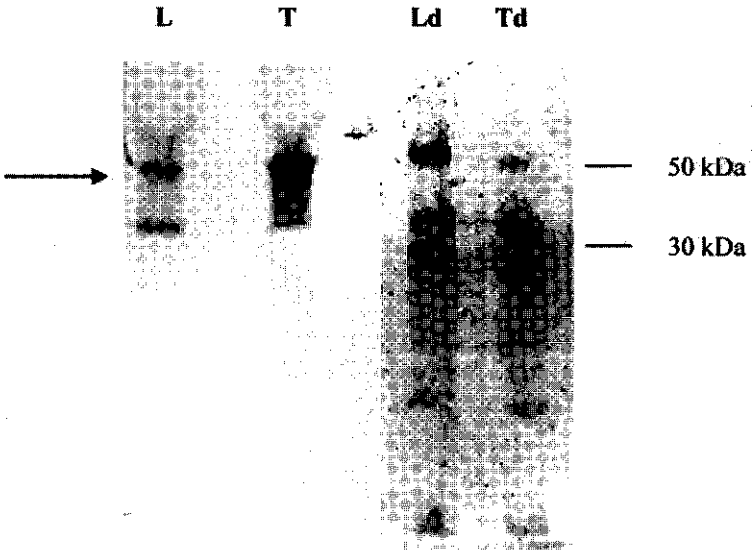

Fig. 8 Cassava AGPase undergoes degradation under normal conditions of isolation and SDS PAGE analysis. Protein (5μg/lane) from cassava tuber and leaf were separated by SDS-PAGE and visualised by immunoblotting, using the AGPase B antibody. Size markers are shown in kDa. The position of the AGPase B protein is at approximately 50 kDa - indicated by arrow. L, leaf, T, tuber, Ld degraded Leaf protein, Td, degraded tuber protein

AGPAse activity is highest in cassava leaves

AGPase activity in cassava leaves, roots, petioles and tubers was determined by assaying spectrophotometrically for NADH formation. The AGPase enzyme activity was highest in leaves with young leaves having an absolute value of .0039 uM/gFW/min which was considerably higher than that found in tubers (0.001 uM/gF/min (Fig. 9; 0 Pi/ 3PGA stage).

Addition of 5mM 3-PGA gave rise to a three-fold increase in AGPAse activity in the leaf tissue resulting in young leaf lobes having absolute activity of 0.13 uM/gFW/min (Fig. 9). No such response was obtained with the tuber enzyme as activity only increased 0.5 times with the addition of 5mM 3-PGA (Fig. 9). There was a considerable decrease in leaf enzyme activity (by 90%) and tuber enzyme activity (by 80%) due to the addition of the inorganic phosphate, Pi (Fig. 9).

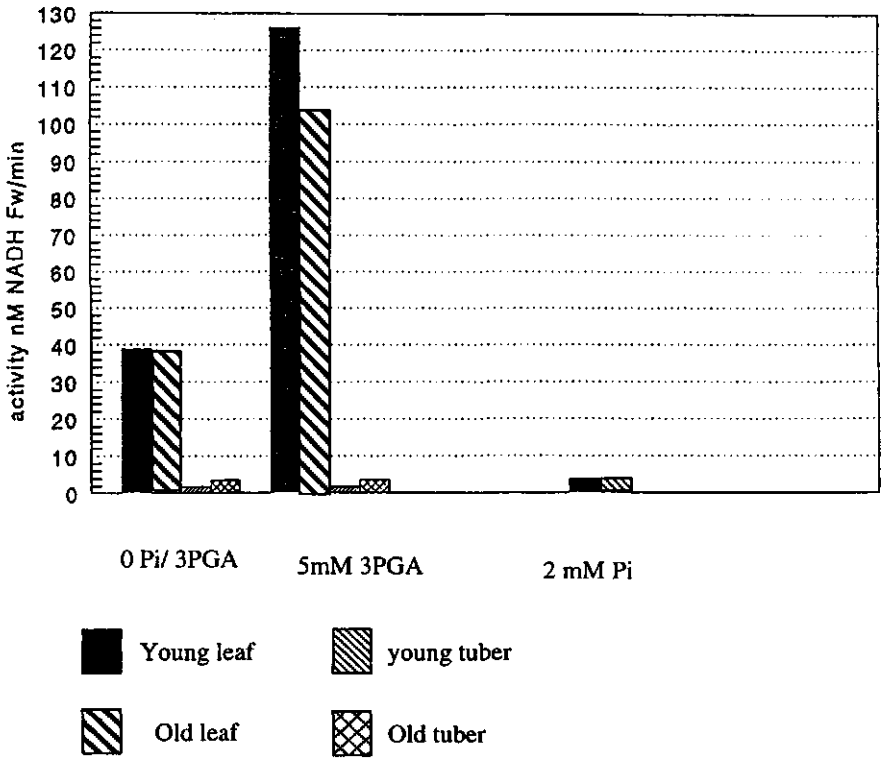

Fig. 9 AGPase activity in cassava leaves, and tubers at different developmental stages. The AGPase enzyme was extracted as described by Weber et al., 1994. The tissue used consisted of young leaves (5-6 cm long), old leaves (10-15 cm long), young tubers (1-3 cm in diameter), old tubers (4-8 cm in diameter). The absolute activities were determined by spectrophotometrically assaying for NADH.

Discussion

Two groups of cDNA clones, designated AGPase B and AGPase S, were isolated from a cassava tuber specific cDNA library using DNA probes encoding the small and large subunit respectively of the potato AGPase. Following cloning and restriction analysis the largest cDNAs from each group were sequenced. Sequence analysis showed that they represented full length AGPase transcripts. AGPase B and S exhibit only 34% homology at the nucleotide level and 54% homology at the amino acid level, to each other (Fig. 3). The derived amino acid sequences of the mature proteins of AGPase B and AGPase S showed significant homologies with other cloned AGPase polypeptides from bacterial and plant species. The AGPase B cDNA shared close identity (74-94%) at amino acid level with already cloned small subunit cDNAs from potato, pea, bean, and sweetpotato. The AGPase S cDNA is very similar to genes encoding other AGPase large subunit cDNAs (70 -84% identity) than to genes encoding small subunit AGPases. All this is in agreement with investigations carried out by Smith-White and Preiss, (1992) when they compared the sequences of known AGPase genes. They found that there was greater similarity amongst the small subunit genes from different species than between the small and large subunit genes from within the same species. This is significant when we look at the dendrogram derived after comparison of AGPase genes from several plant species (including cassava) and those of bacteria and cyanobacteria (Fig. 4). The weighted dendrogram reveals the way in which the AGPase genes may possibly have evolved. According to Smith-White and Preiss (1992) the small and large subunits probably arose from a duplication of a common ancestral gene with subsequent divergence. Our analysis of the amino acid sequences of full length AGPases cloned thus far corroborates this hypothesis and further shows that the cyanobacterial gene is more closely related to the small subunit than to the large subunit (Fig. 4). This would mean that the small subunit and cyanobacterial enzyme are derived from a common ancestral progenitor which diverged from the large subunit. Further evidence for the relatedness of the small subunit to the cyanobacterial enzyme comes from the cross reaction of the cyanobacterial enzyme to antibodies specific to the spinach leaf subunit (Iglesias et al., 1991).

With all these changes in the basic sequence of the AGPase genes there remained certain domains which were conserved and thus unaltered. These domains are those that are involved in the enzyme substrate complex such as, Box IV, SGIVTVIKDALIPSGTV which has been shown to be the binding site for the activator 3-PGA in higher plant enzymes (Smith-White and Preiss, 1992).

The isolation of two different groups of cDNAs encoding the AGPase enzyme would indicate that in cassava the AGPase enzyme is composed of two distinct subunits as has been shown to be the case in maize (Bhave et al., 1990) and other plant species (Bhave et al., 1990, Bae et al., 1990, Müller-Röber et al., 1990). Southern analysis revealed that both cassava AGPase subunits are encoded by genes that are present as only a few copies within the cassava genome. In the case of AGPase S we were able to map it to the female derived linkage group E on the cassava genetic map. The cassava AGPase B gene showed a more complex segregation pattern and appeared to be localised on more than one linkage group. Such a complex organisation for the small subunit genes has been observed previously in potato (Anderson et al., 1990). Although in our case we were able to isolate only one type of cDNA corresponding to the large and small subunit cDNA, this does not preclude the possibility of isoforms of these genes being present in cassava, especially as in all the plant species examined such as maize, potato, sweet potato, bean, and arabidopsis, isoforms of the small and large subunit of AGPase have been found (Weber et al., 1995, Müller-Röber et al., 1997, Prioul et al., 1994).

The expression patterns of the AGPase genes were determined by Northern hybridisation using RNA from leaves, petioles, stems, roots and tubers from greenhouse grown cassava M.Col 22 plants. AGPase B showed the highest expression in leaves, the expression signal in tubers being some four fold less (Figure 5). Little or no expression was found in petioles, stems and roots. It, therefore, seems that the AGPase B cDNA is specific for leaf and tuber tissue. The AGPase S was expressed to equal levels in all the tissues examined. The significance of these results is that while it would seem that AGPase S is constitutively expressed in all tissues, AGPase B is highly expressed in tissue specifically involved in starch synthesis i.e. leaves and tubers.

Expression of a 1 kb BamHI/HindII AGPAse B fragment in *E.coli* resulted in a 40 kDa protein being purified. Polyclonal antibodies to this protein were found to be specific for a 50 kDa protein in cassava leaves, tubers, roots and petioles. This indicated that the AGPase B protein in all cassava tissues was of the same size. This size falls well within the values determined for other small AGPase subunits from other plant species (Preiss et al., 1991, Okita et al., 1990, Nakamura and Kawaguchi, 1992). However storage of the crude protein extract at 0-4 °C resulted in a high rate of proteolytic degradation of this protein in both leaf and tuber tissue. Such degradation has been reported for the large AGPase subunit of barley endosperm (Kleczkowski et al., 1993). Degradation was still

observed even when extraction was carried out in the presence of denaturing agents such as TCA or SDS.

The relative abundance of AGPase B polypeptide was the same in all tissues despite the differences in expression level. The difference in the quantities of mRNA produced specifying AGPase B and the corresponding polypeptide amounts may indicate some post-transcriptional control of AGPase B polypetide levels in leaf and to some extent in tuber tissue. This post-transcriptional control may manifest itself as a high turnover of any excess AGPase B mRNA in leaves (by proteolysis) hence the discrepancy between the high level of mRNA expression and polypeptide abundance in this tissue. Such a possibility of post transcriptional control of polypetide levels has been demonstrated by Nakata and Okita (1995) for the leaf AGPase small subunit in potato.

AGPase activity was found to be highest in young leaves of cassava. There was considerably less enzyme activity in tubers and older leaves, there was virtually little or no AGPase activity in petioles, stems and non tuberous roots. An increase in AGPase activity with increased levels of 3-PGA added showed that the cassava leaf AGPase was allosterically activated by 3PGA. A corresponding increase in the levels of Pi reduced the leaf enzyme activity by 85%. Such a scenario whereby the levels of 3-PGA and Pi increase and decrease occurs in plants during the daytime (where they carry out photosynthesis) and during the night. These results confirm to what is generally known about plant leaf AGPases which in general have been shown to respond to allosteric inhibition and activation by Pi and 3-PGA respectively (Preiss et al., 1991). The only exception is the barley endopserm AGPase which is relatively insensitive to these effectors (Kleczkowski, 1993). In the non-photosynthetic cassava tuber the enzyme activity was relatively unaffected by changes in the level of 3-PGA although the Pi further reduced the already low AGPase activity in this tissue. There is a close correlation between enzyme activity and mRNA steady state levels of the small subunit further indicating that there is a combination of transcriptional as well as post-transcriptional factors affecting the small subunit of cassava and consequently overall cassava AGPase activity.

Salehuzzaman et al. (1993) have already shown that potato plants transformed with a cassava GBSS cDNA in antisense orientation produce almost completely amylose free starch. Thus by cloning the cassava AGPase B we have opened up more options and opportunities to alter starch quantity, as well as the nature of products stored within the cassava tubers. The introduction of the AGPase B gene in sense orientation in starch producing plants would lead to increased expression of this key enzyme of starch

biosynthesis and increased yield of starch as shown for bacterial AGPase in potato (Stark et al., 1992). Plants with an AGPase gene introduced in antisense orientation may have a reduced capacity to produce starch and may be used to accumulate other storage products such as lipids, sucrose (Müller-Röber et al., 1992), cyclodextrins (Oakes et al., 1991) or fructans (van der Meer et al., 1994) in their storage organs which are potentially of great nutritional benefit and commercial value.

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Characterization of potato (*Solanum tuberosum*) plants containing and expressing the cassava (*Manihot esculenta*) small subunit ADP glucose pyrophosphorylase gene in antisense orientation.

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Characterization of potato (*Solanum tuberosum*) plants containing and expressing the cassava (*Manihot esculenta*) small subunit ADP glucose pyrophosphorylase gene in antisense orientation.

Abstract

Data are presented on the analysis of diploid and tetraploid potato (*Solanum tuberosum*) plants that have been transformed with the cassava ADP-glucose pyrophosphorylase small subunit gene, AGPase B, in antisense orientation under the control of the CaMV 35S promoter. While the 244 antisense AGPase B plants did not differ in appearance from the control non transgenic plants 45 of them, however, had on average 4 times more tubers. These tubers were half the mass of those from control plants. Northern analysis of the 45 transgenic plants revealed that 7% of the antisense AGPase plants had reduced levels of AGPase B mRNA (Category I plants). In addition the tubers from these plants had 1.5 to 3 times less starch than tubers from the control plants. The levels of the soluble sugars, sucrose, glucose and fructose, in the antisense plants, increased by at least five times when compared to that found in control plants. There was a correlation between a high number of T-DNA inserts, as revealed by Southern analysis, and the presence of an antisense effect at mRNA, starch, and soluble sugar levels. The results showed that it is possible to induce an antisense effect, in potato, by expressing a cassava AGPase B gene and at the same time confirm the significant role of this gene in the biosynthesis of starch in plants.

