Plant Biotechnology Journal (2006) **4**, pp. 453–465 doi: 10.1111/j.1467-7652.2006.00195.x

Genetic modification of cassava for enhanced starch production

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Received 20 January 2006;
revised 30 March 2006;
accepted 4 April 2006
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Summary

To date, transgenic approaches to biofortify subsistence crops have been rather limited. This is particularly true for the starchy root crop cassava (*Manihot esculenta* Crantz). Cassava has one of the highest rates of CO_2 fixation and sucrose synthesis for any C3 plant, but rarely reaches its yield potentials in the field. It was our hypothesis that starch production in cassava tuberous roots could be increased substantially by increasing the sink strength for carbohydrate. To test this hypothesis, we generated transgenic plants with enhanced tuberous root ADP-glucose pyrophosphorylase (AGPase) activity. This was achieved by expressing a modified form of the bacterial *glgC* gene under the control of a Class I patatin promoter. AGPase catalyses the rate-limiting step in starch biosynthesis, and therefore the expression of a more active bacterial form of the enzyme was expected to lead to increased starch production. To facilitate maximal AGPase activity, we modified the *Escherichia coli glgC* gene (encoding AGPase) by site-directed mutagenesis (G336D) to reduce allosteric feedback regulation by fructose-1,6-bisphosphate. Transgenic plants (three) expressing the *glgC* gene had up to 70% higher AGPase activity than control plants when assayed under conditions optimal for plant and not bacterial AGPase activity. Plants having the highest AGPase activities had up to a 2.6-fold increase in total tuberous root biomass when grown under glasshouse conditions. In addition, plants with the highest tuberous root AGPase activity had significant increases in above-ground biomass, consistent with a possible reduction in feedback inhibition on photosynthetic carbon fixation. These results demonstrate that targeted modification of enzymes regulating source–sink relationships in crop plants having high carbohydrate source strengths is an effective strategy for increasing carbohydrate yields in sink tissues.

Keywords: ADP-glucose pyrophosphorylase, *Agrobacterium*-mediated transformation, starch, transgenic cassava.

Introduction

The starchy roots of cassava (*Manihot esculenta* Crantz) are a valuable source of calories for about 600 million people in the developing tropical countries where food deficiency and malnutrition are often common. The leaves and tender shoots of cassava are also eaten in many parts of Africa as a source of vitamins, minerals and proteins (Cock, 1982; Balagopalan, 2002; Nweke *et al*., 2002). Cassava is valued in many areas for the food security it provides. It tolerates low soil fertility and drought well and is resistant to many herbivores as a result of the presence of cyanogens (Koch *et al*., 1994; Siritunga and Sayre, 2003). In addition, the tuberous roots of cassava can be left in the ground for several years prior to harvest, providing security against famine (Lynam, 1993; Nweke *et al*., 2002).

Unlike many of the world's major crop plants, cassava is not particularly amenable to genetic improvement through sexual crosses. Many varieties flower rarely and seed production is often low. In the field, cassava is typically propagated

clonally by stem cuttings. This propagation strategy is ideal for molecular approaches to crop improvement as gene segregation through outcrossing is limited. In 1996, the first stable genetic transformation of cassava was reported using *Agrobacterium* (Li *et al*., 1996) and microparticle-mediated delivery of DNA to plants (Schopke *et al*., 1996). Additional reports of the genetic transformation of cassava have followed (Sarria *et al*., 2000; Zhang *et al*., 2000a,b); however, only recently have transgenic plants been generated with enhanced agronomic traits. In the last 2 years, there have been several reports of genetically modified cassava with enhanced agronomic traits. In 2003, Siritunga and Sayre introduced an antisense CYP79D1 and CYP79D2 construct into cassava to suppress the expression of the cytochrome P450s that catalyse the first dedicated step in cyanogenic glycoside synthesis. Transgenic plants having less than 1% of the normal root cyanogen levels were generated; however, these plants were unable to grow without supplemental reduced nitrogen (Siritunga and Sayre, 2004). In 2003, Zhang *et al*. reported the expression of an artificial storage protein gene (*ASP1*) in cassava leaves and roots (Zhang *et al*., 2003), however, its expression had little effect on the overall amino acid composition of leaf proteins. More recently, Siritunga *et al*. (2004) have reported the overexpression of hydroxynitrile lyase in tuberous roots leading to accelerated cyanogen removal and food detoxification.

Several attributes of cassava's carbohydrate metabolism suggest that it has unrealized potential for enhanced starch production. For a C3 plant, cassava has an unusually high rate of photosynthetic carbon assimilation (43 μmol $CO_2/m^2/s$) as well as a high temperature optimum (45 °C) for photosynthesis (Hunt *et al*., 1977; Edwards *et al*., 1990; Angelov *et al*., 1993). In addition, cassava has been reported to have one of the highest rates of CO_2 assimilation into sucrose of any plant measured (Hunt *et al*., 1977; Angelov *et al*., 1993). For these reasons, we hypothesized that cassava was an excellent candidate for enhancing carbohydrate allocation to sink tissues through transgenic approaches.

Our objective was to engineer cassava plants with enhanced starch yield or with a shorter crop production cycle. Our strategy was to increase sink (tuberous root) strength by the expression of a modified bacterial ADP-glucose pyrophosphorylase (AGPase) gene in cassava tuberous roots. AGPase plays a critical role in the regulation of starch synthesis in plants, not only because it catalyses the first dedicated step in starch synthesis, but also because it is the rate-limiting step in starch synthesis. Antisense-mediated inhibition of AGPase expression has been shown to lead to a severe decrease in starch production in potato tubers (Muller-Rober *et al*., 1992) as well as cassava tuberous roots (Munyikwa *et al*., 1998).

AGPases have been characterized from several different species and have been shown to have different structures, catalytic rates and allosteric regulation. The plant AGPase holoenzyme is a heterotetramer and is formed from two distinct polypeptides which comprise the large and small subunits (Copeland and Preiss, 1981). The large subunit is 54–60 kDa in size, and the small subunit is 51–54 kDa (Copeland and Preiss, 1981; Martin and Smith, 1995). Multiple isoforms of the subunits have been found in plants (Okita, 1992; Martin and Smith, 1995). Both subunits are required for maximal enzyme activity in plants (Frueauf *et al*., 2001; Tiessen *et al*., 2002). These requirements make the genetic manipulation of the plant AGPase more challenging, as it potentially requires modification of the expression or activity of one or more AGPase genes in transgenic plants. In addition, the plant AGPase is activated by 3-phosphoglycerate (3-PGA), inhibited by inorganic phosphate (Pi) and regulated by the redox state of the cell (Ballicora *et al*., 2000; Tiessen *et al*., 2002; Geigenberger, 2003).

