Characterization of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase*

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The aim of the work described in this paper was to characterize the tubers of potato (*Solanum tuberosum* var. *Prairie*) plants that had been transformed with the *Escherichia coli* ADPglucose pyrophosphorylase (EC 2.7.7.27) gene, *glgC-16*, under the control of a patatin promoter. Over 30 lines of transformed plants with increased ADPglucose pyrophosphorylase activity were obtained. The tubers of six of these lines were compared with those of control plants expressing the gene for β -glucuronidase. The average increase in pyrophosphorylase activity was 200 %, and the highest was 400 %. Western immunoblotting of tuber extracts showed that the amounts of *glgC-16* protein were linearly related to the extractable activity of the ADPglucose pyro-

phosphorylase. Cell fractionation studies showed that the increased activity of the pyrophosphorylase in the *glgC-16* tubers had a similar intracellular location, the amyloplast fraction, to that found in the control tubers. No pleiotropic changes in the maximum catalytic activities of the following enzymes could be detected in the *glgC-16* tubers: sucrose synthase, fructokinase, UDPglucose pyrophosphorylase, phosphofructokinase, soluble starch synthase, starch branching enzyme, phosphoglucomutase and alkaline inorganic pyrophosphatase. The *glgC-16* tubers are held to be suitable for the study of the role of ADPglucose pyrophosphorylase in the control of starch synthesis.

INTRODUCTION

Although there is appreciable evidence for a central role for ADPglucose pyrophosphorylase (EC 2.7.7.27) in the regulation of starch synthesis in chloroplasts, its role in amyloplasts is less well understood [1–3]. In an attempt to remedy this deficiency we have studied the metabolism of potato tubers in which the amount of ADPglucose pyrophosphorylase has been increased by transformation with the *Escherichia coli* gene *glgC-16*. The latter encodes a mutant form of ADPglucose pyrophosphorylase that shows a reduced response to allosteric effectors [4]. This gene was expressed specifically in tubers by placing it under the control of a patatin promoter and was targeted to the plastid by using a transit peptide from the small subunit of ribulose-bisphosphate carboxylase. As controls we used tubers from plants that were expressing the gene for β -glucuronidase (GUS-control). We chose to transform the tubers with a foreign gene in order to reduce the risk of co-suppression and because it is easier to manipulate the *E. coli* enzyme, which is a homotetramer, than the potato enzyme, which is a heterotetramer. We knew that a similar approach had been taken by Stark et al. [5], but argue that their data cannot be used for control analysis because no measurements of flux to starch or of the activity of ADPglucose pyrophosphorylase were reported.

Our argument is that if ADPglucose pyrophosphorylase plays a key role in the control of starch synthesis in potato tubers, then increasing the amount of this enzyme as just described should lead to an increased rate of synthesis. The availability of a range of transgenic tubers with differing activities of the pyrophosphorylase might allow calculation of the control coefficient of this enzyme in respect of starch synthesis. Our approach

depends crucially upon adequate characterization of the transgenic plants [6]. The aim of the work described in the present paper was to provide that characterization.

The first essential was to determine the extent to which the maximum catalytic activity of ADPglucose pyrophosphorylase had been altered. We stress that it is the activity of an enzyme that is paramount in determining its role in control, and that measurements of its mRNA or even the total amount of enzyme protein, though important, are not satisfactory substitutes as they may not be proportional to enzyme activity. The second essential was to check whether the increased pyrophosphorylase activity had the same location as the native enzyme. Finally it was essential to investigate whether any change in ADPglucose pyrophosphorylase activity had been accompanied by pleiotropic changes in other enzymes involved in starch synthesis. In making these checks we took particular care to avoid the pitfalls inherent in measuring enzymes in extracts of potato tubers [7]. For each enzyme we optimized the assay and checked that losses of activity did not occur during extraction and assay. We did this by determining the recovery of samples of pure enzymes added to the tissue sample before extraction.