Introduction

The conversion of glucose-1-phosphate to ADP-glucose by ADP-glucose pyrophosphorylase (AGPase) is an important regulatory step in the synthesis of starch in plants. Plant AGPases are heterotetrameric enzymes composed of two small and two large subunits encoded by different genes (Okita et al., 1993). We have cloned the cDNAs encoding both the large and small subunits of the cassava (*Manihot esculenta*) AGPase enzyme (Chapter 3, this thesis). Functional analysis and verification of the identity of these putative AGPase cDNAs from cassava can now be carried out by genetic transformation of cassava and other starch producing plants. Although a transformation and regeneration system for cassava has been developed recently (Raemakers et al., 1996; Schöpke et al., 1996) the procedures involved still remain labour intensive, inefficient and need to be optimised. Potato (*Solanum tuberosum*) on the other hand has a starch biosynthetic pathway which in many ways typifies higher plant starch metabolism and is mediated by essentially the same enzymes as in cassava (Salehuzzaman et al., 1993). In addition, regeneration and transformation of potato by *Agrobacterium tumefaciens* is a relatively simple procedure (Visser, 1991). This makes potato an ideal candidate in which to test the cassava AGPase cDNAs as has been confirmed by Salehuzzaman et al. (1993) using the cassava GBSSI gene. In a previous experiment Müller-Röber et al. (1992) introduced the potato antisense AGPase B gene in antisense orientation into the potato cultivar Desiree. This resulted in transgenic potato plants having reduced AGPase B mRNA levels, and smaller, more numerous tubers. These tubers stored lower levels of starch but higher levels of sucrose (40% of dry weight) and glucose (8% of dry weight). This paper describes the results of the transformation of the potato genotypes cv Kardal, cv Karnico, and RV with different constructs of the cassava AGPase B gene in antisense orientation and the subsequent analysis of tubers from these plants.

Materials & Methods

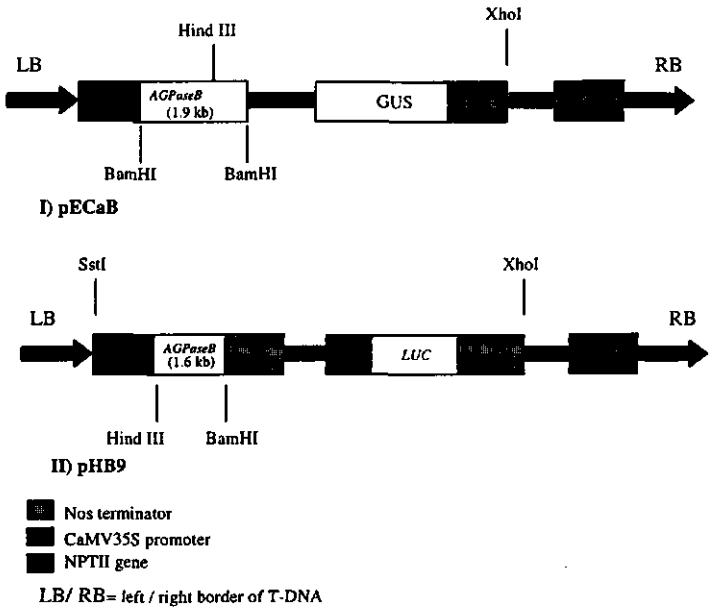

Fig. 1 Design of the cassava AGPase B constructs used in the transformation experiments.

I) Construct pECaB contains the full length cassava AGPase B cDNA (1.9 kb) cloned in antisense orientation into the BamHI between the left and right borders (LB and RB) of pECaGus (Irma Straatman, unpublished). II) A 1.6 kb fragment of the cassava AGpase B gene was restricted from pB45-1 (Chapter 3, this thesis) using the enzymes HindIII and BamHI. The DNA fragment was inserted in antisense orientation into the BamHI/ HindIII site of pJIT100 (Guerineau and Mullineaux, 1993) behind the CaMV35S promoter. PHB9 also contains the luciferase fire-fly (LUC) gene but this was not used in these experiments. Selection of the transgenics was based on kanamycin resistance which is conferred by the neomycin phosphorylase gene (NPTII).

Bacterial strains and DNA manipulations

Agrobacterium tumefaciens LBA4044 and *Escherichia coli* strain DH5a (Bethesda Laboratories, Gaithersburg, USA) were cultivated using standard techniques (Sambrook et al., 1989). All DNA manipulations were largely as described in Sambrook et al. (1989).

The cassava AGPase B antisense constructs

The cassava AGPase B cDNA was recently isolated from a tuber specific cassava cDNA library and cloned into pUC19 forming the plasmid pB45-1 (Chapter 3, this

thesis). In order to verify the function of the cassava AGPase B cDNA using the antisense technique, two antisense constructs pECaB, and pHB9 were made. The constructs are depicted in Fig. 1.

Construct pECaB contained the full length cassava AGPase B cDNA (1.989 kb) in antisense orientation. It was made by restricting pB45-1 with BamHI to release the complete AGPase B cDNA. The cDNA was then ligated into the BamHI site of pECaGUS (kindly provided Irma Straatman, Plant Breeding Dept., Wageningen Agricultural University) behind a double CaMV 35S promoter (Fig. 1). An internal Hind III site 1.6 kb from the N terminal of the AGPase B cDNA (1989 kb) was used to determine the orientation of the inserted gene.

The construct pHB9 was made by digesting cassava pB45-1 with HindIII and BamHI resulting in a 1.6 kb and 0.3 kb HindIII/BamHI fragment. The 1.6 kb fragment was chosen for use because it contained the three conserved domains of the AGPase small subunit gene (Chapter 3, this thesis). This DNA fragment was fused in antisense orientation, in the HindIII/BamHI site between a double 35S promoter and a CaMV35S poly A tail (Fig 1). Plasmid pHB9 also contains the luciferase firefly gene. This was, however, not used in the selection of transgenic plants. The constructs were transferred to *Agrobacterium tumefaciens* strain LBA4404 (Ooms et al., 1982) by triparental mating as described by Hoekema et al. (1983)

Plant material, transformation and selection of transgenic potato-plants

Three *Solanum tuberosum* genotypes were used for transformation. These were: the tetraploid genotypes Karnico (cv) and Kardal (cv); and the diploid potato clone RV (kindly provided by Ir. R. Eijlander of the Plant Breeding Department Wageningen Agricultural University). They were chosen for their ease of transformation or ability to form tubers readily. The in vitro plants were cultured on solid MS20 medium containing Murashige and Skoog (1962) salts and vitamins, 30 g/l sucrose, 8 g/l agar at 21 EC and 14 h light. Stem segments derived from 3 week old plants of the genotypes above were transformed using *Agrobacterium tumefaciens* strain LBA 4404 carrying the plasmids pECaB1, pECaB2 and pHB9 according to the method of Visser (1991). The explants were selected on plates containing the following regeneration medium (MS20, 200mg/l claforan, 200mg/l vancomycin, 1 mg/l zeatine, 100 mg/l kanamycin, 8% agar, pH 5.8) and transferred to fresh medium every two weeks. Regenerated shoots

were cut off and transferred to tubes containing MS20 medium containing 100 mg/l kanamycin and 200 mg/l claforan and vancomycin. Following root induction the plants were transferred to the greenhouse, and tubers were harvested after 18 weeks. In vitro tubers were induced according to Hovenkamp-Hermelink et al. (1988)

Analysis of potato plants.

Observations of the phenotype of the transgenic plants as compared to the control plants were carried out during growth of the plants in the greenhouse. Factors such as flowering time, appearance of tubers and location of tubers on the stolon were considered.

Tuber fresh and dry weight

Upon harvesting of the plants the number of tubers per plant and their weight were noted. The average weight per tuber was calculated for each plant. The dry weights of the tubers were determined by placing randomly selected tubers at 80 °C until there was no change in the weight.

Iodine staining of tubers

Iodine staining with Lugol's solution (I_2:KI) of *in vitro* as well as greenhouse grown tubers was used to visualise the presence or absence of starch. At least two tubers from each plant were cut into 5 mm thick slices and stained with Lugol/H20 (1:1). The stained starch granules (including those from the isolated starch samples, see below) were examined with a light microscope.

Isolation of starch, starch granule size and amylose content

Potato tubers were first weighed, washed and cut into pieces. The pieces were then put into a blender with milliQ water containing a knife tip of $Na_2S_2O_3.5H_20$ and blended. The pulp was filtered through cheese-cloth and the filtrate collected in a beaker. The remaining pulp in the cheesecloth was blended in milliQ water and filtered through the cheesecloth once again. The filtrate was left for 2-3 hours for the starch to settle down and the supernatant was poured off. The starch was washed once again by resuspending it in milliQ water and letting it precipitate. After discarding the water the starch was allowed to air dry at room temperature.

The starch was first sieved through a micropore mesh and then suspended in water. The size and distribution of the starch granules within the different samples was then determined with the Coulter multisizer IIe according to the manufacturer's instructions. Granule morphology as well as iodine staining characteristics were checked microscopically.

Starch and soluble sugar content of the starch granules

The starch, sucrose, glucose and fructose content of the potato tubers were determined using kits supplied by Boehringer Mannheim Co., Germany). The volumes used were reduced 100 fold to enable measurements to be carried out using a microplate reader (Bio-Rad Model 3550-UV).

DNA /RNA isolation and hybridisation

DNA (from potato leaves) and RNA (from tubers) were isolated and prepared for Southern and Northern analysis respectively as described in Chapter 2.

Western analysis and AGPase activity

Protein isolation from potato tubers, blotting and immuno detection were carried out as described in Chapters 2 and 3 of this thesis. Immuno assays were carried out using antiserum raised against the cassava AGPase B protein (Chapter 3, this thesis). AGPase activity in potato tubers was determined as described previously (Chapter 3, this thesis).

Results

Antisense AGPase B plants have smaller and more numerous tubers

A total of 244 independent kanamycin resistant shoots were produced by *Agrobacterium* mediated transformation of the three potato genotypes with the constructs pECaB1, pECaB2 and pHB9. For each genotype (whether diploid or tetraploid) at least 50 independent shoots derived from each of the antisense constructs and at least 15 shoots from the control non transgenic plants were isolated, rooted and transferred to the greenhouse. The transgenic clones were named

Table 1: Identity of transgenic potato plants derived from the transformation of the potato genotypes Kardal, Karnico and Rv with different cassava AGPase B constructs

Genotype	Construct	Name of Derived plant	#. of plants
Kardal	pECaB	KBn	34
	PHB9	KHBn	26
		KDC	15*
Karnico	pECaB	NBn	22
	PHB9	NHBn	28
		KNC	15*
RV	pECaB	RVBn	70
	pHB9	RHBn	64
		RVC	20*

n = each transgenic clone in a series is identified by a number after the name (e.g KB23) * =KDC, KNC, and RVC control non transgenic plants

= number of independent transgenic plants obtained.

according to their original background and the construct that they carried (Table 1).

Observations of the transgenic potato plants carrying the antisense cassava AGPase B gene during 5 months of growth in the greenhouse showed that they did not differ in appearance and time of flowering from the non transgenic potato plants.

Upon harvesting, however, it was found that 45 out of the 275 transgenic plants produced smaller and more numerous tubers than control non transgenic plants. They had on average 4 times more tubers than the control plants. These tubers were twice as small as those from control plants and consequently they had a considerably lower average tuber fresh weight (Table 2). Antisense plants exhibiting this phenomenon had three or four tubers per stolon compared to one tuber per stolon for the control plants (results not shown).

Iodine staining of the tubers showed that all the antisense plants invariably contained starch. There was little difference in the amount of stain retained and, therefore, it was not possible to categorise the antisense plants with this method.

High number of TDNA inserts is correlated with reduced mRNA levels

About 92% (206 out of 224 plants) of the transgenic plants contained between 1 and 2 inserts of either pECaB or pHB9 (Table 2) as revealed by southern analysis of genomic DNA from these plants. Northern analysis of total tuber RNA from these plants, using the potato AGPase B gene as a probe, revealed that they had AGPase B mRNA expression levels which were similar to those of the control plants (see plant RVB17; Fig. 2 a and b). Some 7.4% (17 out of 224 plants) of the transgenic plants had between 3 and 7 inserts of either pHB9 or pECaB (Table 2). These plants had clearly reduced levels of AGPase B mRNA expression (e.g. RVB27, Fig. 2 a and b). This indicated a correlation between a high number of pECaB or pHB9 T-DNA inserts and a reduction in AGPase B mRNA level. No plant was found which had complete absence of AGPase B mRNA, i.e. total inhibition of AGPase B mRNA expression.

Table 2: Transformation of diploid and tetraploid genotypes of potato with the cassava AGPase B antisense construct leads to transformants that have tubers with a reduced average tuber weight.

Genotype	# of Transformants	# TDNA-inserts	AGPaseB mRNA	Av. # of tubers	Av. .tuber fresh weight per plant(g)	Starch granule size (μm)	Category* I/II/III
Tetraploid genotypes							
Construct pECaB							
KB1-32	32	1-2	normal	2	9.4	32.64-33.04	III (30),II(2)
NB50-72	20	1-2	normal	2	9.9	32.10-34.65	III (18),II(0)
KB33	1	4	reduced	8	5.0	27.48	I
KB44	1	5	reduced	12	5.5	28.37	I
NB73	1	5	reduced	10	4.6	27.36	I
NB74	1	3	reduced	10	5.4	28.65	I
KNC	15	0	normal	2	10.0	29.74	III
KDC	15	0	normal	2	9.45	32.41	III
Construct pHB9							
KHB1-22	26	1-2	normal	2	11.3	31.64-32.84	III (21), II (1)
NHB30-58	27	1-2	normal	2	10.9	30.55-35.91	III(27), II(0)
NHB33	1	4	reduced	7	4.6	26.81	I

Table 2 (cont'd)

clone	# of Transformants	# TDNA-inserts	AGPaseB mRNA	Av. # of tubers	Fresh weight per plant (g)	Starch granule size (μm)	Category I/II/III
Diploid genotype							
Construct pECaB							
RVB11-81	60	1-2	normal	2	5.4	28.50-31.00	III (61), II (9)
RVB12	1	3	reduced	8	2.0	27.83	I
RVB14	1	7	reduced	5	2.0	27.32	I
RVB23	1	3	reduced	14	2.8	26.6	I
RVB24	1	4	reduced	4	3.2	28.40	I
RVB27	1	5	reduced	8	2.0	20.94	I
RVB55	1	6	reduced	6	2.4	26.00	I
RVB71	1	4	reduced	5	2.7	29.15	I
RVB74	1	3	reduced	4	2.0	26.82	I
RVB77	1	5	reduced	7	1.6	25.42	I
RVB79	1	5	reduced	7	1.4	29.32	I
Construct pHB9							
RHB90-154	63	1-2	reduced	6	3.6	24.4-27.2	III (42) II (5)
RHB112	1	4	reduced	6	3.5	26.8	I
Control RV RVC	20	0	normal	2	5.6	29.50-33.50	III

The transgenic plants were identified as illustrated in Table I

*Plants were further classified according to three categories: Category I: these transgenic plants had reduced levels of AGPase B mRNA level in addition to having smaller average tuber weights and more numerous tubers than control plants. Category II plants: these exhibited mRNA levels similar to those of control plants but had more numerous and smaller tubers than control plants. Category III plants had no reduction in AGPase B mRNA levels and had average tuber fresh weights and numbers per plant similar to those of control plants. The number of plants within each category are shown in parenthesis.