The bacterial AGPase is allosterically regulated by effectors different from those of the plant AGPase. The bacterial AGPase is activated by fructose-1,6-bisphosphate (FBP) and is inhibited by adenosine monophosphate (AMP) (Preiss, 1988). The *Escherichia coli* (bacterial) AGPase is also a single gene (*glgC*) product and, importantly, its specific activity is several hundred-fold greater than that of the plant enzyme. Several residues have been identified as important allosteric regulatory sites (Kumar *et al*., 1989; Frueauf *et al*., 2001). A glycine-336 mutant (G336D) has been shown to have high activity with or without the activator FBP, higher substrate (ATP and glucose-1-phosphate) affinity and reduced affinity for the inhibitor AMP (Meyer *et al*., 1998).

Previously, the bacterial AGPase has been expressed in transgenic plants but with mixed outcomes. Expression of the G336D mutant (*glgC*16) form of the bacterial AGPase in potato tubers has been reported to result in a 36% increase in AGPase activity and a corresponding 35% increase in tuber starch content (Stark *et al*., 1992). This increase in tuber starch production occurred only when the *glgC*16 gene was expressed in the tuber (driven by the patatin promoter). In contrast, expression of the *glgC*16 gene in all tissues led to weakly performing plants, presumably as a result of improper allocation of carbohydrate between phototrophic and heterotrophic tissues (Stark *et al*., 1992; Edwards *et al*., 2000; Gibson *et al*., 2003). Sweetlove *et al*. (1996) reported even higher AGPase activities (200%–400%) in transgenic potatoes expressing the *glgC*16 gene (under the control of the

patatin promoter), however, they observed no increase in starch yield. They attributed this result to higher rates of starch turnover in the transgenic tubers. These results suggest that starch steady-state levels in transgenic tubers may be influenced by the sink–source demands of other tissues as well as by potential rates of ADP-glucose synthesis.

We hypothesized that enhanced production of ADP-glucose by a bacterial AGPase with modified allosteric regulation would increase cassava tuberous root starch production as a result of cassava's enhanced capabilities to produce photosynthate (sucrose). As described below, we have generated transgenic plants expressing the modified (G336D) *E. coli glgC* gene. These plants have nearly two-fold higher AGPase activities and two-fold greater root and top (stem and leaf) biomass than wild-type plants. We propose that the enhanced top biomass production represents a release of feedback inhibition on photosynthesis (dry matter accumulation), similar to that observed when expressing the *glgC* gene in potato tubers (Stark *et al*., 1992) and wheat (Smidansky *et al*., 2002) and rice (Smidansky *et al*., 2002; Sakulsingharoj *et al*., 2004) endosperm.

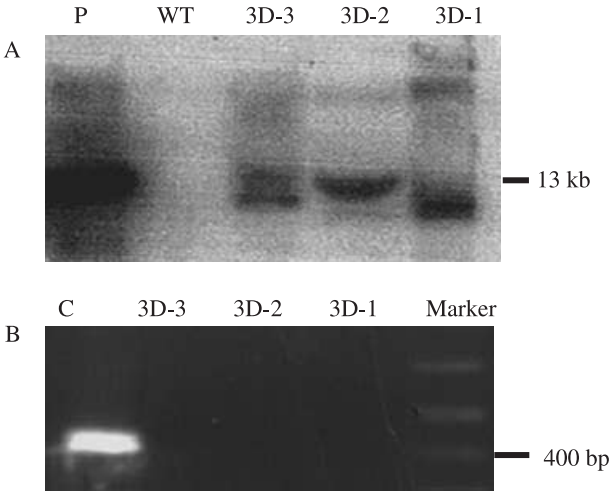

Figure 1 (A) Southern blot of wild-type and transgenic cassava plants demonstrating the integration of the *glgC* gene. Cassava genomic DNA (20 μg) was electrophoresed on 1% agarose gel and probed with a ^{32}P-labelled fragment of the *glgC gene-nos* terminator. P, 3D plasmid DNA control; WT, wild-type cassava (TMS 71173); 3D-1–3D-3 are cassava plants (TMS 71173) transformed with *Agrobacterium* harbouring the 3D plasmid containing the *glgC* gene. (B) Polymerase chain reaction (PCR) analysis of the presence of the *Agrobacterium virG* gene in cassava plants. 3D-1–3D-3, transformed cassava plants; C, *Agrobacterium* control.

Results

Production of transgenic cassava

Germinating somatic embryos of cassava cultivar TMS 71173 were transformed via *Agrobacterium*-mediated transformation with a modified *glgC* gene having a single mutation (G336D), which eliminates feedback regulation by FBP. The modified *glgC* gene also included a pea *rbcs* chloroplast transit peptide sequence at its 5′ end to target the enzyme to the amyloplast, and was expressed under the control of a Class I patatin promoter. Transformed cells were selected initially for paromomycin resistance and underwent somatic embryogenesis in 4–6 weeks. Paromomycin-resistant embryos converted to plantlets in 1–2 months and, after 4 months, were transferred to the glasshouse. We recovered 26 paromomycin-resistant embryos from 872 TMS 71173 somatic embryo explants. Five of the lines were confirmed to be transformed by polymerase chain reaction (PCR) analysis for the integration of the *glgC* gene. Based on the analysis of relative AGPase activity, three of the transgenic lines (3D-1, 3D-2 and 3D-3), representing the maximum and minimum levels of AGPase activity, were used in the analyses presented in this paper.

Confirmation of transformants

The presence of the modified *glgC* gene in paromomycin-resistant plants was confirmed by PCR analysis of an 850-bp patatin promoter–*glgC* gene fragment. Importantly, three independent wild-type plants were regenerated through the same tissue culture system used for transgenic plants, but without paromomycin selection. Thus, differences in AGPase expression levels between wild-type and transgenic plants could not be attributed to differences in plant regeneration or propagation protocols. To verify further the integration of the T-DNA into the plant genome, Southern blot analyses were carried out using cassava genomic DNA digested with *Kpn*I, which does not restrict the T-DNA, and probed with the radiolabelled *glgC* gene (Figure 1A). As expected, the bacterial *glgC* probe did not hybridize to wild-type DNA. The *glgC* hybridization patterns for the three transformed plants were different, indicating that they were independent transformants. The apparent copy number of *glgC* genes integrated into the cassava genome varied from one (3D-2) to two (3D-1 and 3D-3) copies. To confirm that the AGPase gene had integrated into the plant genome and was not harboured in contaminating *Agrobacterium*, transformed plants were screened for *Agrobacterium* contamination by PCR amplification of the *Agrobacterium virG* gene product. No diagnostic *virG* PCR products were detected from any of the primary transformed plants, indicating that the regenerated plants were not contaminated with *Agrobacterium* (Figure 1B). All PCR products were also sequenced to confirm their identity.