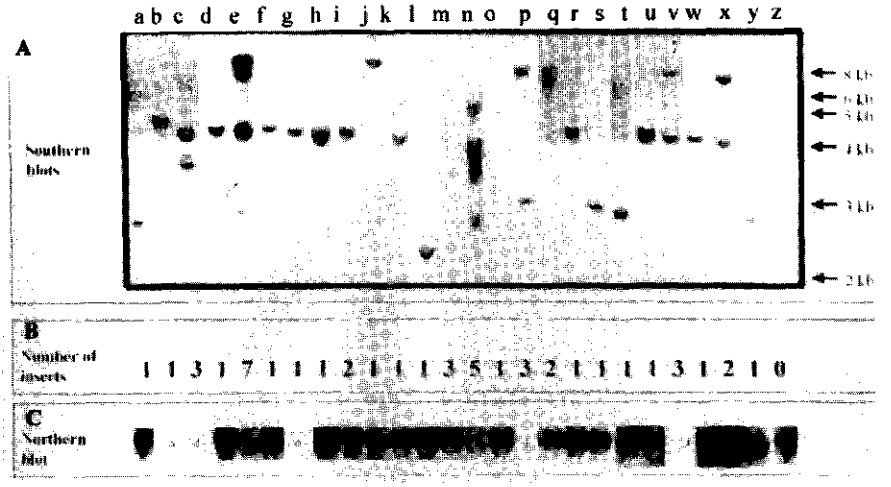

Fig 2A: Southern analysis of genomic DNA from transgenic potato plants derived from the diploid genotype RV containing the cassava antisense AGPase B gene construct pECaB. A total of 10ug of DNA was loaded per lane. A 0.5 kb fragment of the NPT II gene was used as a probe to confirm the presence of the cassava AGPase B containing T-DNA construct. Lanes a-z contain DNA from the transformants: RVB15, RVB16, RVB12, RVB17, RVB14, RVB16, RVB17, RVB19, RVB23, RVB22, RVB25, RVB21, RVB24, RVBB27, RVB35, RVB55, RVB71, RVB68, RVB65, RVB72, RVB74, RVB76, RVB80, RVB81, RVB87 and Control non transgenic RV, respectively

Fig 2B: Minimum number of T-DNA inserts observed on southern blot in A for each transgenic plant.

Fig 2C: Northern analysis of total RNA from tubers of transgenic potato plants transformed with the cassava AGPase B gene. There is a clear correlation between a high number of T-DNA inserts (Fig. 2a) and a reduction in the level of AGPase B mRNA (Fig. 2b). The symbol **d** indicates that the sample was not included in this particular blot but was analysed with other samples. 20μg of total RNA was loaded per lane and the potato AGPase B gene was used as a probe. The transcript size was about 1.9 kb. The lanes are demarcated and contain total RNA from plants described above.

Over three quarters (14 out of 17) of the plants with reduced AGPase B mRNA levels were derived from explants transformed with the full length cassava AGPase B cDNA (pECaB) while the rest carried the 1 kb AGPase B cDNA fragment of pHB9 (Table 2). Reductions in mRNA levels were more pronounced in transformants derived from the diploid genotype RV, that had 13 (7%) plants exhibiting this phenomenon as compared to 5 (4%) derived from the tetraploid genotypes (Table 2).

Correlation between reduced AGPase B mRNA expression and increased number of tubers

All the 17 plants with reductions in AGPase B mRNA expression also belonged to the group of 45 antisense plants having more numerous and smaller tubers. They were classified as category I antisense plants (Table 2). The remaining 18 antisense plants exhibited AGPase B mRNA levels similar to those of control plants but had more numerous and smaller tubers than the control plants were classified as category II antisense plants. Antisense plants (199 plants) with tuber numbers and weight similar to those found in control plants were designated as category III plants. The transgenic plants in Category I had smaller tubers than those in category II and III. This indicates that the size of the potato tubers is influenced by AGPase B mRNA expression.

Detection of AGPase small subunit protein in antisense AGPase B plants

Immunoblotting of total protein from the antisense plants showed that the plants with reduced mRNA levels had hardly any detectable AGPase B protein (Fig. 3). On the other hand the plants classified in category II cross reacted with the cassava AGPase B polyclonal antibody to the same extent as the category III plants. This indicates a clear correlation between mRNA levels and the production of the AGPase B protein.

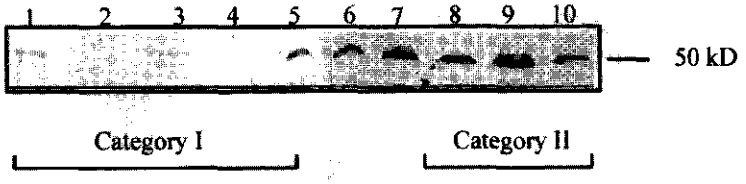

Fig 3: Immuno detection of the AGPase B protein in potato plants containing and expressing the cassava AGPase B gene. Total protein from tubers was separated by SDS-PAGE (20 ug/ lane) and analysed using the cassava AGPase B antibody. The protein detected was about 51 kDa. Lanes 1-5 contain protein from tubers of transgenic clones RVB14, RVB27, RVB25, RVB73 and RVB23 respectively. Lanes 6 and 7 contain protein from control plants RVC and KDC respectively. Lanes 8 to 10 contain protein from the transformants RVB25, RVB73 and RVB65 respectively.

Low starch levels and high soluble sugar content are associated with reduced AGPase B mRNA levels

A reduction in the amount of AGPase B mRNA expression due to the introduction of an antisense AGPase cDNA in potato was expected to result in a decrease in the

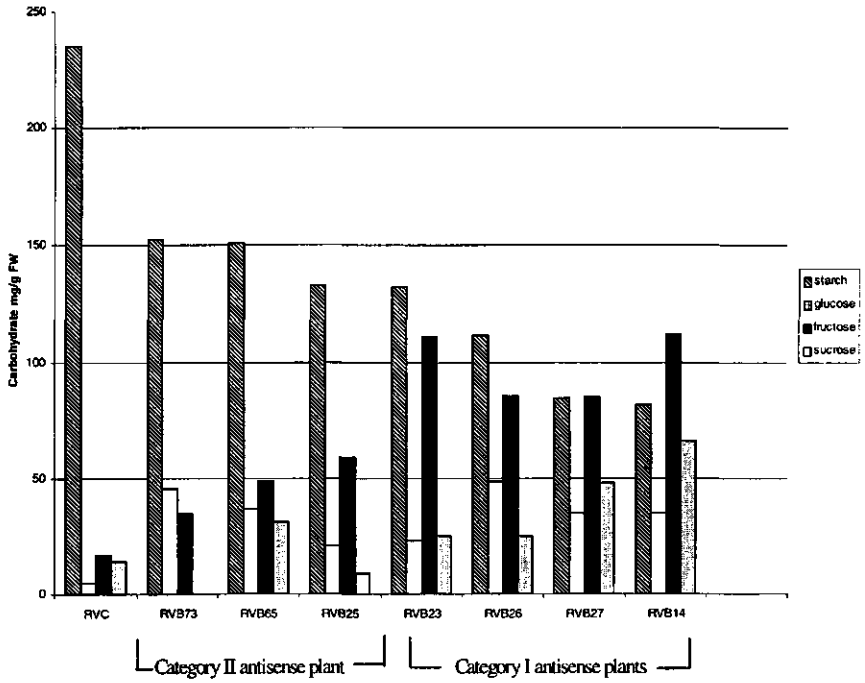

Fig 4: Low starch levels are associated with high soluble sugar (glucose, fructose and sucrose) content. The values of the starch and sugars were determined from triplicate samples obtained from 2 to 3 tubers per plant. The values are given in mg/g fresh weight. Similar observations were made when dry weights were used. In plant RVB73 the level of sucrose was below detectable limits

amount of AGPase enzyme formed. Consequently this should have a negative effecton ADP-glucose formation and ultimately should result in less starch or no starch being formed in the plant because the pathway catalysed by AGPase is the predominant way by which starch is formed in potato. In order to verify this expectation the amount of starch in the transgenic as well as control plants was determined.

The dry weight of the tubers varied between 15% to 23% of tuber fresh weight for the category I plants and between 24% to 26% of tuber fresh weight for the control plants and category III plants. The general trend in terms of starch and soluble sugar content was the same as with fresh weight data.

Category I antisense plants, having reduced levels of AGPase B mRNA, showed dramatic reductions in starch levels. The amount of starch in these plants

ranged between 8% and 16% of the tuber fresh weight compared to 20.8% to 23.5% of the tuber fresh weight in the control and category III plants. An example is the Category I plant RVB27, which had only 8% of the fresh weight of the tuber being starch. Category II plants had a starch level (18% to 23% of tuber fresh weight) that was intermediate between category I and category III plants (Fig 4). These results were also mirrored at the level of the starch granule. The starch granules of category I plants were smaller than the starch granules of Category II and III plants which were found to be similar in size (Table 2). This is clearly illustrated by plant RVB27 that had an average diameter by volume of 20.94 μm compared to an average value of 30.19 μm for category II, III and control starch granules.

A reduction in the level of starch in antisense plants was accompanied by an increase in the level of the soluble sugars sucrose, fructose and glucose. This increase was most pronounced in category I antisense plants where for plant RVB27 there was 10 times more glucose, 6 times the amount of fructose, and 5 times the amount of sucrose, per gram fresh weight than in the control plants (Fig. 4). Category I and II antisense plants had higher levels of fructose and glucose compared to sucrose. In plant RVB73 (category II plant) the level of sucrose was below detectable limits in contrast to the high level of fructose (35 mg/g FW) and glucose (45 mg/g FW) contained in the tubers (Fig 4).

Discussion

Introduction of the cassava AGPase B cDNA in antisense orientation into potato led to the suppression in expression of the native AGPase B mRNA in 17 out of 224 plants (Table 2). The reduction in AGPase B mRNA was accompanied by a reduction in the level of the AGPase B protein (Fig. 4) and subsequently by a dramatic reduction to 32% of the normal starch levels in plant RVB27 (i.e. 84 mg/g FW instead of 257 mg/g FW). These results reconfirm the major role of the AGPase enzyme in starch formation. Research on starch mutants in maize, amongst other plants, have shown that the reductions in starch level were the result of low endosperm AGPase activity caused by mutations at the independent AGPase loci *Shrunken-2* and *Brittle-2* (Hannah et al., 1980). More recently Müller-Röber et al. (1992) showed that a dramatic reduction in potato starch levels could be obtained through the antisense expression of the potato AGPase B. The antisense effect was observed in plants that showed suppression of

AGPase B mRNA expression and was most severe in plants which showed no AGPase B mRNA expression. The frequency of transgenic plants exhibiting an antisense effect was about 23%. In the case of the potato plants carrying the cassava gene no plant with complete inhibition of AGPase B mRNA was found and the frequency of plants having an antisense effect was only 5%. This low frequency could be due to the use of a heterologous gene, cassava AGPase B, which exhibits only 68% amino acid homology (Chapter 3, this thesis) to the potato AGPase B gene. In cases of antisense suppression with heterologous genes it has been noted that the frequency of transformants showing antisense activity is lower than in cases where a homologous gene is used (Kuipers, 1994). For cassava GBSSI which exhibits 74% homology to potato GBSSI Salehuzzaman et al. (1993) found that only 23 % of the transformants had an antisense effect as compared to 50 % when the potato gene was used (Kuipers, 1994). It would be necessary to analyse more plants carrying the cassava antisense AGPase B gene in order to find the plant exhibiting complete inhibition of AGPase B mRNA expression.

The plants that showed reductions in AGPase B mRNA expression had at least 3 T-DNA inserts indicating the strong influence of a high number of T-DNA inserts on the inhibition of AGPase B expression. A similar copy number effect was found in potato plants transformed with the potato antisense GBSS I gene (Kuipers et al., 1995). The antisense effect was more frequently observed in transformants of the diploid RV (12 plants) than in those of the tetraploid cultivars further indicating the copy number effect as far as the gene which is being suppressed is concerned. Ninety percent of the antisense plants that had reduced AGPase B mRNA levels were derived from the construct pECaB, that has the full length AGPase B cDNA. The rest carried a 1 kb HindIII/BamHI fragment of the N terminal region of cassava AGPase B.

Antisense plants with reduced starch levels due to suppression of AGPase B expression had more numerous tubers per stolon, and smaller tubers than control plants (Table 1). Why this occurs still remains unclear but according to Müller-Röber et al. (1992) this may be due to changes in sink strength and /or nitrogen ratios. Even the sizes of the starch granules of plants with severe reduction in starch levels (plants RVB27 and RVB26) were smaller than those of normal plants. This indicates a role for AGPase in determining starch granule size and ultimately influencing the tuber-forming process.

Reductions in tuber starch level were accompanied by an increase in the level of

the soluble sugars sucrose, fructose and glucose. This was most pronounced in antisense plants showing clear reductions in AGPase B mRNA expression such as for plant RVB14 that had up to 10 times more glucose, 6 times the amount of fructose, and 5 times the amount of sucrose than that found in the control plants. These results are in agreement with those of Müller-Röber et al. (1992) who found high levels of sucrose (30% to 40% of dry weight) and glucose (8% of dry weight) in plants with reduced starch levels. The only difference is the high proportion of fructose and glucose as compared to sucrose levels in the potato plants carrying the cassava antisense AGPase B gene. This difference in the type of sugar accumulated may be as a result of metabolism of any accumulated sucrose to its components i.e. fructose and glucose. In general, sucrose is cleaved into uridine-5-diphosphate (UDPGlc) and fructose by sucrose synthase (Susy) or it can be hydrolysed by invertase resulting in glucose and fructose (Morrell and ap Rees, 1986). Sucrose synthase was found to have a high constant expression level in the antisense cassava AGPase B potato plants (results not shown), indicating that sucrose breakdown via the pathway catalysed by this enzyme was probably taking place. However, the role of invertase in degrading sucrose into fructose and sucrose cannot be entirely discounted. The actual differences in amount of sugars accumulated may also be influenced by the potato genotype used, i.e. cv Karnico in our case versus cv Desiree (used by Müller-Röber et al. 1992) and the conditions under which the plants were grown and harvested. What is clear from comparison with control plants is that these sugars accumulate as a result of the inhibition of AGPase activity due to suppression of AGPase B expression.

The presence of osmotically active sugars instead of the osmotically inactive starch is thought to directly affect sink strength allowing for the formation of more than one tuber per stolon. However, the expression of levan sucrase from *Erwinia amylocora* which by producing the polymer fructan was thought to be able to reverse the accumulation of sugars only resulted in a further decrease in yield and in higher fructose levels (Röber et al., 1996). Recently Sonnewald et al., (1997) have shown that, under greenhouse conditions, cytosolic expression of a yeast invertase in potato leads to a reduction in tuber size and potato yield. In contrast when the invertase was targeted to the extracellular spaces, larger and fewer tubers than those from control plants were produced. Sonnewald et al. (1997) have put forward two hypotheses to account for the changes in tuber size. The first hypothesis is based on the reduced turgor pressure due to

breakdown of sucrose in the extracellular spaces leading to sucrose unloading through the plasmodesmata. The converse would happen when invertase was expressed in the cytosol. The second hypothesis looks at glucose accumulated in the extracellular spaces as being capable of acting as a signal to trigger cell division leading to tuber enlargement while glucose accumulated in the cytosol apparently does not have the capacity to act as a signal molecule. Much work still needs to be carried out to determine how tuber size is regulated. What is clear from our results and those of others is that the accumulation of sugars and their location within the tuber (sink) has a profound impact on tuber size and yield.