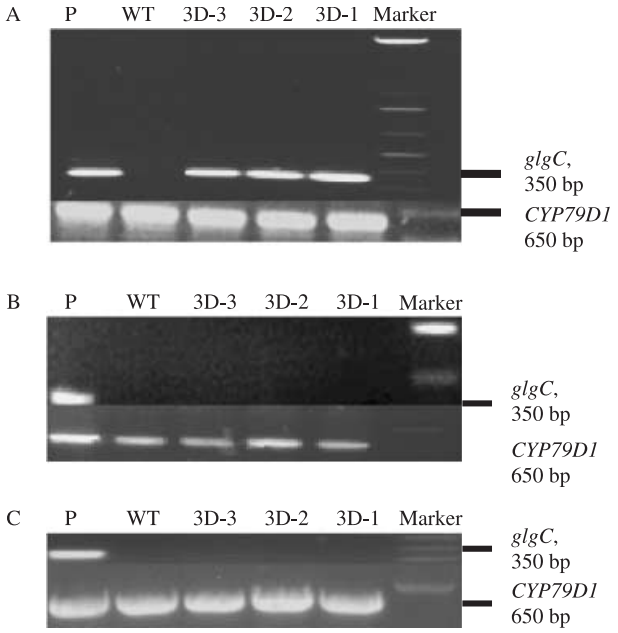

Figure 2 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *glgC* gene expression in roots, leaves and stems of three transgenic and wild-type cassava plants. (A) RT-PCR analysis of *glgC* gene expression in roots of transgenic and wild-type cassava plants and RT-PCR of cytochrome P450 (*CYP79D1*) expression for loading control. Lane P, 3D plasmid control; WT, wild-type cassava (TMS 71173); 3D-1–3D-3, cassava TMS 71173 transformed with the *glgC* gene. (B) RT-PCR analysis of *glgC* gene expression in leaves of three transgenic and wild-type cassava plants and RT-PCR of cytochrome P450 (*CYP79D1*) expression for loading control. Lane P, 3D plasmid control; WT, wild-type cassava (TMS 71173); 3D-1–3D-3, cassava TMS 71173 transformed with the bacterial *glgC* gene. (C) RT-PCR analysis of *glgC* gene expression in stems of three transgenic and wild-type cassava plants. Lane P, 3D plasmid control; WT, wild-type cassava (TMS 71173); 3D-1–3D-3, cassava TMS 71173 transformed with the bacterial *glgC* gene. RT-PCR of cytochrome P450 (*CYP79D1*) expression was used as loading control.

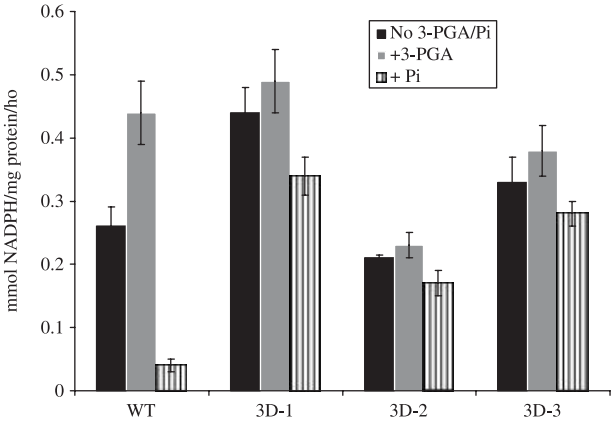

Figure 3 Total tuberous root ADP-glucose pyrophosphorylase (AGPase) activities for 6-month-old wild-type and transformed (*glgC* gene) plants grown in the glasshouse. Assays were carried out in the presence and absence of allosteric regulators of the plant AGPase enzyme. Assays were repeated three times for each of three independent plant extracts for each transgenic and wild-type line (see 'Experimental procedures'). Control, wild-type cassava (TMS 71173); 3D-1–3D-3, cassava TMS 71173 transformed with the bacterial *glgC* gene. 3-PGA, 3-phosphoglycerate; Pi, inorganic phosphate.

Gene expression analysis

To determine the patterns of tissue-specific expression of the *glgC* gene in transgenic plants, non-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using root, stem and leaf (negative control) total RNA obtained from primary transgenic plants and one of the wild-type cassava regenerants as a template. Prior to cDNA synthesis, the RNA was treated with DNase to eliminate false positives from DNA contamination of the RNA. The transgene was amplified using *glgC*- and *nos* terminator-specific oligonucleotide primers. Each of the primary transformed cassava plants identified by PCR and Southern blot analysis was confirmed positive for the expression of the *glgC* gene in root tissues (Figure 2A). The RT-PCR results showed no detectable expression of the bacterial *glgC* gene in the leaves of cassava (Figure 2B). The RT-PCR analysis of *glgC* expression in stems was also negative, indicating that the *glgC* gene was expressed only in roots (Figure 2C). All PCR products were confirmed by DNA sequence analysis.

AGPase activity in wild-type and transgenic tuberous roots

Plant material from 6-month-old glasshouse-grown plants was used for AGPase enzyme assays. Three independent plants subcloned from the original primary transformants were used for assay purposes, and each plant was assayed three times. As shown in Figure 3, transformed plants had up to a 70% increase in total root AGPase activity (0.21–0.44 μmol NADPH/mg protein/h) relative to wild-type plants (0.26 μmol NADPH/mg protein/h) when assayed under conditions (pH 7.5 and temperature of 25 °C) which were sub-optimal for the bacterial (37 °C) enzyme. The 3D-2 transformed plant had the lowest AGPase activity (0.21 μmol ADP-glucose/mg protein/h) of the transgenic plants, equivalent to wild-type plants and significantly lower than the 3D-1 and 3D-3 transformed plants. The relative differences in AGPase activity between the three transformants could be the result of position effects with regard to the site of insertion of the transgene into the genome, or could reflect apparent differences in copy number of the integrated *glgC* genes (Sarria *et al.*, 2000; Zhang *et al.*, 2000a). Transgenic plants having higher total root AGPase activity had two apparent copies of the *glgC* gene, whereas plants having lower AGPase activity had

one apparent copy of the *glgC* gene integrated into the genome. Importantly, there was little variation in tuberous root AGPase activity between the independent clones from any transgenic or wild-type plants, indicating that potential variance possibly attributed to chimerism in AGPase expression patterns was unlikely. Similar results have been observed for other cassava transformants generated from transformed somatic embryos (Siritunga and Sayre, 2003, 2004; Siritunga *et al*., 2004).

To demonstrate that the increase in AGPase activity was largely attributable to the expression of the bacterial AGPase, and not a result of elevated plant AGPase activity, we measured the effects of various plant-specific AGPase activators and inhibitors on total tuberous root AGPase activity. It would be expected that these allosteric regulators would have substantial effects on the AGPase activity of the wild-type plant enzyme and no effects on bacterial AGPase activity. This is a result of the insensitivity of bacterial AGPase to the plant AGPase allosteric regulators, 3-PGA and Pi. As shown in Figure 3, the addition of the plant AGPase activator, 3-PGA (2.0 mM), increased total AGPase in tuberous root extracts of wild-type plants by up to 70%, but only slightly enhanced (10%–15%) the AGPase activity obtained from tuberous root extracts of transgenic plants. The limited enhancement of tuberous root AGPase activity by 3-PGA in transgenic plants is consistent with the co-expression of both the wild-type and bacterial forms (not subject to 3-PGA activation) of the enzyme in tuberous roots. Similarly, the addition of the plant enzyme-specific AGPase inhibitor, Pi, inhibited (84%) AGPase activity in crude extracts obtained from tuberous roots of wild-type cassava, but had a reduced effect (19%–23% inhibition) on the AGPase activity from extracts of transgenic tuberous roots (Figure 3). Overall, these results are consistent with the co-expression of both the bacterial and plant forms of AGPase in tuberous roots of transgenic plants.

Analysis of wild-type and transgenic cassava yield in the glasshouse

To determine the impact of the root-specific expression of the *glgC* gene in transgenic plants on total mass (fresh weight) and biomass (dry weight) allocation to different organs, we measured the number, fresh weight and dry weight of leaves, stems and roots from wild-type and transgenic plants grown in the glasshouse. Transgenic plants (3D-1 and 3D-3) expressing the highest AGPase activity in tuberous roots had greater average tuberous root fresh weights, tuberous root numbers per plant, leaf fresh weights per plant and stem fresh weights per plant than did wild-type plants following 7 months of growth in the glasshouse (Table 1). The average fresh weight yield of tuberous roots from transgenic cassava plants expressing bacterial AGPase was 52%–166% greater than that of wild-type plants. The average number of tuberous roots per transformed plant was also greater than that of wild-type plants (8–12 tuberous roots per plant for the transgenic plants vs. seven tuberous roots per plant for the wild-type), although the values were only significantly different for the transgenic plants (3D-1) expressing the highest levels of tuberous root AGPase activity compared with the wild-type. Similarly, the total tuberous root yields of the transformants (3D-1, 198 g fresh weight per tuberous root; 3D-3, 123 g fresh weight per tuberous root) having the highest AGPase activities were approximately two- to three-fold greater than those of wild-type cassava (74 g fresh weight

Table 1 Fresh weight and organ number of *glg*C-transformed (3D-1, 3D-2, 3D-3) and wild-type (WT) cassava plants. The plants were grown in the glasshouse for 7 months. Three independent replicates of wild-type and transformed plants were used in the analysis

	WT	3D-1	3D-2	3D-3
Leaf (g)	220.67 ± 45^{b}	335 ± 26^{a}	284.33 ± 4.9^{ab}	299 ± 38^{a}
	(100)	(156)	(127)	(135)
Number of leaves	92.3 ± 9.4^{b}	123 ± 10^{a}	108.6 ± 18^{ab}	114 ± 13^{ab}
	(100)	(134)	(116)	(124)
Stem (g)	374 ± 75^{c}	584 ± 47^{a}	431.67 ± 85^{b}	529 ± 60^{ab}
	(100)	(156)	(116)	(144)
Stem–root junction (g)	59 ± 7.5^{a}	86.33 ± 5.4^{a}	81.33 ± 12^{a}	63.67 ± 15^{a}
	(100)	(146)	(137)	(106)
Root (g)	74.33 ± 19^{c}	198.67 ± 29^{a}	113.33 ± 31^{bc}	123 ± 23^{b}
	(100)	(266)	(152)	(166)
Number of roots/plant	7 ± 0.8^{b}	12 ± 3.3^{a}	8 ± 2.0^{b}	11 ± 0.8^{a}
	(100)	(174)	(114)	(157)

Numbers with the same letter represent no statistical difference; different letters indicate statistically significant difference at the 95% confidence level. Values in parentheses are percentages of wild-type values.

per tuberous root) (Table 1) after 7 months of growth in the glasshouse. Interestingly, the average leaf fresh weight per plant for the 3D-1 (335 g fresh weight leaf per plant) and 3D-3 (299 g fresh weight leaf per plant) transgenic plants was also significantly greater than that of wild-type plants (220 g fresh weight leaf per plant). This trend was not followed for transformant 3D-2 which had tuberous root AGPase activities similar to those of wild-type plants. The tuberous root and leaf fresh weight values for the 3D-2 transgenic plants were also not significantly different from those of the wild-type plants.

Interestingly, the non-root total biomass, including stems and leaves, was significantly greater for the transgenic plants expressing the highest AGPase levels than for wild-type plants. The total stem fresh weight for the transgenic plants ranged from a high of 584 g for transformant 3D-1 to a low of 431 g for transformant 3D-2. Wild-type plants had an average stem fresh weight of 374 g. Transformed plants also produced more leaves than wild-type plants. The average number of leaves produced by the transformed cassava plants ranged from a low of 108 (3D-2) to a high of 123 (3D-1). The number of leaves produced by the 3D-1 and 3D-3 plants was significantly greater than that of the 3D-2 (108) and wild-type (92) plants (Table 1).

The trends in dry weight or biomass yields for the tuberous roots and stems from transformed and wild-type plants were similar to those observed for the fresh weight yields, except for the fact that there was no statistical difference between the dry weights of the leaves of the different cultivars (Table 2), possibly reflecting efficient reallocation of carbohydrate from source to sink tissues in the transgenic plants. The total dry weights of the stems of transgenic lines 3D-1 (147 g) and 3D-3 (134 g) were also not significantly different from each other, but differed significantly from that of the wild-type plants (89 g). The total dry weights of the root–stem junction of 3D-1 (25 g) and 3D-2 (25 g) plants were also significantly different from that of the wild-type (16 g), similar to the trend observed for the stem biomass. Similar to the results observed with fresh weight measurements for tuberous roots, the root dry weights of transgenic lines expressing the highest levels of AGPase activity (3D-1, 48 g dry weight per tuberous root; 3D-3, 28 g dry weight per tuberous root) were 2.7- and 1.5-fold greater, respectively, than that of wild-type plants.

Statistical analyses of the relationship between the fresh weight values of the leaves and tuberous roots of 3D-1 transgenic plants indicated that there was a significant ($P < 0.05$) positive correlation ($r = 0.94$) between leaf and tuberous root fresh weight biomass, stem and tuberous root fresh weight mass ($r = 0.67$) and the number of leaves and tuberous root mass ($r = 0.85$). There was, however, no correlation between tuberous root number and tuberous root fresh weight biomass ($r = 0.19$) (data not included) (SAS Institute Inc., 1985). The correlation coefficient for leaf fresh weight and tuberous root mass of 3D-1 plants was greater than that for wild-type cassava plants ($r = 0.94$ vs. $r = 0.57$). In addition, stem and tuberous root mass fresh weight were positively correlated ($r = 0.67$) for 3D-1 plants, but were negatively correlated ($r = -0.91$) for wild-type cassava. These results indicate that the greater tuberous root yield of the *glgC*-expressing cassava plants is correlated with greater leaf and stem biomass, unlike wild-type cassava.