The accumulation of fructans in starch deficient tubers (Röber et al., 1996) is an example of one way in which starch forming plants can be modified to store sucrose derivatives. It will, however, not be possible to knock out the AGPase activity completely by just inhibiting the synthesis of the AGPase B subunit. There will always be some residual AGPase activity due to the presence of isoforms of the small subunit in many plant species, which may be capable of combining with the large subunit to form an active heterotetrameric enzyme. In addition there is always the possibility that an active homotetrameric enzyme may be formed by the large AGPase subunit as shown by the expression of each subunit alone in *E.coli* (Igleasias et al., 1993). There is great potential to use the cloned cassava AGPase B cDNA to modify starch synthesis in cassava, in order to influence productivity as well as to produce new storage sinks.

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Pinpointing towards improved transformation and regeneration of cassava (*Manihot esculenta* Crantz)

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submitted

Pinpointing towards improved transformation and regeneration of cassava (*Manihot esculenta* Crantz)

Abstract

Friable embryogenic callus (FEC) of the cassava genotype 60444 was transformed by particle bombardment with DNA from the plasmid constructs pHB1 and pJIT100. Both plasmids contained the luciferase (LUC) marker gene under the control of the CaMV 35S promoter. In addition pJIT100 had the CaMV35S driven phosphino acetyl transferase (PPT) gene while pHB1 contained the cassava cDNA coding for the small subunit of ADP glucose pyrophosphorylase (AGPase B) in antisense orientation under the control of a double CaMV35S promoter. Two weeks after bombardment LUC positive FEC units (spots) were isolated and subcultured separately for further proliferation. Four weeks later those cultures having at least 4 positive LUC spots were subjected to three different selection regimes namely: stringent LUC selection, non stringent LUC selection and combined LUC/phosphinothricin (PPT) selection. Sixteen weeks after bombardment, stringent LUC selection gave rise to cultures in which 92% of the FEC units were LUC positive. Within the same time period non stringently LUC selected cultures and LUC/PPT selection had only 1% and 41% of the units being LUC positive, respectively. The number of LUC positive mature embryos formed was directly proportional to the percentage of LUC positive FEC units, within a culture, found with each selection method. Stringent LUC selection enabled transgenic plants to be produced in 28-36 weeks compared to 32-41 weeks for LUC/PPT selection and 53-78 weeks for non stringent LUC selection. This indicates that stringent selection is a more efficient and reliable method for obtaining transgenic cassava plants. Southern blot analysis of transgenic cassava plants revealed that they had between one to seven copies of the pHB1 and pJIT100 construct. The production of the first cassava plants carrying an agronomically important trait affecting starch biosynthesis is reported. Expression of the antisense AGPase B gene resulted in cassava plants with extremely low levels of starch, compared to control plants, as shown by iodine staining of *in vitro* thickened stems. In plants exhibiting the highest AGPase B antisense effect, starch formation was limited only to the epidermal layer of *in vitro* thickened stems.

Introduction

The cultivation of cassava, a major crop in the tropics, is beset by many problems including diseases, pests, the high cyanide content of the roots and the low nutritional quality and commercial value of the starch. Improvement of the cassava germplasm by traditional breeding methods has been hampered by the non-availability of necessary genes in the germplasm, the alloploidy nature of the plant, and the low fertility of cassava, amongst other factors (for reviews see Thro et al., 1996 and references therein). The newer techniques of genetic transformation, whilst offering greater hope for improvement of cassava, have in the past been hindered by the lack of a reproducible transformation and regeneration system for this crop (for review see Raemakers et al., 1997a). The breakthrough came with the development of systems of regeneration based on friable embryogenic callus (Taylor et al., 1996) or on adventitious shoot formation (Li et al., 1995). This enabled certain cassava genotypes to be transformed using particle gun delivery of DNA into friable embryogenic callus (FEC) cultures (Schöpke et al., 1996, Raemakers et al., 1996) as well as electroporation of FEC derived protoplasts (Raemakers et al., 1997b). In addition Li et al. (1996) have described a method that allows transformation of cassava with *Agrobacterium tumefaciens* infection of cotyledon explants cultured for adventitious shoot formation.

The methods used for selecting transgenic tissue, in FEC transformation, can basically be divided into procedures involving: 1) chemical selection with aminoglycosides such as paromomycin (Schöpke et al., 1996); 2) non invasive selection with the firefly luciferase gene (Raemakers et al., 1996); and 3) a combination of chemical selection with phosphinothrycin and use of the luciferase marker gene (Snepvangers et al., 1997). While these methods have established the route by which cassava will be transformed in future they are not, however, optimally efficient for universal application. The main disadvantages of the methods being that they are genotype dependent, time and labour consuming, inefficient with respect to selection and /or regeneration.

The FEC cultures used in the above mentioned studies were derived from the African cultivar called 60444. This cultivar was developed in a breeding programme led by Beck in the 1950's using material originating from Ghana and East Africa during the same period (Dr R. Dixon, IITA Nigeria, personal communication). Successful use of FEC derived from only this genotype means that much work still remains to be done to produce FEC from the important cassava cultivars world-wide.

For each of the methods described above it took from 8 months to more than a year to produce transgenic plants after the initial transformation procedure. This invariably implies that a lot of labour goes into maintaining the cultures on fresh media. The longer the time spent in tissue culture, the greater too is the expense of the procedure. Chemical selection with aminoglycosides such as paromomycin seemed to have a negative effect on the ability of transgenic FEC to regenerate into plants (Schöpke et al., 1997). Use of the luciferase gene for selection of transformed tissue remained labour intensive, since no selective advantage was given to the transgenic tissue over the non transgenic tissue. The use of the selection agent phosphinothricin (PPT) while not being detrimental to the ability of FEC to form plants, however, led to escapes and added extra steps to the whole procedure of producing transgenic plants (Snepvangers et al., 1997). There was a need to obtain an effective, reliable and less labour intensive transformation and regeneration method that can be applied universally.

All the transgenic plants derived from the afore mentioned experiments carry marker genes such as β glucuronidase (GUS), luciferase (LUC), phosphino acetyl transferase (PPT), hygromycin phosphotransferase (HPT) or neomycin phosphotransferase II (NPTII) and no genes which confer new traits to the cassava germplasm. The cassava gene encoding the small subunit of ADP glucose pyrophosphorylase has been cloned (Chapter 3, this thesis). Use of this gene in antisense orientation in potato resulted in plants having reduced starch and elevated sugar levels in their tubers (Chapter 4, this thesis). This opened the possibility of increasing the commercial and nutritional value of cassava tubers by producing elevated levels of products such as fructans, cyclodextrins, and nutritionally important proteins in cassava tubers deficient in starch. This paper describes improvements in the procedure of cassava transformation using only luciferase selection and regeneration of FEC into plants. The plants produced carried the cassava AGPase small subunit gene in antisense orientation, in addition to the luciferase selection marker gene.

Materials and Methods

Plant material and media

The FEC culture used in the experiment was from the genotype 60444 (International Institute of Tropical Agriculture) and was kindly provided by Dr N. Taylor. The following media were used: **Solid FEC proliferation medium** (GD2) which consisted of Gresshof and Doy (1972) salts and vitamins (1972), 8 g/l micro agar, 20 g/l sucrose and 10 mg/l picloram, pH adjusted to 5.7 before autoclaving; **Liquid FEC proliferation medium** (SH6) made up of Schenk and Hildebrandt (1972) salts and vitamins, 60 g/l sucrose and 10 mg/l picloram, pH adjusted to 5.7;

Liquid (SH6) to solid (GD2) transfer medium (GD4) is identical to GD2 with 40 g/l sucrose added; **Maturation media** made up of Murashige and Skoog (1972) vitamins and salts, 20 g/l sucrose (MS2), 8 g/l Micro agar and 1 mg/l NAA or 1 mg/l Picloram; **First cycle secondary somatic embryogenesis medium** consisted of solid MS20 plus 8 mg/l 2,4D; Second cycle secondary embryogenesis medium was made up of liquid MS2 and 1 mg/l NAA; **Germination medium** made up of MS2 plus 1 mg/l BAP; **Rooting medium:** the shoots were rooted on solid (8 g/l Micro agar) MS2 medium.

Constructs and particle bombardment

Standard molecular biology techniques were used in DNA manipulations (Sambrook et al., 1992). The constructs used were pHB1 (8.0 kb) and pJIT100 (6.7 kb). The plasmid pJIT 100 contained the gene coding for luciferase (LUC) and phosphino acetyl transferase (PPT) both under the control of the CaMV 35S promoter and terminated by the CaMV polyadenylation region (Guerineau and Mullineaux, 1993). This construct was kindly provided by J. Guerineau of the John Innes Research Institute Norwich, U.K. Plasmid pHB1 (Fig. 1) was made by introducing a 1.3 kb fragment of the cassava AGPase B cDNA (cut with BamHI/HindIII from plasmid pB45-1) in antisense orientation (HindIII 5' and BamHI 3') between the CaMV 35S promoter and the LUC gene of pJIT 100. Both constructs contained the ampicillin resistance gene for selection of bacteria carrying these plasmids. For particle bombardment 20 μg of plasmid DNA was coated on to 10 μg of gold particles having an average size of 1.6 μm. The method of coating the gold particles and bombardment of the FEC cultures was as described by Raemakers et al. (1996).

Plasmid pHB1 was used together with pJIT100 for the transformation

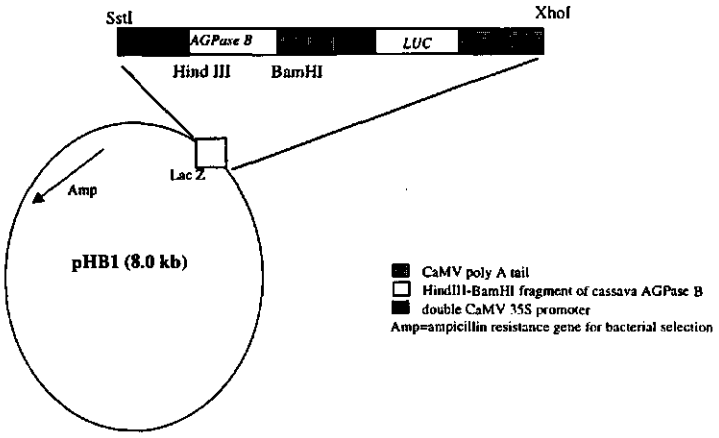

Figure 1: Construct pHB1 carrying the cassava antisense AGPase B gene.

A 1.6 kb BamHI and HindIII fragment of the cassava AGPase B gene from pB45-1 (Chapter 3, this thesis) was cloned in antisense orientation into the HindIII and BamHI site of pJIT100 (Guerineau and Millineau, 1993) behind a CaMV35S promoter. Selection of transgenic tissue was based on the light emitting activity of the luciferase enzyme, encoded by the luciferase (LUC) gene

experiments. The main interest, however, was in the plants transformed with pHB1 because these contained an agronomically important gene (cassava AGPase B) unlike pJIT100 derived plants with only the marker/selection genes.

Luciferase assays

In order to select transgenic tissue, the firefly luciferase gene was used as a reporter gene. The luciferase gene encodes the enzyme luciferase (LUC) which converts the substrate luciferin into oxyluciferin in the presence of ATP, O_2 and Mg^{2+} emitting light (de Luca and McElroy, 1978). Cassava FEC cultures, mature embryos or plants were sprayed with 0.25 mg/ ml luciferin (Promega, E160). LUC activity was determined by measuring the amount of photons emitted by the explants, using a VIM intensified CD camera and an Argus-50 photon counting image processor (Himamatsu Photonic Systems). Superimposition of the electronic image of LUC activity with the normal image of the explant enabled the LUC positive tissue to be identified

and isolated.

Selection of transgenic tissue

Prior to bombardment new suspension cultures were initiated by transferring established FEC cultures from solid GD2 into liquid SH6 proliferation medium for two weeks. The new suspensions were then collected and spread out on solid (8 g/l Micro agar) SH6 medium. In total 50 petri-dishes were bombarded with pHB1 and 40 Petri-dishes with the construct pJIT100. The bombarded FEC cultures were then placed in 250 ml pots (diameter 10 cm) containing 50 ml of SH6 medium. After two weeks the cultures were collected on solid GD4

medium and assayed for LUC activity. Putatively transgenic tissue was identified as LUC positive spots. Each spot plus the surrounding tissue (about 1 cm diameter around the LUC positive spot) was subcultured separately and grown for a further four weeks in 250 ml pots (diameter 10 cm) containing 50 ml SH6 medium. Those cultures in which the number of LUC positive spots had increased to more than 4 were used for the following selection regimes:

a) Non stringent luciferase selection

Non stringent selection was applied on 16 cultures bombarded with DNA from pHB1 and 10 cultures bombarded with pJIT100. For this selection procedure LUC positive FEC cultures were subcultured on GD2 medium and every 2 weeks LUC positive tissue plus the surrounding tissue (0.5 to 1 cm diameter around the LUC spot) was subcultured. After 10 weeks (16 weeks after bombardment) the total LUC activity of the cultures as well as the proportion of LUC positive FEC units was determined.

b) Stringent luciferase selection (pinpointing)

Stringent LUC selection was applied on 8 cultures bombarded with pHB1. For this selection procedure LUC positive FEC cultures were subcultured on GD2 medium (discarding non transgenic FEC). Two weeks later LUC positive tissue plus the surrounding (0.5 to 1 cm diameter) was again selected and divided as fine as possible in individual FEC units. These selected embryogenic units were grown for a further two weeks on GD2 medium and then assayed for LUC activity. Because the FEC had been

spread out finely on the plates individual LUC positive FEC units in each culture could be pinpointed microscopically, isolated, collected

together on fresh GD2 medium (positive selection). The cultures were grown for a further six weeks on GD2 medium and assayed every 2 weeks. Sixteen weeks after bombardment total LUC activity and the proportion of LUC positive FEC units was determined.

c) combined luciferase and PPT selection

Six weeks after bombardment 10 FEC cultures, transformed with pJIT100, were placed on GD2 medium containing 20 mg/l PPT. Every two weeks LUC positive tissue was identified and subcultured on to fresh medium. This process was repeated for 10 weeks after which the proportion of LUC positive tissue in each culture was determined.

Maturation of FEC

Two sets of experiments were conducted to determine the best conditions of maturation of the transgenic and non transgenic FEC. In the first set of experiments 0.1 g of FEC, derived sixteen weeks after bombardment from all three selection regimes, was subcultured in Petri-dishes on solid MS2 medium (8 g/l Micro agar) supplemented with 1 mg/l NAA (Schöpke et al., 1997) or 1 mg/l Picloram (Raemakers et al., 1997c) and 0, 0.01, 0.1, 1 and 10 mg/l ABA. The cultures were transferred after 4 weeks to medium without ABA.

In the second set of experiments, FEC derived from non stringent selection was further cultured and assayed for LUC activity for 10 weeks. After which highly LUC active FEC was transferred to liquid proliferation medium for 2 weeks. Then it was cultured on solid MS2 medium plus 1 mg/l BA for maturation.

All FEC cultures were transferred to fresh maturation medium every two weeks until mature embryos appeared (up to 12 weeks). The mature embryos were harvested and assayed for LUC activity.

Germination of mature embryos

LUC positive mature somatic embryos were first multiplied on solid MS2 + 8 mg/l 2,4 D and then grown in liquid MS2 + 10 mg/l NAA. The resulting mature somatic embryos were desiccated and then cultured for germination on MS2 + 1 mg/l BA as described by

Raemakers et al. (1996).

Southern and Northern analysis

DNA, RNA, and protein from LUC positive cassava leaves were isolated, blotted and assayed as described in Chapter 2. The LUC and cassava AGPase B genes labelled with ^{32}P dCTP were used to probe Southern and Northern blots respectively.

Iodine staining of *in vitro* thickened stems

Thickened stems were induced by growing transgenic and non transgenic cassava plants *in vitro* for 4 weeks on solid MS medium supplemented with 80 g/l sucrose as described by Salehuzamman et al. (1993). The presence or absence of starch was visualised by iodine staining of cross sections of the *in vitro* thickened stems with Lugol's solution (I_2:KI). The stained stem sections were visualised microscopically.

Results

Effect of selection regime on the proportion of luciferase positive tissue

Two weeks after bombardment LUC assays revealed a mean number of one LUC positive spot per pHB1 culture and two LUC positive spots per pJIT100 bombarded culture. Each spot was cultured separately and grown for the next four weeks in liquid SH6 medium. At the end of that period 26 of the 50 pHB1 and 46 of the 80 pJIT100 LUC positive spots, had disappeared and, 24 pHB1 and 18 pJIT100 cultures had more than four LUC positive spots (indicating continued growth of the transgenic tissue). The pHB1 bombarded cultures were used in the experiments with the stringent and also the non stringent LUC selection regimes. Ten of the 20 pJIT100 bombarded cultures were used for the combined LUC/PPT selection and for the non stringent LUC selection.

After ten weeks of selection (16 weeks after bombardment) dramatic differences were observed in the LUC activities of the cultures. Stringently selected cultures had 50 to 100 times higher LUC activity than non stringently selected cultures and seven times higher LUC activity than cultures from the combined LUC and PPT selection (Table 1).

Table 1: The effect of selection regime (non stringent and stringent luciferase selection, and combined luciferase and phosphinothricine based selection) on the efficiency of producing transgenic cassava plants by particle bombardment with the constructs pHB1 or pJIT100.

Selection Regime applied	# of cultures used	Total LUC activity/dish (photons/min) after 10 weeks selection	%LUC+ FEC units/dish	Mature somatic embryos # tested	%LUC+	Time taken to form transgenic plant (weeks)
Non stringent LUC (pHB1)	16	$0.04.10^6$	< 1	1250	<1	53-78
Stringent LUC (pHB1)	8	$1.91.10^6$	92	432	90	28-36
LUC/phosphinothricin (pJIT100)	10	$0.29.10^6$	41	638	45	32-41
Non stringent LUC (pJIT100)	10	$0.02.10^6$	< 1	845	<1	53-78

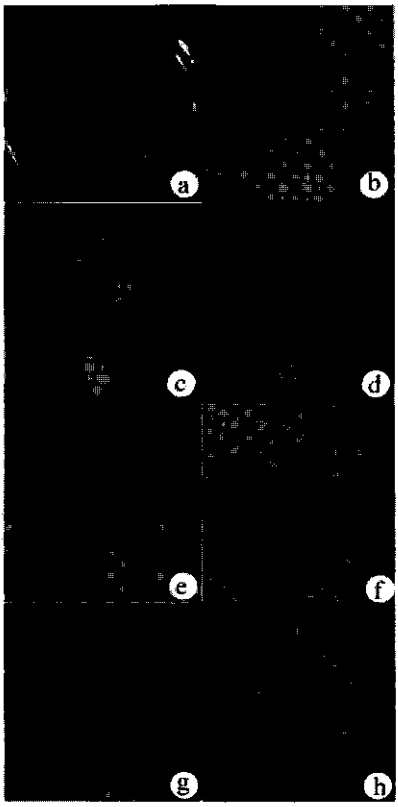

Figure 2: Stringent luciferase selection for the production of transgenic cassava friable embryogenic callii (FEC) and plants.

Panel a: A friable embryogenic culture (BH6) bombarded with the construct pHB1, expressing luciferase, two weeks after bombardment. **Panel b**) close up (magnification X 20) of luciferase positive FEC (8 weeks after bombardment) that was selected from the surrounding non transgenic FEC and spread out finely on solid GD2 for further culture. **Panel c**) view of stringently selected and well spread out FEC units under normal light, 16 weeks after bombardment. The precise pinpointing of LUC positive FEC units was possible, as shown in **panel d**. Using stringent selection it was possible to obtain cultures with >95% LUC positive FEC units, 16 weeks after bombardment as shown in **panel e** (normal view) and **panel f** (viewed in the dark, after adding luciferin. Following mature embryo formation and germination stringently selected cultures gave rise to luciferase positive plants shown under normal light (**panel g**) and expressing luciferase (**panel h**)

Microscopic examination of the cultures revealed that over 90% of the FEC units in a stringently selected culture were LUC positive. This is considerably higher than the 45% LUC positive FEC units obtained in combined LUC and PPT selection. Non stringent selection gave rise to cultures with less than 1% LUC positive FEC units. The process and results of the stringent LUC selection regime are shown in Fig. 2a-h.

Mature embryo formation and regeneration of transgenic cassava shoots

Regeneration of plants from FEC starts with the formation of mature somatic embryos. The percentage of LUC positive mature embryos after using the different selection regimes was directly proportional to the percentage of LUC positive FEC units (Table 1). An amount of 0.1 g FEC cultured on maturation medium supplemented with 1 mg/l NAA or 1 mg/l Picloram yielded 66 and 124 mature embryos respectively while the addition of ABA did not have a positive effect on this number (results not shown).

For mature embryo formation the best results were obtained with FEC cultures that were grown first in liquid SH6 medium without refreshing for two weeks and then transferred to maturation medium. The FEC units became primed for maturation i.e. instead of initiating new FEC units they became bigger. These primed cultures gave rise to high numbers of mature embryos.

About half of the mature embryos had a morphology similar to that observed for mature embryos derived from secondary somatic embryogenesis (Raemakers et al., 1993). The mature somatic embryos possessed two cotyledons that were in most cases fused together. Malformations that were observed included mature embryos with pinnulate, oval, and serrated cotyledons. In many cases a Petri-dish (with FEC derived from one transgenic line) contained predominantly mature somatic embryos from one aberrant type while another Petri-dish, from the same transgenic line and on the same medium contained normal looking mature embryos. Although this was not investigated systematically there seemed to be a relation between the morphology of the mature somatic embryos and their ability to form secondary embryos. However, all transgenic lines yielded secondary embryos that were cultured for germination

Efficiency of production of transgenic plants

The time required to obtain transgenic plants was dependent on the amount of transgenic FEC necessary for mature embryo formation and the time taken for mature embryo

Table 2: Identity of transformants obtained after introduction of the plasmids pHBI and pJIT100 into cassava friable embryogenic callii.

Plant	Stage of Regeneration	Minimum # of inserts	Starch Inhibition Level	Morphology of regenerants # of plants in parenthesis
HB-1	plant	4	I	D
HB-2	plant	1	I	C
HB-3	plant	1	I	N
HB-4	plant	1	II	C
HB-6	plant	1	II	C
HB-7	plant	2	II	A
HB-8	plant	7	II	N
HB-9	plant	2	II	N
HB-10	plant	1	II	N
HB-11	plant	1	II	C
HB-12	plant	1	II	D
HB-13	plant	2	III	N
HB-14	plant	1	III	D
HB-15	plant	1	III	N
HB-16	plant	1	III	D
HB-17	plant	2	nd	D
HB-18	plant	3	nd	D
HB-19	plant	1	nd	A
HB-20	plant	nd	nd	N
HB-21	plant	nd	nd	C
HB-22	mature embryos	1	nd	nd
HB-23	mature embryos	6	nd	nd
HB-24	mature embryos	nd	nd	nd
JT1-20	plants	1-6	III	N (10), A (1) B (2) C (4) D (3)
FEC1-20	plants	0	III	N (10), A (3), B (7)
60C1-20	plants	0	III	N (20)

Key: Starch inhibition level :

I - completely inhibition in cortex region, some starch in endodermal layer

II - incomplete inhibition in cortex region No inhibition in endodermal layer

III - no inhibition in cortex and endodermal regions

Morphology of regenerated plants

N = normal
A = zig-zag
B = curled leaves
C = thick stems and numerous shoots
D = stunted growth with tiny leaves

formation and germination. The chance that a mature embryo was transgenic was the only factor that differed amongst the three selection regimes used. Using stringent LUC selection it was possible to get large numbers of transgenic FEC and mature embryos within a period of 16 weeks compared to those obtained by the other two selection regimes (Table 1). Stringent selection led to a reduction in the time required to obtain transgenic FEC and mature embryos. The time required to produce transgenic plants with stringent selection and improved maturation and germination was only 28-36 weeks compared to 32-41 weeks for LUC/PPT and 53-78 weeks for non stringent selection (Table 1).

Morphology of transgenic plants

In total 21 of the 24 pHB1 luciferase positive cultures (Table 1) yielded a transgenic plant carrying the LUC selection marker gene. The other three lines are currently cultured for germination. The morphology of these plants was compared with those from three control groups, that is: 20 pJIT100 plants carrying the luciferase gene (AGPase control), non transgenic FEC derived plants (regenerant control), and non transgenic controls propagated *in vitro* via cuttings (overall control). Most of the transgenics and the regenerant controls did not grow as vigorously as the overall control. Some of the transgenics had an aberrant growth type: highly branched (probably due to a carry-over effect of BAP as this phenotype disappeared with subsequent micropropagation), zig-zag stem (overall control had a straight stem), plants with curled leaves, and fleshy thick stems with small shoots. All these phenotypes, except the last one, were also observed in the regenerant control plants. None of the aberrations was observed in the overall control *in vitro* propagated by cuttings. Ninety percent of the overall control and 40% of the transgenics and the regenerant controls survived the transfer to the greenhouse

Evidence of an antisense AGPase effect in cassava stems

The introduction of the AGPase B gene in antisense orientation is expected to result in a decrease in AGPase B expression. This should have a negative impact on starch formation, as reduced levels of AGPase would be available for ADP-glucose formation. To test this expectation it was necessary to check for starch formation in cassava roots. However, in the greenhouse it takes at least four months before any substantial cassava roots are formed. It was necessary to analyse some other part of the antisense AGPase B plants that had large amounts of starch and for this purpose *in vitro* thickened stems were

used.

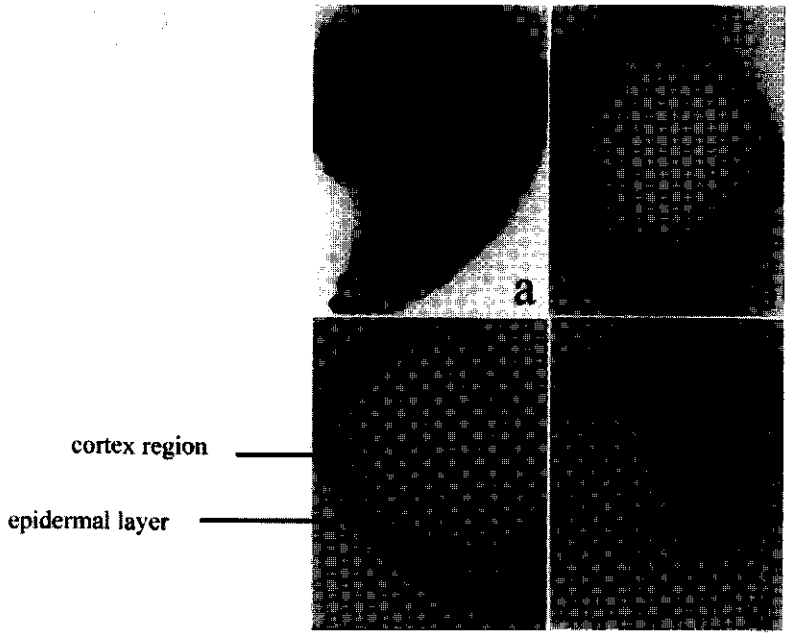

Figure 3: *Iodine staining of in vitro thickened stems of cassava plants containing* the pHB1 construct

a) Cross section of the *in vitro* thickened stem of HB-10, a category III plant stained with iodine. Note the strong staining of all tissue layers in the cortex and epidermal areas indicating the presence of starch. b) Category II transgenic plant (HB-10) had little or no staining in the cortex region but intense blue / black staining in the epidermal layer. c) category I transgenic plants (example is HB1) had no staining in the cortex region and very few starch granules in the epidermal region as shown in the close up (magnification X 50) transverse section in panel d.

Sections of induced thickened stems of 16 of the 21 antisense AGPase B plants and controls (20 pJIT100 transgenics, 20 non transgenic regenerant and 20 overall control plants) were stained with iodine to determine the presence or absence of starch. The antisense AGPase B plants could be divided into three categories based on iodine staining of in vitro thickened stems, as shown in Fig. 3. Of the 16 transgenic plants containing the AGPase B antisense gene there were four which belonged to Category III (Table 2). These plants exhibit staining characteristics similar to the three groups of control plants i.e. all tissue in the cortex region and epidermal area stained blue / black with iodine (Fig. 3a).