The total biomass (dry weight per plant) was also correlated with the tuberous root AGPase activity ($r = 0.67$). The 3D-1 plants had a 61% increase in total (root and shoot) biomass relative to wild-type plants. These results suggest that the increase in apparent sink strength resulting from higher AGPase activity releases possible feedback inhibition on overall photosynthesis or carbon fixation. The 3D-1 plants also had the highest percentage of tuberous root biomass (27%). The tuberous root biomass for wild-type plants was 15%. The 3D-3 and 3D-2 plants having intermediate tuberous root

Table 2 Dry weight (biomass) comparison of *glgC*-transformed (3D-1, 3D-2 and 3D-3) and wild-type (WT) (TMS 71173) plants. Harvested materials were dried in the oven at 80 °C for 3 days until a constant weight was attained. Three independent replicates of wild-type and transformed plants were used in the analysis

	WT	3D-1	3D-2	3D-3
Leaf (g)	55.1 ± 8.3^{a}	68.55 ± 4.0^{a}	60.4 ± 0.8^{a}	62.45 ± 9.4^{a}
	(100)	(123)	(109)	(112)
Stem (g)	89.69 ± 5.0^{c}	147.78 ± 8.8^{a}	114.02 ± 25^{bc}	134.4 ± 13^{ab}
	(100)	(165)	(128)	(150)
Stem–root junction (g)	16.66 ± 1.5^{b}	25.36 ± 2.5^{a}	25.16 ± 2.7^{a}	23.1 ± 3.6^{ab}
	(100)	(156)	(156)	(143)
Root (g)	18.9 ± 4.3^{c}	48.23 ± 9.3^{a}	24.89 ± 7.8^{bc}	28.52 ± 8.7^{b}
	(100)	(266)	(133)	(155)

Numbers with the same letter represent no statistical difference, different letters indicate statistical difference at the 95% confidence level. Values in parentheses are percentages of wild-type values.

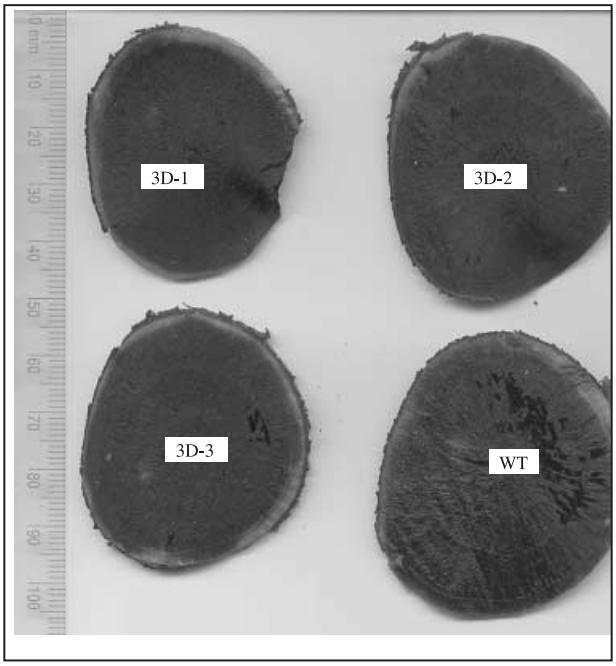

Figure 4 Iodine assay of cassava tuberous root starch. WT, cassava wild-type plant TMS 71173; 3D-1–3D-3, TMS 71173 plants transformed with *patatin-TP-glgC* cassette.

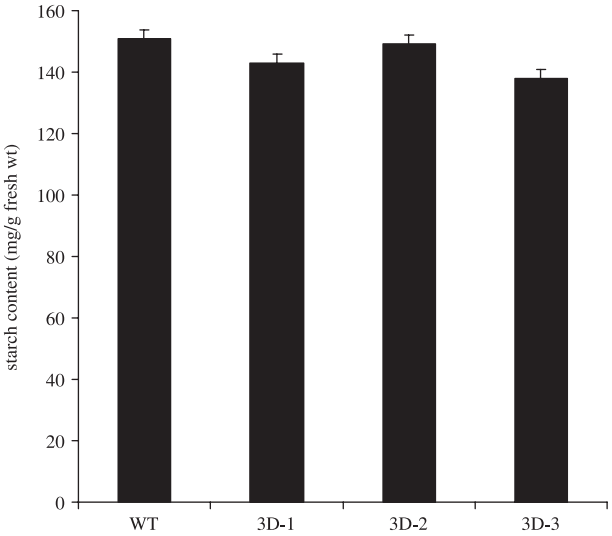

Figure 5 Starch analysis of tuberous root of wild-type and transgenic cassava plants transformed with the *patatin-TP-glgC* plasmid construct. WT, cassava wild-type plant TMS 71173; 3D-1–3D-3, TMS 71173 plants transformed with *patatin-TP-glgC* cassette.

AGPase activity had biomass allocations to tuberous roots which were of intermediate values: 20% and 19%, respectively. Overall, these results demonstrate that moderate increases in tuberous root AGPase activity are associated with a greater carbon allocation to tuberous roots as well as a greater total biomass accumulation per plant.

Starch assay

The analysis of the distribution of starch in the tuberous roots of wild-type and transgenic plants by iodine staining indicated no differences in the pattern of starch distribution between wild-type and transgenic tuberous roots (Figure 4). To determine whether the density of root starch varied between wild-type and transgenic plants, we quantified enzymatically the total starch per gram fresh weight. As shown in Figure 5, there was no statistical difference between wild-type and transgenic plants. The tuberous root starch contents per gram fresh weight for wild-type (TMS 71173) and transgenic (3D-1, 3D-2 and 3D-3) plants were 151, 143, 149 and 138 mg per gram fresh weight (Figure 5).

Given that the ratio of dry weight to fresh weight was also identical for wild-type and transgenic plants, these results indicated that the density of starch was not altered in the transgenic plants relative to the wild-type plants. Therefore, the increase in total tuberous root starch content in transgenic plants could not be attributed to increased starch content per cell, but to an increase in tuberous root size and number.