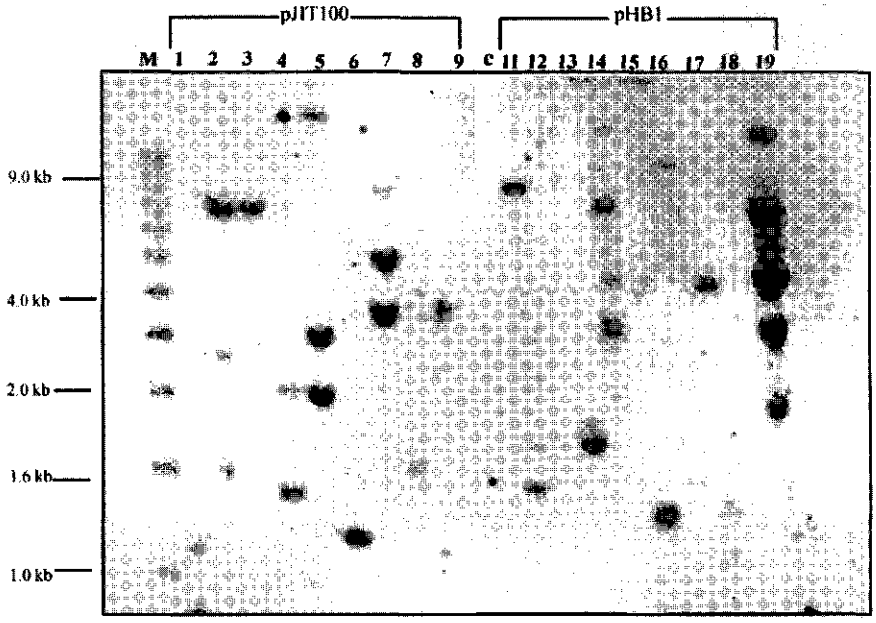

Figure 4: Southern blot analysis of transgenic cassava plants containing the plasmids pJIT100 and pHB1.

An amount of 10 μg of DNA was applied per lane, blotted on to a hybond filter and hybridised with a ^{32}P labelled 1.5 kb luciferase gene. M= 1 kb DNA marker lane. Sizes are as shown on the left hand side of the panel. C = control DNA from non transgenic in vitro propagated 60444 plants. Lanes 1-9 contain DNA from the transformants J1 to J9. Lanes 11 to19 contain DNA from the transformants HB 10, HB19, HB13, HB8, HB2, HB5, HB3, HB18, HB23. DNA from all the transformants was derived from leaves of in vitro plantlets except for lane 19 (HB23) where DNA isolated from mature embryos was used

There were nine plants (56%) designated as category II antisense plants (Table 2) with little or no staining in the cortex region but retaining more stain in the epidermal layer (Fig. 3b). Category I plants of which there were three transgenic plants (Table 2) had no staining at all in the cortex region (Fig. 3c). They showed some staining in the epidermal layer which was less than 10% of that found in category II sections. When examined microscopically only a few iodine staining starch granules were found in the cells of the epidermal layer as shown in Fig 3d for plant HB1.

Staining of the induced *in vitro* thickened stems, revealed that in total 74% of the AGPase B antisense plants had reduced levels of starch compared to the three groups of control plants. The number of pHB1 inserts in the antisense plants was determined by

Southern analysis using the LUC gene as a probe. This revealed that the plants contained between one to seven inserts of the AGPase B antisense construct and between one to six inserts of the pJIT100 construct (Fig. 4). There was no clear correlation between a high number of pHB1 inserts and the degree of antisense effect (Table 2).

Discussion

Twenty one independent transgenic cassava plants were produced by particle bombardment mediated transformation of cassava 60444 FEC with the plasmid pHB1. This plasmid carried the cassava starch gene AGPase B in antisense orientation under the control of a double CaMV 35S promoter. This is the first reported transformation of cassava with a gene conferring a new trait to the crop other than the previously described transgenic cassava plants, which carried reporter or marker genes (Li et al., 1996; Raemakers et al., 1996, Snepvangers et al., 1997, Schöpke et al., 1996). Southern analysis revealed that these plants carried between one and seven inserts of the pHBI gene construct. LUC assays conducted on the plants and explants showed that they were indeed transgenic. In actual fact it was even possible to visualise the light emitted by these plants in the dark, upon spraying them with luciferin, using the naked eye. This phenomenon has not been previously reported by other researchers working with the LUC firefly gene in plants. This result is clearly correlated to the high activity of the luciferase enzyme in the antisense AGPase cassava plants.

The time taken to produce transgenic cassava plants using the improvements in stringent LUC selection and improved maturation of transgenic FEC was only 28-36 weeks compared to 32-41 weeks for combined LUC/PPT selection and to 53-78 weeks for non stringent LUC selection. The stringent selection procedure based on two cycles of microscopic identification, selection and growth of LUC positive FEC units was found to have clear advantages over the methods used previously of non stringent LUC selection (developed by Raemakers et al., 1996) and LUC/PPT selection (Snepvangers et al., 1997), see Table 1. An MS2 medium supplemented with 1mg/l NAA did not improve embryo maturation, as compared to MS2 medium supplemented with 1 mg/l picloram.

A higher efficiency rate of maturation was accomplished by using FEC which were "primed" for maturation. This was done by leaving the FEC cultures in liquid proliferation medium without refreshing, over a period of two weeks, and then transferring

the cultures on to maturation medium. These primed cultures gave rise to relatively high numbers of mature embryos. Further research is required to determine whether this priming effect is due to nutrient starvation or to density.

Most of the trangenic and non trangenic FEC derived plants did not grow as fast as the overall control, in vitro propagated, plants. In about 50% of the cases continued in vitro propagation resulted in growth rates similar to those of overall control plants. The other 50% of the plants had more serious aberrations such as zig-zag stems, stunted growth, curly leaves, thick stems and numerous small shoots. The latter two were only observed in the transgenics. It remains unclear whether or not this is due to the introduction of the gene constructs, or to somaclonal variation induced by the regeneration process.

The transgenic plants containing the construct pHB1 potentially contain the AGPase B gene in antisense orientation. The AGPase B gene encodes the small subunit of the heterotetrameric AGPase enzyme. This enzyme plays a critical role in starch formation where it is involved in forming ADP-glucose from ATP and glucose-1-phosphate (Preiss et al., 1991). Suppression of the expression of this gene would lead to reduced levels of the enzyme and ultimately to a reduction in starch formation. Analysis of the starch present in *in vitro* thickened stems of the antisense AGPase B plants revealed that 74% of the transgenic plants had reduced levels of starch compared to the control non transgenic and pJIT100 transformed plants. This functionally confirms the identity of the AGPase B cDNA cloned from a cassava tuber specific library (Chapter 3, this thesis). It is also a clear indication that the introduction of the antisense AGPase B gene blocks the formation of ADP-glucose, the major glucosyl donor for starch formation (Preiss et al., 1991). The high frequency (74%) of transgenic plants exhibiting an antisense effect is in stark contrast to what was observed when the same cDNA was introduced into potato where the frequency of plants exhibiting an antisense effect was only 5% (as judged by reduced starch levels), [Chapter 4, this thesis]. This difference in response can be attributed to the reduced sequence homology between the cassava and potato AGPase B genes of only 68% hence probably the reduced response when the heterologous cassava gene was introduced into potato.

Interestingly in the three plants exhibiting the highest antisense effect a cell layer within the stem still had some starch. This could be due to the fact that the CaMV35S promoter is not active in this tissue layer. The reduction in starch levels in AGPase

antisense cassava plants is similar to what was observed with the introduction of antisense cassava AGPase B in potato. This gave rise to plants with reduced starch levels and increased levels of fructose and glucose in their sugars (Chapter 4, this thesis). Similar reductions in starch levels and increase in glucose levels had been reported in potatoes carrying the antisense potato AGPase B gene (Müller-Röber et al., 1992). From the antisense work in potato it can be inferred that sweet cassava plants have been produced. This opens the way to be able to utilise such sweet cassava plants for sugar production in much the same way as sugar beet is used in Europe. The cassava plants may also be made to produce novel compounds such as fructans or cyclodextrins using the excess soluble sugars available.

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General Discussion

6

Cassava Biotechnology: Impact of growing transgenic cassava in the field

Cassava (*Manihot esculenta* Crantz) is a crop that in the very recent past has discarded its label as a poor man's crop. This has been brought about not only because of the recognition of its importance in the tropics where it is a staple food for millions but also because of its growing use in starch based industries such as the food and beverages industries amongst others. As a result, the study of cassava starch metabolism, diseases and pests, and other factors affecting cassava production have thus been stimulated. This revival period for cassava research has coincided with great leaps in the field of plant molecular biology with gene cloning, and transformation being readily applicable techniques. This has enabled the formulation of novel strategies to improve the cassava production of existing varieties. Much emphasis in cassava research is focused on improved tissue culture techniques for rapid propagation of planting material and increasingly on the cloning and transformation of cassava with genes for resistance to viral and other diseases, quality and quantity of cassava starch, and the cyanide pathway (Thro et al., 1996).

Research into cassava starch biosynthesis has led to the cloning of the major genes involved in starch formation. These include the genes coding for: the large and small subunits of ADP-glucose pyrophosphorylase (Chapter 3), the two isophorms of granule bound starch synthase; GBSSI (Salehuzzaman et al., 1993) and GBSSII (Chapter 2), and branching enzyme (Salehuzzaman et al., 1992). Evidence from the characterisation of these genes and their products has revealed that, as expected, the process of starch formation, in cassava, is essentially the same as in other higher plants (Salehuzzaman et al., 1993, Munyikwa et al., 1997). Early verification of the biological role of these genes has been possible through transformation and analysis of transgenic potato plants. It has thus been possible in potato to produce plants with almost no amylose by expressing an antisense cassava GBSS cDNA (Salehuzzaman et al., 1993). Potato plants transformed with an antisense cassava AGPase B gene had reduced starch levels and stored more soluble sugars (Chapter 4). The possibilities are thus opened to use these genes to produce new cassava varieties with modified starch quality and quantity. Until recently it was not possible to transform and regenerate cassava. This has changed in the last year with the production of the first transgenic cassava plants by several research groups. These plants carry marker genes such as the luciferase (LUC) firefly gene, neomycin phosphotransferase gene (NPTII) and the β glucuronidase gene (GUS), (Raemakers et al.,

1996; Schopke et al., 1996, Li et al., 1996). The first transgenic cassava plants containing an agronomically important gene have recently been produced (Chapter 5, this thesis). These plants are the forerunners to new cassava varieties containing genes designed to improve existing cassava varieties.

Novel cassava varieties

In future it should be possible to produce cassava plants containing genes for resistance to viral, bacterial and insect disease. It will also be possible to produce cassava varieties with altered starch quality and quantity. Cassava varieties with low amylose may be produced through down regulation of GBSS. The starch from these plants would have a higher commercial value while also being beneficial nutritionally due to the low fat uptake of amylose free starch. Cassava varieties with high solid mass can be produced through expression of an AGPase gene modified in such a way that it would be largely insensitive to 3PGA/Pi regulation, hence increasing starch synthesis as has been shown for the *Escherichia coli* enzyme in potato (Stark et al., 1993). This would be beneficial for farmers who would have excess material for consumption as well as for selling to the starch industries. Expression in cassava of an antisense AGPase would lead to tubers having less starch and accumulating large amounts of soluble sugars. These starchless tubers could be used to produce high value products such as fructans and dextrins as has been done in potato. Expression of important nutrients such as vitamins in cassava would go a long way in improving the dietary value of the crop. Such a possibility has been demonstrated by the expression of an intermediate of provitamin A, phytoene, in rice endosperm (Burkhardt et al., 1997).

Transformation and plant breeding

All this is however dependent on the transformation and regeneration of cassava being universally applicable and reproducible. The method described by Li et al., 1996 makes use of of somatic embryos that are cultured for adventitious shoot organogenesis. This method is relatively easy as plants can be produced in one step. However, at this moment only one lab has managed to obtain transgenic plants using this method and at least some of the transgenic plants were chimaeric (Li et al., 1996).

The advent of the friable embryogenic callus (FEC) system (Taylor et al., 1996) and microprojectile mediated transformation of cassava has opened up another avenue by

which efficient transformation and regeneration of cassava can be achieved. However, the procedures developed up to date (Raemakers et al., 1996; Schopke et al., 1996) for transforming cassava were genotype dependent, time and labour consuming, and inefficient with respect to selection and /regeneration.

The ideal transformation and regeneration procedure is one in which the transgenic callus is easily selectable and regenerated into plants. Stringent luciferase selection of transgenic material transformed with constructs carrying the luciferase firefly gene offers the possibility to drastically reduce the time required for transformation and regeneration of transgenic plants (Chapter 5, this thesis). While the system is easily manageable this is currently offset by the initial high cost of the detection equipment required. This limits the applicability of this method to labs in developed countries or international research institutions. However, with time and improvements in procedures for detecting transgenic tissue, transgenic cassava plants will soon be produced in national research institutions in developing countries. Besides, cassava is a tropical crop so any field trials will certainly have to be carried out in tropical countries. Hence transgenic cassava crops will eventually find their way into the tropics.

The genotype 60444 while highly manageable for FEC production and transformation is actually not part of the current breeding population in many breeding programmes. This genotype was produced by Beck during the 1950s in a breeding programme involving cassava varieties from Nigeria and Ghana (Dr R.Dixon, IITA Nigeria, personal communication). Hence any new traits introduced by the transformation of 60444 will have to be transferred to existing varieties, by crossing, in order for new traits to be introduced into breeding material. However for 60444 this may prove to be difficult because observations of the number of chromosomes in some of the regenerants from transformation experiments with cassava AGPase B (Chapter 5) have revealed that they have 35 instead of 36 somatic chromosomes (Fig.1). These plants thus exist as aneuploids making it more difficult to cross with breeding populations. It will be necessary to produce and transform FEC from existing plant varieties which are already adapted for particular agro-ecological regions within the tropics.

Benefits and biosafety implications

There are clear advantages in introducing transgenic cassava in Southern countries. These have mainly to do with the introduction of engineered novel traits such as disease and pest

resistance and improved cassava tuber or starch production. This should lead to more stable productivity,

Fig. 1 DAPI stained chromosomes of cassava transformant HB18 showing 35 metaphase chromosomes instead of the expected 36 chromosomes found in most genotypes of *Manihot esculenta*

improved nutritional quality, and increased income for resource poor farmers in the south. Transgenic plants carrying herbicide resistance to biodegradable pesticides such as phosphinothrycin may help reduce the amount of chemicals which are used under normal circumstances enabling better environmental conditions to be created.

However, there are also some disadvantages of introducing transgenic cassava into Southern tropical countries. The main one is the lack of knowledge by the majority of people in these countries about genetically modified organisms (GMOs) and their products. People in southern countries have in the main not been sensitised about GMOs and have not been involved in any public discussions about biosafety and durability of GMOs. In contrast in Northern countries there exists various consumer groups, environmental organisations and regulatory offices all dealing with and giving voice to issues pertaining to transgenic crops.

Biosafety regulations are now on the statute books of several developing countries

including Thailand, Brazil, and South Africa. However, these regulations may not stop individuals bringing in transgenic plants illegally since this material in most cases does not differ from non transgenic material. There is also a danger of genetic flow whereby outbreeding of transgenic cassava with closely related relatives, such as other Euphorbia species, may result in the transfer of antibiotic or herbicide resistance genes into the wild. This may, in theory, lead to the development of new weeds which are difficult to control. Such a possibility of gene transfer has been demonstrated by Mikkelsen et al. (1996) who showed that it was possible for genes to flow quickly from a *Brassica napus* crop to its wild weedy relatives. For cassava, quantitative studies would need to be carried out to determine the potential gene flow into wild relatives of the crop before any large scale distribution of transgenic material can take place.