Discussion

We have generated transgenic cassava lines expressing a plastid-targeted and modified bacterial AGPase expressed under the control of the patatin promoter. Analyses of tuberous root AGPase activity indicated that the best-performing transformed plant (3D-1) had a 70% increase in AGPase activity relative to the lowest-performing transformed (3D-2) and wild-type plants. Transformed plants (3D-1 and 3D-3) having significant increases in AGPase enzyme activity also had significantly higher tuberous root yields (3D-1: 266% of the wild-type dry weight yield) than transformed plants (3D-2) having little or no apparent increase in AGPase enzyme activity (Figure 3, Tables 1 and 2). The relative differences in AGPase activity between transformants could be the result of position effects with regard to the site of insertion of the transgene into the genome (Sarria *et al*., 2000; Zhang *et al*., 2000a), or to variation in the apparent copy numbers of the *glgC* gene inserted into the cassava genome. The transformed plants (3D-1 and 3D-3) having two apparent copies of the *glgC* gene had higher AGPase enzyme activity and higher root yields than the transformed plant (3D-2) having only one apparent copy of the bacterial *glgC* gene.

In higher plants, 3-PGA activates and Pi allosterically inhibits AGPase catalytic activity (Ballicora *et al*., 2004). We observed that 3-PGA substantially enhanced the activity of

AGPase isolated from wild-type tuberous roots, but had little effect on the AGPase activity isolated from transgenic tuberous roots; these results were consistent with the expression of the bacterial form of the enzyme in transgenic plants (Figure 3). Similarly, we observed substantial Pi-mediated inhibition of AGPase activity in wild-type tuberous roots, but not in transgenic plants, again consistent with the expression of the bacterial enzyme in transgenic tuberous roots.

The overall increase in tuberous root AGPase activity in transgenic plants was not great, however. The increase in AGPase activity observed in transgenic cassava tuberous roots was of a similar order of magnitude to that observed in transgenic potato (tubers), rice or wheat (seed) expressing either a bacterial or plant-encoded AGPase gene (respectively). Transgenic rice expressing a modified maize AGPase large subunit gene (*Sh2r6hs*) had only a 42% increase in AGPase activity relative to wild-type plants (Smidansky *et al*., 2003). Similarly, transgenic wheat overexpressing the AGPase large subunit had only a 24% increase in enzyme activity relative to wild-type plants (Smidansky *et al*., 2002). Stark *et al*. (1992) reported a 36% increase in AGPase activity in transgenic potato expressing the bacterial *glgC* gene under the control of the patatin promoter. Stark *et al*. (1992) also observed, however, that expression of the *glgC* gene in leaves resulted in poorly performing plants, presumably because of an inability to adequately mobilize starch from source to sink. These results suggest that there may be limits to the ability to reallocate carbohydrate between different organs. Sweetlove *et al*. (1996) noted a 400% increase in AGPase activity in transgenic potato plants relative to wild-type plants; however, this did not result in any increase in tuber biomass, presumably because of increased starch metabolism in transgenic tuberous roots.

Given these potential constraints, however, we observed that transgenic cassava plants expressing the highest tuberous root AGPase activity (3D-1: 70% of the wild-type) also had the greatest tuberous root biomass (166% greater than that of the wild-type; Figure 3, Tables 1 and 2). Transformed rice plants also had a 22% greater total (top and root) biomass and were taller than wild-type plants (Smidansky *et al*., 2003). A similar result was reported for transgenic wheat (Smidansky *et al*., 2002) that overexpressed AGPase, in which the transgenics produced 31% more total biomass than the wild-type plants. These results most likely reflect higher rates of root starch synthesis, greater rates of sucrose utilization and reduced feedback inhibition on photosynthesis in transgenic plants (Worrell *et al*., 1991; Martin and Smith, 1995; Smidansky *et al*., 2002).

The starch contents per gram fresh weight for wild-type (TMS 71173) and transgenic (3D-1, 3D-2 and 3D-3) plants were 151, 143, 149 and 138 mg per gram fresh weight, respectively (Figure 5). These values are similar to those observed by Edwards *et al*. (1999) for transgenic potatoes (100–120 mg starch per gram fresh weight) with reduced expression of starch synthase II and III gene. However, they are substantially less than the value of 230 mg per gram fresh weight reported for potatoes (clone RV) by Munyikwa *et al*. (2001).

The relative roles of source and sink strength in determining starch yields in sink tissues requires the analysis of metabolic flux rates to determine the relative contributions to steady-state starch levels. Although we have not analysed carbohydrate flux rates in transgenic cassava, we can draw upon observations made in other transgenic crops overexpressing AGPase activity in sink tissues. In *Arabidopsis*, Sun *et al*. (2002) observed that plants with higher rates of starch biosynthesis in leaf tissues generally had greater photosynthetic capacities as a result of reduced feedback inhibition of photosynthesis. Thus, steady-state starch levels in sink tissues were determined both by increased rates of carbohydrate conversion into starch as well as increased rates of carbohydrate production through photosynthesis. This pattern of starch allocation is most consistent with the altered patterns of dry mass allocation we observed between wild-type and transgenic cassava overexpressing bacterial AGPase in tuberous roots. We observed a positive correlation between top and root biomass with increased AGPase activity in transgenic plants, consistent with an increase in overall photosynthesis rate. The relative impact of increased total biomass production on tuberous root yield is not known, however. In potato tubers, it has been suggested that source strength primarily determines (80%) biomass allocation to tubers (Stark *et al*., 1992). Relative to potatoes, however, cassava has an exceptionally high rate of photosynthesis and sucrose production, suggesting that source strength may be maximized in cassava.

In addition to an increase in total dry weight accumulation in transgenic plants, we also observed an increase in tuberous root number in transgenic plants expressing the highest AGPase levels. The increase in tuberous root number (Table 1) was similar to that reported for potatoes transformed with a cytosolic invertase gene, which could also presumably increase sink strength (Sonnewald *et al*., 1997). Potato plants transformed with the gene encoding cytosolic invertase, however, had smaller potatoes than did wild-type plants. Transgenic cassava expressing the modified bacterial *glgC* gene had an increase in both tuberous root number and tuberous root dry weight, but no increase in tuberous root starch density, indicating that starch density may be maximized in wild-type cassava tuberous roots.

In summary, moderate increases in root-specific AGPase activity resulted in corresponding increases in both tuberous root and total plant biomass. The high photosynthate potential of cassava provides a strong source of carbohydrate for meeting the demands of increased sink strength, resulting in a net increase in overall biomass accumulation, presumably reflecting a reduction in feedback control of photosynthate production.

Experimental procedures

Plant material

Cassava cultivar TMS 71173, from the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria, was used for transformation purposes. Somatic embryogenesis was induced with apical leaf cultures on Murashige–Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose, 8 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 10 mg/L 100× Gamborg's B-5 vitamins (Gamborg *et al*., 1968), 50 mg/L casein hydrolysate and 0.5 mg/L $CuSO_4$, pH 5.7. Germplasm maintenance cultures were maintained on a 12 h per day photoperiod at 28 °C at a light intensity of 50 μmol photons/m^2/s. Somatic embryogenesis cultures were covered with four layers of cheese cloth. Somatic embryos were transferred to germination medium [MS basal medium supplemented with 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 2% (w/v) sucrose, 0.01 mg/L 2,4-D, 1.0 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L gibberellic acid (GA), pH 5.7] after 4–6 weeks to generate embryo cotyledons.

Site-directed mutagenesis of *E. coli glgC* gene

Site-directed mutagenesis of the *glgC* gene (glycine 336 to glutamate, G336D) was carried out as described by Sayers *et al*. (1988) using the mutagenic primer 3′ CA*AAG*GCT-GCCAACA 5′. To verify that there were no unintended additional mutations and to confirm the introduction of the intended G336D mutation, the entire coding region of the *glgC* gene was sequenced.

Construction of Ti-plasmid binary vector

A pBI121 Ti-plasmid (Mountain View, Clontech) was used for transfer of the *glgC* (G336D) gene into cassava. The cauliflower mosaic virus (CaMV) 35S promoter was substituted with a gene fragment containing a 1.0-kb Class I patatin promoter from potato (Kim *et al*., 1994), followed by a 400-bp pea chloroplast transit peptide (TP) (Anderson and Smith, 1986) fused to the 5′ end of the 900-bp *E. coli glgC* gene containing the G336D mutation (Baecker *et al*., 1983; Kumar *et al*., 1989). An *Agrobacterium nos* terminator was added to the 3′ end of the modified *glgC* structural gene (Bevan, 1984). The T-DNA also included an *npt*II selectable marker driven by a CaMV 35S promoter. The *npt*II confers resistance to kanamycin and its analogue paromomycin. This plasmid construct was designated 3D.

The 3D plasmid was transformed into *E. coli* and confirmed by PCR analysis. The forward primer used for PCR analysis was TTTCTCAACTTGTTTACGTGCCTA and the reverse primer targeting the 3′ end of the patatin promoter and the 5′ end of the *glgC* gene was ACCTTTTTCGACGTGATCGATAAGC. The PCR conditions were as follows: 3 min at 94 °C, 30 s at 94 °C, 45 s at 54 °C and 50 s at 72 °C, for 30 cycles.

Transformation of cassava with *Agrobacterium* and selection of putative transformants

Plasmid 3D (100 ng) was used to transform the LBA4404 strain of *Agrobacterium tumefaciens* from Invitrogen (Rockville, MD, USA). Kanamycin- and streptomycin-resistant colonies were screened by PCR with *npt*II-specific primers used to amplify an 800-bp piece to confirm transformation. The forward primer was CTTCGTGGCCGTGACCCGCGCGGC and the reverse primer was CCGAATTCATAGATGACCCGCGC. DNA amplification was carried out for 30 PCR cycles of 94 °C for 3 min, 94 °C for 30 s, 55 °C for 60 s (annealing temperature), 72 °C for 50 s (extension temperature) and 72 °C for 4 min.

The cotyledons of germinated somatic embryos were used for transformation (Arias-Garzon, 1997). *Agrobacterium* was co-cultivated with cassava somatic embryo cotyledons on MS basal medium plus 100 μM acetosyringone for 2 days. The tissues were then transferred to MS medium containing 8 mg/L 2,4-D, 75 mg/L paromomycin and 500 mg/L carbenicillin to eliminate *Agrobacterium* and to select for transformants. Somatic embryos were grown under a 12 h per day photoperiod at a light intensity of 50 μmol photons/m^2/s at 28 °C. Clumps of somatic embryos formed after 4 weeks of culture and were transferred to cassava germination medium containing 75 mg/L paromomycin and 500 mg/L carbenicillin for a further 4 weeks. Following germination, individual plantlets were transferred to cassava micropropagation medium [MS salts plus 2% (w/v) sucrose, 0.04 mg/L BAP, 0.05 mg/L GA, 0.02 mg/L naphthaleneacetic acid (NAA), 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, pH 5.7] without antibiotics for root induction. Independent wild-type plants used for biochemical experiments were regenerated from somatic

embryos using the same protocol as that employed to regenerate transgenic plants but without antibiotic selection.

Verification of transformants by Southern blot and PCR

Genomic DNA was isolated from glasshouse-grown plants according to the methods of Soni and Murray (1994). Southern blot analysis of wild-type and transgenic cassava was carried out according to the methods of Sambrook *et al*. (1989). Genomic DNA (20 μg) was restricted with *Kpn*I, followed by gel separation of the restriction fragments. The gel was depurinated with 250 mM HCl for 10 min whilst shaking slowly. This was followed by 1 h denaturation with 1.5 M NaCl and 0.5 M NaOH, and then 1 h neutralization with 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl with slow shaking. The genomic DNA transfer to ZetaBind nylon membrane (Life Science Products, Inc., Denver, CO, USA) was performed overnight with 20 × SSC (3 M NaCl, 0.3 M sodium citrate buffer, pH 7.0). The membrane was then stored dry at room temperature until prehybridization with CHURCH buffer [0.5 M $NaHPO_4$ (pH 7.2), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) bovine serum albumin (BSA), 7% (w/v) sodium dodecylsulphate (SDS)] at 50 °C for 3 h. The probe for the Southern blot was made by PCR amplification of the *glgC/nos* terminator fragment. The *glgC* forward primer (0.4 μmol, TTCTCGCGCGTTCGCGTGAATT) and *nos* terminator reverse primer (0.4 μmol, ATCGCAAGACCGGCAACAGGATTC) were suspended in a total volume of 50 μL, including 5 μL 10 × PCR buffer, 100 ng 3D vector, 0.1 mmol each of dATP/dTTP/dGTP, 100 μCi ^{32}P-dCTP (Amersham, Piscataway, NJ, USA), 2.5 units Taq polymerase (Life Technologies, Grand Island, NY, USA) and $MgCl_2$ at a final concentration of 1.5 mM. PCR was run under the following conditions: 3 min at 94 °C, 30 s at 94 °C, 45 s at 58 °C and 30 s at 72 °C, for 30 cycles. The probe was purified with a Qiagen PCR Kit (Qiagen Inc., Valencia, CA, USA), boiled at 100 °C for 5 min, quickly cooled on ice and added to the hybridization buffer (same as the prehybridization buffer) overnight. Subsequently, the membrane was washed with 1 × SSC, 0.1% (w/v) SDS for 30 min at 50 °C, followed by two washes with 0.1 × SSC, 0.1% (w/v) SDS for 30 min at 50 °C. The membrane was wrapped with Saran wrap and incubated overnight with a phosphor imager that was prebleached for 30 min with light.

To determine whether transgenic plants were contaminated with *Agrobacerium*, we performed PCR using a total genomic DNA extract as a template and *Agrobacterium*-specific *virG* gene primers. The forward and reverse *virG* primers were GCCGACAGCACCCAGTTCAC and CCTGCCGTAAGTTTCACCTCACC, respectively. The PCR conditions were as follows: 3 min at 94 °C, 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, for 30 cycles, followed by 4 min at 72 °C. The PCR products were separated on a 0.8% (w/v) agarose gel and the products were confirmed by DNA sequence analysis.