If transgenic crops become very successful in Southern countries this may force farmers to move from their normal practise of intercropping cassava with other crops such as cowpeas or beans (in Africa and Indonesia) to extended monoculture of transgenic cassava. This may in turn lead to wholesale breakdown of disease resistance in transgenic crops.

When transgenic cassava reaches the tropical southern countries there is no doubt about the profound effect these new varieties will have on the lives of the inhabitants of these countries. It is important that measures are taken by governments, international and national research institutions, and non governmental organisations to inform and initiate discussions with farmers, consumers and researchers about the advent, benefits and possible harmful effects of producing transgenic crops.

Intellectual property rights (IPR)

Lastly cassava is a crop which has been grown and bred over many generation by farmers in the developing countries. However, the development of transgenic cassava, and patenting of the genes, products and transformation processes (intellectual property rights) currently takes place in developed countries. It is important that some form of international legal arrangements are made to ensure that resource poor farmers in developing countries benefit from crops, like cassava, which after all they have nurtured over the centuries. This may involve the free transfer of transgenic material by commercial companies to communities as long as the material is being used for local consumption. Only when large-scale production for export is undertaken would farmers be obliged to pay for any IPR

levy. The money from such a levy could then be used to help bolster agricultural research in developing nations either through national or international research institutions.

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Summary

Cassava (*Manihot esculenta* Crantz) is a tropical crop grown for its starchy thickened roots, mainly by peasant farmers, in the tropics, for whom it is a staple food. There is an increasing demand for the use of cassava in processed food and feed products, and in the paper and textile industries amongst others. This thesis describes research on the cloning of the genes encoding ADP-glucose pyrophosphorylase small and large subunits (AGPase B and S, respectively) and granule bound starch synthase II (GBSSII). These genes and their products were extensively characterised to determine their role in starch biosynthesis in cassava. Functional verification of the genes was carried out by transforming potato and cassava followed by analysis of the starch produced by the transgenic plants.

In Chapter 1 cassava production in the world in general and in Zimbabwe in particular is examined against the backdrop of new cloning and transformation strategies to improve starch quality and quantity. The development of cassava cultivars whose starches have novel physico-chemical properties by genetic modification of the process of starch biosynthesis is examined therein. The main criteria for these new cultivars to emerge are set forth as being: the availability of cloned and characterised starch biosynthesis genes, a universally applicable transformation and regeneration procedure for cassava, transfer to appropriate cassava cultivars, and biosafety analysis of transgenic cassava plants before disbursement to farmers.

The cloning of the cassava starch biosynthesis genes encoding granule bound starch synthase II (GBSSII) and the large and small subunits of ADP-glucose pyrophosphorylase (AGPase) is described in Chapters 2 and 3. The cloning of GBSSII reveals that there is indeed a second isoform of this enzyme in cassava as in other plants species. While sharing very little amino acid sequence homology with cassava GBSSI the GBSSII isophorm shares high amino acid sequence homology to other GBSSII genes from pea and potato. Cassava GBSSII seems to be more important in leaf tissue where it is more highly expressed than in tuber tissue where GBSSI predominates. Mapping of GBSSII revealed that this is a single copy gene located on the male derived linkage group T of the cassava mapping population.

Cloning of the cassava genes coding for the small (B) and large subunit (S) of

AGPase revealed interesting aspects about the cassava enzyme. The cassava AGPase is likely to be heterotetrameric in constitution as had been found in other plant species. Comparison of the cassava AGPase sequences with those of already cloned AGPases revealed that AGPase B is more similar to small subunit genes from other plants than to cassava AGPase S coding for the large subunit (Chapter 3). Segregation analysis of a cassava mapping population revealed that AGPase S is a single copy gene that is localised on the female derived linkage group E of the cassava genetic map. Both genes are expressed in all cassava tissues but AGPase B was shown to have a higher steady state mRNA level than AGPase S especially in leaf and tuber tissue. Post-transcriptional control of small subunit polypeptide levels could be inferred from the discrepancy between AGPase B mRNA and polypeptide levels. The AGPase enzyme activity was much higher in young cassava leaves than older leaves and tubers. Cassava leaf AGPase activity was increased 3 fold by the addition of 3-PGA (3-phospho-glycerate) and inhibited by up to 90% in the presence of inorganic phosphate (Pi). The tuber enzyme was relatively unaffected by 3PGA, but was highly inhibited by Pi.

In order to verify the biological role of the AGPase B gene antisense constructs were made of the cassava AGPase B behind a CaMV35S promoter (chapter 3). This was transferred into potato plants by *Agrobacterium tumefaciens*. While the 224 transgenic antisense AGPase B potato plants did not differ in appearance from normal potato plants, 45 transgenic plants, however, had more numerous and smaller tubers than control plants. Antisense plants with reduced AGPase B mRNA levels had 1.5 to 3 times less starch than tubers from the control plants. The levels of the soluble sugars in the antisense plants increased significantly (up to 10 times more glucose, 6 times the amount of fructose, and 5 times the amount of sucrose) when compared to those found in control plants. These results show that a heterologous gene from cassava can have an antisense effect in potato, but that the number of plants required to find plants exhibiting maximum antisense effect has to be very large. This is probably due to sequence homology differences between the cassava AGPase B and potato AGPase B genes which share only 68% amino acid sequence homology.

Chapter 5 describes the further development of an efficient, time and labour saving protocol for transforming cassava based on stringent selection of the luciferase

(firefly) marker gene. In addition the first reported transformation of cassava with a gene (AGPase B) other than a marker gene is described. An antisense construct was made for transforming cassava. This consisted of the cassava AGPase B gene which was placed in antisense orientation behind the CaMV35S promoter. This was then coupled to the luciferase gene driven by another CaMV35S promoter. After particle bombardment of cassava FEC transgenic tissue was selected using three different selection regimes: non stringent luciferase selection, stringent luciferase selection and combined chemical (phosphinothrycin) and luciferase selection. Stringent luciferase selection whereby luciferase positive FEC units were precisely pinpointed, isolated and cultured was found to be the most effective and time saving method. It was possible to generate cultures having more than 90% luciferase positive FEC tissue after 12 weeks of stringent LUC selection, compared to 45% and <1% for combined selection and non stringent selection respectively. The number of luciferase positive mature embryos generated was directly proportional to the percentage of luciferase positive tissue in the original FEC culture. Stringent luciferase selection enabled the time taken for production of transgenic cassava plants to be reduced to 28-36 weeks as compared to 8 months to a year with no stringent selection or LUC/PPT selection.

Cassava plants carrying the AGPase B antisense gene had extremely low levels of starch, compared to control plants, as shown by iodine staining of in vitro induced thick stems. In plants exhibiting the highest AGPase B antisense effect, starch formation was limited only to the epidermal layer. These results functionally confirm the identity of cassava AGPase B as well as emphasising the critical role of AGPase in starch formation in cassava.

A discussion about the significance and implications of cloning cassava genes and producing transgenic cassava for culture in developing countries is carried out in Chapter 6. While there are clearly many economic and nutritional benefits to producing transgenic cassava, for resource poor farmers, many people in the South are not aware of the biosafety implications of growing transgenic crops. It is further emphasised that discussions and debate should be initiated to make local communities aware of the issues surrounding transgenic crops and their products. In addition it is recommended that some form of international legal framework be set up to ensure that resource poor

farmers are not disadvantaged by the patenting of material originating from their communities by individuals and companies in the North. This thesis clearly demonstrates how it will be possible in the near future to produce new cassava cultivars carrying the appropriate genes to affect pronounced changes on tuber productivity and starch quality.

Samenvatting

Cassave (*Manihot esculenta* Crantz) is een gewas dat geteeld wordt in de tropen door kleine boeren voor wie de zetmeelrijke verdikte wortels basisvoedsel zijn. Er is een toenemende vraag naar het gebruik van cassave bij onder andere de productie van levensmiddelen en diervoeders en in de textielindustrie. Dit proefschrift beschrijft het onderzoek naar het kloneren van de genen die coderen voor de kleine en grote subeenheid van ADP-glucose pyrophosphorylase (respectievelijk AGPase B en S) en korrelgebonden zetmeelsynthase II (granule bound starch synthase II, GBSSII). Deze genen en hun producten zijn uitvoerig gekarakteriseerd met betrekking tot hun rol in de zetmeelsynthese van cassave. De functie van de genen werd geverifieerd door transformatie van aardappel en cassave gevolgd door analyse van het zetmeel dat door de transgene planten werd geproduceerd.

In Hoofdstuk 1 wordt de cassave productie in de wereld, en in het bijzonder in Zimbabwe, bestudeerd tegen de achtergrond van nieuwe klonerings- en transformatietechnieken die verbetering van de zetmeelkwaliteit en -kwantiteit mogelijk maken. Ook wordt de ontwikkeling van cassave cultivars besproken waarvan het zetmeel nieuwe fysisch-chemische eigenschappen heeft door genetische modificatie van de zetmeelbiosynthese. De belangrijkste criteria voor de ontwikkeling van deze nieuwe cultivars zijn: de beschikbaarheid van gekloneerde en gekarakteriseerde genen uit de zetmeelbiosynthese, een universeel toepasbaar transformatie- en regeneratiesysteem voor cassave, toepassing in de meest geschikte cassave cultivars en een veiligheidsanalyse van transgene cassave planten voordat deze beschikbaar komen voor de landbouw.

De klonering van de cassave genen coderend voor zetmeelsynthase II (granule bound starch synthase II, GBSSII) en de kleine en grote subeenheid van ADP-glucose pyrophosphorylase (respectievelijk AGPase B en S) is beschreven in de Hoofdstukken 2 en 3. De klonering van GBSSII laat zien dat er in cassave een tweede isoform van dit enzym bestaat, zoals dat ook in andere plantensoorten werd gevonden. Terwijl de sequentiehomologie op aminozuurniveau met GBSSI erg laag is, vertoont GBSSII een hoge sequentiehomologie met de GBSSII genen van erwt en aardappel. In bladweefsel lijkt GBSSII van cassave een belangrijker rol te spelen dan GBSSI, aangezien GBSSII sterker tot expressie komt, terwijl in knolweefsel voornamelijk GBSSI wordt

aangetroffen. Door kartering is aangetoond dat er één kopie van het GBSSII-gen bestaat die op de genetische kaart van cassave is gelocaliseerd binnen koppelingsgroep T, die van mannelijke afkomst is.

Door de klonering van de cassave genen die coderen voor de kleine (B) en grote (S) subeenheid van AGPase zijn enkele interessante aspecten van dit enzym aangetoond. De AGPase van cassave is waarschijnlijk heterotetrameer van opbouw zoals ook voor andere plantensoorten is gevonden. Vergelijking van de cassave AGPase sequenties met die van al eerder gekloneerde AGPases heeft aangetoond dat AGPase B meer overeenkomst vertoont met genen voor de kleine subeenheid van andere planten dan met het cassave gen dat codeert voor de grote subeenheid (Hoofdstuk 3). Door splitsingsanalyse van een karteringspopulatie van cassave is aangetoond dat er één kopie van het AGPase S-gen bestaat die op de genetische kaart van cassave gelocaliseerd is binnen koppelingsgroep E, die van vrouwelijke afkomst is. Beide genen komen tot expressie in alle weefsels van de cassaveplant, terwijl AGPase B, vooral in blad- en knolweefsel, een hoger steady-state mRNA niveau bleek te hebben dan AGPase S. Uit de discrepantie tussen de AGPase B mRNA en polypeptide niveau's kon worden geconcludeerd dat het eiwitniveau van de kleine subeenheid onder post-transcriptionele controle staat. De AGPase enzymactiviteit was veel hoger in jong cassaveblad dan in ouder blad en knollen. De AGPase activiteit in blad nam toe met een factor 3 na de toevoeging van 3-phospho-glyceraat (3-PGA) en kon voor maximaal 90% worden geïnhibeerd in aanwezigheid van anorganisch fosfaat (Pi). Hetzelfde enzym uit de knol was relatief ongevoelig voor 3PGA, maar werd sterk geïnhibeerd door Pi.

Om de biologische rol van het AGPase B gen te bepalen werden antisense constructen gemaakt van het cassave AGPase B gen achter een CaMV 35S promoter (Hoofdstuk 3). Deze constructen werden ingebracht in aardappelplanten door transformatie met *Agrobacterium tumefaciens*. Terwijl de 224 transgene antisense AGPase B aardappelplanten in uiterlijk niet afweken van ongetransformeerde aardappelplanten, bleken 45 van deze planten meer en kleinere knollen te vormen dan controle planten. Antisense planten met gereduceerde AGPase B mRNA niveau's hadden anderhalf tot driemaal minder zetmeel dan knollen van de controleplanten. De hoeveelheid oplosbare suikers in de antisense planten nam significant toe (tot 10 maal meer glucose, 6 maal meer fructose en 5 maal meer sucrose) ten opzichte van

controleplanten. Deze resultaten tonen aan dat een heteroloog gen van cassave een antisense effect kan bewerkstelligen in aardappel, maar dat het aantal transformanten dat nodig is om planten met een maximaal antisense effect te vinden erg groot zal moeten zijn. Dit wordt waarschijnlijk veroorzaakt door de relatief lage sequentiehomologie (68%) tussen de AGPase B genen van cassave en aardappel.

Hoofdstuk 5 beschrijft de verdere ontwikkeling van een efficiënt transformatieprotocol voor cassave dat is gebaseerd op stringente selectie van het luciferase (vuurvlieg) merkergen. Bovendien wordt in dit hoofdstuk voor het eerst de transformatie van cassave met een ander gen (AGPase B) dan een merkergen beschreven. Voor de transformatie van cassave werd een antisense construct gemaakt waarin het cassave AGPase B gen in antisense oriëntatie achter de CaMV35S promoter werd geplaatst. Dit werd vervolgens gekoppeld aan het luciferase gen dat door een andere CaMV35S promoter wordt aangedreven. Na particle bombardment van cassave werd FEC (friable embryogeen callus) transgeen weefsel geselecteerd met behulp van drie verschillende selectie regimes: niet-stringente luciferase (LUC) selectie, stringente luciferase selectie en gecombineerde chemische (phosphinothrycin; PPT) en luciferase selectie. De meest effectieve en snelste methode bleek de stringente luciferase selectie te zijn, waarbij luciferase positieve FEC-units precies konden worden gelocaliseerd, geïsoleerd, en verder worden opgekweekt. Hierbij was het mogelijk om cultures te verkrijgen met meer dan 90% luciferase positief FEC-weefsel na 12 weken van stringente LUC selectie, tegenover 45% en <1% voor respectievelijk gecombineerde selectie en niet-stringente selectie. Het aantal luciferase positieve volgroeide embryo's stond rechtstreeks in verhouding tot het percentage luciferase positief weefsel in de oorspronkelijke FEC cultuur. Door stringente luciferase selectie werd de benodigde tijd voor de productie van transgene cassave planten verminderd tot 28-36 weken, een duidelijke reductie ten opzichte van de 8-12 maanden die nodig zijn voor niet-stringente selectie of LUC/PPT selectie.