RT-PCR analyses

Total RNA was extracted from root, leaf and stems (100 mg) using a Qiagen Plant RNA Extraction Kit (Qiagen Inc.). The RNA was treated with 1.0 unit of DNase (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature to eliminate DNA contamination, and the DNase was inactivated by treating with 25 mM EDTA followed by heat inactivation at 65 °C. The first-strand cDNA synthesis was carried out with 10 μg of total RNA using 1 × reverse transcription buffer, 0.3 mM dNTP, 0.5 μg $oligodT_{(12–18)}$ primers and 200 units of SuperScript II RT (Life Technologies, Rockville, MD, USA). The mixture was incubated at 65 °C for 5 min without RT, followed by incubation at 42 °C for 1 h with RT.

The cDNA was amplified by PCR using *glgC*-specific forward (TTCTCGCGCGTTCGCGTGAATT) and reverse (ATCGCAAGACCGGCAACAGGATTC) primers. The PCR conditions were as follows: 3 min at 94 °C, 30 s at 94 °C, 45 s at 58 °C and 30 s at 72 °C, for 30 cycles, followed by 4 min at 72 °C. The PCR product was run on 0.8% (w/v) agarose gel. Control experiments included no cDNA product (negative control for genomic DNA contamination) and RT-PCR amplification of the *CYP79D1* gene [positive standard, according to Siritunga and Sayre (2003), which encodes a cytochrome P450 enzyme that catalyses the first dedicated step in linamarin synthesis] (Anderssen *et al*., 2000). The RT-PCR analysis was non-quantitative; the bands from the first amplification were used to repeat the PCR analysis.

AGPase enzyme extraction and assays

All isolation steps were conducted at 4 °C unless indicated otherwise. Three independent plants obtained from each initial transformant, or from three independently regenerated wild-type plants, were used for the enzyme extractions. The tuberous root sample (5 g) was sliced and placed into a Warring blender containing 50 mL of 0.05 M Tris-HCl (pH 7.5), 10 mM glutathione, 1.0 mM EDTA and 0.04 mL of 10% (w/v) sodium bisulphite, pH 6.0. The tissue was homogenized for two 15-s slow-speed periods followed by two 15-s fast-speed blending periods. The suspension was filtered through miracloth and the extract was centrifuged for 20 min at 27 000 ***g*** (Sowokinos, 1976; Kalt-Torres and Huber, 1987). The supernatant was used for all enzyme assays. Reaction assays for

AGPase contained, in 1 mL, 2.0 μmol ADP-glucose, 5 μmol $MgCl_2$, 80 μmol glycylglycine, 10 μmol NaF, 1 unit of P-glucomutase (Sigma-Aldrich, St. Louis, MO, USA) (4.4 μg), 20 μmol cysteine, 0.02 μmol glucose-1,6-diphosphate, 0.75 units glucose-6-phosphate dehydrogenase (Sigma-Aldrich) (2.1 μg), 0.6 μmol NADP and 0.005–0.05 units of pyrophosphorylase, in 50 mM Tris-HCl pH 7.5. The reaction was initiated by the addition of 1.5 μmol inorganic pyrophosphate (PPi). 3-PGA (2 mM) or phosphate (0.5 mM $KHPO_4$) was added to the assay mixture prior to initiation to study the effect of 3-PGA (activator) and Pi (inhibitor) on the plant AGPase. The production of NADPH was monitored spectrophotometrically at 340 nm for 10 min at 25 °C, and is stoichiometrically equivalent to the ADP-glucose consumed (Sowokinos, 1976; Kalt-Torres and Huber, 1987). Quantification of crude protein was performed using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA), according to the manufacturer's specifications, with BSA as a protein standard.

Growth analysis

Three independent replicates of 1-month-old plantlets with well-established roots were transferred to the glasshouse for growth measurements. The plantlets were initially maintained in a growth chamber at 28 °C at a light intensity of 50 μmol photons/m^2/s. Plantlets were transferred to 11 in × 12 in plastic pots filled with Scott's MetroMix soil (The Scott's Company, Marysville, OH, USA) after 30 days and grown in the glasshouse to maturity to generate stakes for glasshouse growth experiments. One foot long stakes of equal mass were planted in 11 in × 12 in plastic pots filled with Scott's MetroMix soil (The Scott's Company). The plants were grown from 23 January 2002 to 27 August 2002 (7 months). Tuberous roots were also harvested from 7-month-old plants for AGPase assays. Following the harvest of transformed and wild-type plants, the leaves, stem, tuberous roots and root–stem junction were separated, weighed for fresh weight values and placed in paper bags and dried in an incubator at 80 °C to determine the dry weight. The samples were weighed every 2 days until a constant dry weight was obtained. Each treatment had a minimum of three replicates.

Iodine assay

Free-hand cross-sections were made of 3D-1, 3D-2, 3D-3 and wild-type cassava tuberous roots of glasshouse-grown plants of similar size and age. The cross-sections were stained with Lugol's solution (I_2–KI)/distilled water (1 : 9) for 30 min. The sections were then washed with water and photographed.

Starch assay

Starch was extracted from 1 g of cassava tuberous root (fresh weight) by boiling in 30 mL of 80% (v/v) aqueous ethanol after grinding. The insoluble residue was dried at room temperature to remove ethanol, homogenized with 10 mL of distilled water and filtered. The suspension was autoclaved, cooled and made up to 16 mL with distilled water. A portion of the suspension (2 mL) was incubated at 37 °C for 2 h with 2 mL of 0.2 M sodium acetate buffer (pH 4.8) containing amyloglucosidase (5.6 units) and α-amylase (0.5 units). The mixture was then centrifuged for 10 min (3500 ***g***) (Stitt *et al*., 1978). The hexoses in the mixture were assayed through the reduction of NADP by a coupled reaction including hexokinase, phosphoglucomutase, invertase and glucose-6-phosphate dehydrogenase (Jones *et al*., 1977; Banks *et al*., 1999). The production of NADPH was measured spectrophotometrically at 340 nm and is stoichiometrically equivalent to the hexose generated.

Statistical analysis

Analysis of variance (ANOVA) and other statistical analyses were carried out using the SAS software program (SAS Institute Inc., 1985). Samples were evaluated using ANOVA for a randomized complete block design. Duncan's multiple range test was used to separate treatment means found to be significantly different by ANOVA. Pearson's correlation coefficient was used. All analyses were performed at a confidence level of $P \leq 0.05$.

Acknowledgements

This research was supported in part by the Rockefeller Foundation, the Centro Internacional Agricultura Tropical (CIAT) and the Ohio State University (RTS).

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