Cassaveplanten met het antisense AGPase B gen bleken, na jodiumkleuring van in vitro geïnduceerde verdikte stengels, extreem weinig zetmeel te bevatten in vergelijking met controle planten. In de planten met het sterkste AGPase B antisense effect bleef de zetmeelvorming beperkt tot de epidermale laag. Deze resultaten bevestigen de functionele identiteit van cassave AGPase B en tonen tevens de essentiële rol van AGPase in de zetmeelvorming in cassave aan.

Een discussie van de significantie en de implicaties van het kloneren van cassave genen en de productie van transgene cassave voor de teelt in ontwikkelingslanden staat beschreven in Hoofdstuk 6. Terwijl er duidelijk veel economische en voedingskundige voordelen zitten aan de productie van transgene cassave door kleine boeren, zijn weinig mensen in het Zuiden zich bewust van de veiligheidsaspecten van de teelt van transgene gewassen. Benadrukt wordt dat discussies en debatten geïnitieerd zullen moeten worden om de lokale gemeenschappen bewust te maken van vraagstukken gerelateerd aan transgene gewassen en hun producten. Een andere aanbeveling is het opzetten van een internationaal wettelijk kader om te garanderen dat de kleine boeren niet benadeeld zullen worden door het patenteren van materiaal afkomstig uit hun gemeenschappen door personen en bedrijven uit het Noorden. In dit proefschrift wordt duidelijk gemaakt hoe het in de toekomst mogelijk zal zijn om nieuwe cassave cultivars te produceren die de juiste genen bevatten om de knolproductiviteit en zetmeelkwaliteit te beïnvloeden.

Pfupiso

Mufarinya (Manihot esulenta Crantz) imbesa, inorimirwa upfu (starch) hurimumidzi mayo, nevanhu vanohuita chikafu vanogara munyika dzinopisa dzema*tropics*. Kudiwa kwemufarinya unoshandiswa muchikafu chevanhu nemhuka nekugadzira mapepa nemachira kuri kukwira. Gwaro iri rinotsanangura wongororo iri maererano nezvekuraurwa kwe[moyo wemhodzi] unobudisa chidimbu chidiki nechikuru che ADP-glucose pyrophosphorylase (AGPase B ne S saizvozvo), netukwichidzi tunoumba hupfu twakabatira patumedu twetsanga (GBSSII). Mwoyo yetsanga iyi nezviumbwa zvayo zvakapenengurwa zvakadzama kuti paonekwe kushanda kwayo mukuwumbwa kwehupfu hwemufarinya. Nzira yekugutsikana nayo nemashandiro emoyo iyi yaive kusima moyo yekusandura mukati membesa yemagwiri nemifarinya zvichiteverwa nekudonongodzwa kwehupfu hunobva mumbeu idzi dzakasimwa moyo yemhodzi.

Muchikamu chekutanga kurimwa kwemufarinya pasirino zvaro nemuZimbabwe kunyanya kunowongororwa maererano neruzivo rwamazuva ano rwekuraura nekusima-kunosandura zvinowedzera huwandu nekukosha kwehupfu hwemufarinya. Kugadzirwa kwembeu tsva dzemufarinya dzine hupfu hune huumbwa hutsva nekusima-kunosandura moyo yembeu kuno wongororwa muchikamu ichochi. Zviyereso zvikuru zvekuti tiwane mbeu tsva idzi ndeizvi: kuvepo kwemoyo yemhodzi yakaraurwa nekupenengurwa, kuvepo kwenzira inoshanda kwese-kwese yekusima-kunosandura mbeu dzemufarinya, kusima-kunosandura mbeu dzakafanira nekuongororwa kwekururama kwembeu dzemufarinya idzi dzakasimwa moyo yemhodzi dzisati dzaparadzirwa kuvarimi.

Kuraurwa kwemoyo yemhodzi yemufarinya inobudisa tukwichidzi tunoumba hupfu twakabatira patumedu twetsanga (GBSS II) nekubudisa chidimbu chidiki nechikuru chetukwichidzi tunonzi ADP-glucose pyrophosphorylase (AGPase B ne S saizvozvo) kunotsanangurwa muchikamu chechipiri nechechitatu. Kuraurwa kwechikwichidzi che GBSSII kunoratidza zvechokwadi kuti kune chikwichidzi chakasiyana chine maitire mamwe sezvakaonenkwa mune zvimwe zvirimwa. Kanahazvo GBSSII neGBSSI dzakasiyana pakurongwa kwema amino asidzi kwadzo, kurongwa kwema amino asidzi *e*GBSSII kwakafanana nemoyo yemhodzi yemamwe maGBSSII inowanikwa mu PEAS nemumagwiri. GBSSII yemufarinya inenge inokosha mumashizha umo inonyanya kuratidzwa pane mumidzi munonyanyo ratidzwa GBSSI. Mukuwongorora mbeu dzemufarinya tichitsvaga nharaunda inowanikwa moyo wemhodzi weGBSSII takawona kuti mhodzi iyi iri yoga uye inowanikwa muchikwata chinonzi T chakarerekera kugono remufarinya.

Kuraura kwe[moyo wemhodzi] unobudisa chidimbu chidiki (B) nechikuru (S) che AGPase zvakaratidza makarekare anoyevedza etukwichidzi twemufarinya. Chkwichidzi che AGPase chemufarinya chnoratidza kuti chingangodaro chakauumbwa nenhengo ina dzakasiyana sezvakaonekwa mune zvimwe zvirimwa. Kuenzaniswa kwehurongwa hweAGPase B yemufarinya nehurongwa hwe mamwe maAGPase akaraurwa kare kwakaratidza kuti AGPase B yakanyanyo fanana nemimwe moyo yemhodzi inopa zvidimbu zvidiki mune zvimwe zvirimwa pane ne moyo wemhodzi unopa chidimbu chikuru che AGPase S (Chikamu chetatu). Mukuwongorora kwembeu dzemufarinya tichitsvaka nharaunda inowanikwa moyo wemhodzi we AGPase S zvakawonekwa kuti mhodzi iyi iri yoga zvakare irimuchikwata chechikadzi chinonzi E. Moyo yemhodzi inowanikwa munhengo dzomufarinya dzose, asi nhumwa dze AGPase B (mRNA) dzakawanda kupfuwura dze AGPase S kunyanya mumashizha nemumhidzi. Kudzorwa kwehuwandu hwe ma shoko anozopa zvikwichidzi zve AGPase B kwakawoneka

nekuwongorora kukusaenderana kwehuwandu we nhumwa ne ma shoko e AGPase B. Kushanda kwetukwichidzi twe AGPase mumufarimya kwakanga kuripamusomoro mumashizha matsva pane ekare nemumhidzi. Kushanda kwe AGPase ye mufarinya mumashizha kwakawedzerwa zvakapetwa katatu nekuiswa kwe 3-PGA uye kushanda uku kwaka dzvanyirirwa zvinosvika makumi mapfumbamwe kubva muzana (90%) painge paiswa inorganic fosifeti (Pi). Chikwichidzi chomumidzi chakaratidzwa kusa kanganiswa ne 3-PGA asi chave chinodzvanyiririswa ne Pi.

Pakutsvaka nzira yekugutsikana kwemashandiro emoyo wemhodzi unopa AGPase B, takavaka nhumwa dzakatakura shoko re AGPase B raka tsveyama dzichitumwa nemutumi anova CaMV35S (Chikamu chechina). Zvivakwa izvi takazvisima mumagwiri tichishandisa Agrobacterium tumefaciens. Miti yemagwiri mazana maviri ane makumi mavirinemana, yakasimwa moyo ye mhodzi inopa AGPase B, yaka ratidza kusasiyana nemagwiri kwawo, asi paive nemiti yemagwiri makumi mana aneshanu ayive nemagwiri akawanda uye arimadiki. Miti yemagwiri yayive nenhumwe dze AGPAse B dzakaderedzwa yayive nemagwiri ane wupfu hwakaderera ne 1.5 kubva mu 3 tichi enzanisa ne wupfu hwemagwiri kwawo. Huwandu hwe tsvigiri dzinonyunguduka hwaka wedzerwa zvakanyanya mumiti yakasimwa nhumwe dze AGPase B dzaka tsveyama (gurukozi yaka kwira kusvika kakapetwa ka gumi, furukutozi kakapetwa rutanatu, ne sukurozi) tichi enzanisa ne wupfu hwemagwiri kwawo. Zvakabuda izvi zvinoratidza kuti moyo wemhodzi unobva mumufarinya unokwanisa kutsveyamisa shoko mumagwiri, asiwo kuti kuno dikanwa kuwongorora miti yemagwiri yakawanda kuti tiwone miti inoratidza kuti shoko rakatsveyamiswa. Izvi zvingangove zvinobva mukusiyana kwemarongerwo ema amino asidzi eAGPase B yemufarinya ne emagwiri anongofanana zvinongova 68% mukurongwa kwazvo.

Chikamu chechishanu chinotsanangura nekugadzira kwenzira isingapedzi nguva, ichidawo vashandi vashoma, yekusima moyo yemhodzi mumufarinya tichishandisa kupenya kunopiwa nemoyo wemhodzi unobva muzvitayitayi kuti tiwone miti yaka simwa moyo yemhodzi unosandura. Akandiko kekutanga kekunyorwa nezveku sima moyo we mhodzi unosandura mumufarinya kunze kwezvakambonyorwa apo kwayishandiswa moyo yemhodzi inopa kuti tiwone kuti nzira iyi yekusimwa kwe moyo yemhodzi inoshanda, zvatinoti zviratidziri. Takavaka nhumwa dzakatakura shoko re AGPase B raka tsveyama dzekusima mumufarinya. Nhumwa idzi dzainge dzichitumwa nemutumi anova CaMV35S uye dzainge dzaka batanidza kuzviratidziri zvezvitayitayi sezviratidziri zvekuwona miti yaka simwa moyo yemhodzi idzi. Mhodzi dzemufarinya dzakasimwa moyo ye mhodzi nekupfura moyo yemhodzi iyi nepfuti ye helium inemabara akazodzwa moyo yemhodzi idzi. Mhodzi dzainge dzapindwa nemabara awa, dzainge dzasimwa moyo yemhodzi iyi, dzaiwonekwa nekutaima. Mhodzi dzaitaima dzakatorwa dzikasarurwa nenzira nhatu dzinova: kusarura nezvakatenderedza mhodzi dzinekutaima, kozowuya kunanga mhodzi dzinekutaima chete, nemubatanidzwa wekusarura nenzira mbiri dzinova yemushonga (phosphinothrycin) nekutaima kwemhodzi. Kusarura kwekunanga tichishandisa kutaima kwemhodzi kwakawonekwa kuti ndiyo nzira inoshanda nemazvo, uye ichingoda vashandi vashoma, pakusima moyo yemhodzi mumufarinya. Zvakaita kuti tikwanise kuwana zvikamu makumi mapfumbamwe kubva muzana zvemhodzi dzaitaima mushure me mavhiki gumi nemaviri ekusarura kwekunanga, tichienzanisa makumi mana nezvishanu kubvamuzana ne chidimbu chimwechete kubva muzana zvemubatanidzwa wekusarura ne kusarura nezvakatenderedza mhodzi dzine kutaima saizvozvo. Huwandu hwemhodzi dzaitaima dzakabva mukusarura kwekunanga hwayinge huchi endererana nekuwanda kwemhodzi

dzayitaima pakutanga kwesarudzo iyi. Kusarura kwekunanga kwakaita kuti nguva inotorwa mukuva nemiti yemufarinya yakasimwa moyo yemhodzi inosandura ideredzwe kubva muma vhiki anobva pa makumi mavirinesere zvichisvika pa makumimatatu anesvitanatu tichienzanisa nemwedzi misere kusvika kugore tichishandisa mubatanidzwa wekusarura kana kusarudza nezvakatenderedza.

Miti yemufarinya yakasimwa moyo yemhodzi ye AGPase B inenhumwa dzaka tsveyama yainge iyine hupfu hushoma mumidzi, tichienzanisa ne miti yemufarinya kwayo. Izvi tichizviwona nekushandisa mushonga unoratidza upfu weiyodini mumhanda dzemifarinya yakasimwa mumabhodoro. Mumiti yemifarinya yayitaridza kutsveya mashoko eAGPase B upfu waingo wanikwa huri mugavi remhanda idzi. Izvi zvinoratidza mashandiro eAGPase B nekukosha kwayo mukugadzirwa kwe hupfu mumufarinya.

Nhaurwa dzakadzika maererano negumisiro rekuraurwa kwe moyo yemhodzi yemifarinya nekugadzira mufarinya yekusimwa moyo yemhodzi munyika dzichiri kubudirira kunokurukurwa muchikamu chechitanhatu. Kunyangwe hazvo zviri pachena kuti kugadzira mifarinya yakasimwa moyo zvinopundutsa upfumi nekudya kunyanya kuvarimi varombo, ruzhinji rwenyika dzekumaodzanyemba haruzivi nezvegumisiro rekurima mbesa dzakasimwa moyo idzodzi. Zvinosimbisiswa zvakare kuti nhaurwa dzakafanira kutangiswa kuti ruzhinji rwevagari vemumaruva vazive nemaererano ezvembesa dzakasimwa moyo nezvadzinopa. Tichivedzera, zvinokurudzirwa kuti pave nenzira yekudzika mitemo yepasi rose yekudzivirira varimi varombo kuti vasarasikirwe nekupamba kwekudhindiswa kwezvinhu zvinobva munzvimbo dzavagere nevanhu vane umbimbindoga nemakambani enyika dzekuchamhembe. Gwaro iri rinoratidza pachena kuti zvinozokwanisika sei munguva inotevera kugadzira zvirimwa zvemidzi zvine moyo yemhodzi kwayo inopa kusanduka kunoonekwa muhuwandu nekukosha kwehuphu hwacho.

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CURRICULUM VITAE

Tichafa Regis Ignatius Munyikwa was born on 28 November, 1967 in Buhera, Zimbabwe. His early schooling was in the city of Bulawayo where he did his High School Certificate at Northlea High School during which time he was elected Bulawayo Junior Town Clerk in 1985-1986. From 1988 to 1990 he studied science at the University of Zimbabwe where he obtained a BSc (Hons) degree in Biological Sciences. Between 1991 and 1992 he studied and obtained an MSc degree in Biotechnology at the University of Zimbabwe on a DAAD (Germany) scholarship. The research component of the MSc programme was carried out between March and September 1992 at the Vrije University in The Netherlands on a Netherlands Fellowship. In 1993 he joined the department of Maths and Science Education of the University of Zimbabwe as a short-term lecturer in Biology for one year. He started his PhD research programme at the Department of Plant Breeding of the Wageningen Agricultural University in December 1993 on sponsorship from the DGIS funded Cassava and Biotechnology Project - Zimbabwe.