EXPERIMENTAL

Materials

Except where stated otherwise, substrates and enzymes were from Boehringer-Mannheim U.K. (Lewes, E. Sussex, U.K.). Glucose 1,6-bisphosphate, kanamycin A and ampicillin were from Sigma Chemical Co. (Poole, Dorset, U.K.). Radiochemicals and the Amersham ECL immunodetection kit were from

Abbreviation used: GUS-control plant, plant expressing the gene for β -glucuronidase.

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* On 3 October 1996, Professor Tom ap Rees was tragically killed while cycling home. We dedicate this paper to his memory.

Amersham International (Aylesbury, Bucks., U.K.). ADPglucose pyrophosphorylase from *E. coli glgC-16* and polyclonal antibodies to it were kindly supplied by Advanced Technologies (Cambridge) Ltd. (Cambridge, U.K.). Antibodies were produced as follows. The *glgC-16* coding sequence [8] was cloned into the pGex vector (Pharmacia Biotech), and the vector was expressed in *E. coli* according to the manufacturer's instructions. The *glgC-16* gene product was purified by absorption on to glutathione-L-Sepharose equilibrated with 50 mM Tris, pH 8.0, and 150 mM NaCl. After washing the resin with equilibration buffer, the protein was eluted in 50 mM Tris, pH 8.0, 5 mM glutathione and 150 mM NaCl. Antibodies to the purified fusion protein were obtained from New Zealand white rabbits as described by Burrell et al. [8a].

Plants

The work was carried out with tubers from GUS-control and *glgC-16*-transformed plants of *Solanum tuberosum* var. *Prairie*. These plants were generously supplied by Advanced Technologies (Cambridge) Ltd. The GUS-control plants were produced by making the plasmid pFW4101 from pBin19 [9] with a patatin promoter made from two genomic clones, *ps3* and *ps27* [10], and the coding sequence for β -glucuronidase. This plasmid was introduced into *Agrobacterium tumefaciens*, strain C58, which was then used to transform potato leaf discs as described by Blundy et al. [10]. Kanamycin-resistant cells were used to regenerate shoots *in vitro* on Murashige and Skoog's medium [11]. Plants expressing the *E. coli glgC-16* gene were produced in the same way, except that the leaf discs were inoculated with *Agrobacterium* that contained plasmid pFW4173. The latter was made from plasmid pFW4101, with the β -glucuronidase coding sequence being replaced by that of a ribulose biphosphate carboxylase transit peptide and the *glgC-16* gene [8]. The regenerated shoots were grown at 22 °C in a 16-h photoperiod and a quantum irradiance of 159 $\mu\text{E/s}$ per m^2 . Microtubers were induced by transferring the shoots to Murashige and Skoog's medium [11] that contained 2.5 mg/l kinetin and 4.7 mg/l ancymidol, and growing at 22 °C in the dark for 4–6 weeks. To obtain tubers, shoots were planted in Fisons Levingtons F1 compost in pots of 63 mm diameter and grown in a greenhouse at 16–20 °C in a 16-h photoperiod of daylight supplemented with artificial light that gave a minimum quantum irradiance of 125 $\mu\text{E/s}$ per m^2 . After 1 week in a propagator the plants were transplanted into a mixture of Perlite and Levingtons M2 compost (1:2, v/v) in 130 mm diameter pots and grown for 2–3 months under the conditions just described. Plant lines were propagated clonally by planting cuttings of shoot tips as we have described above. Experiments were carried out with tubers of 10–50 g fresh weight either immediately after harvest or after a brief period of storage at 4 °C.

Enzyme assays

Tubers were cut into 2 mm-thick slices, which were immediately freeze-clamped and ground to a fine powder in liquid N_2 with a pestle and mortar. The frozen powder was immediately transferred to -180°C and stored at this temperature for up to 2 months before use. To assay enzyme activity, 1 g of the frozen powder was resuspended at 4 °C in 5 ml of extraction medium [100 mM Hepes, pH 7.5, 10 mM EDTA, 5 mM dithiothreitol, 0.5% (w/v) BSA] that contained 0.1% (w/v) polyvinylpyrrolidone. After 5 min the suspension was centrifuged at 10000 g for 5 min at 4 °C and the supernatant was desalted by passage through a column (5 cm $\times$ 1.5 cm) of PD-10 Sephadex (G-25M) equilibrated with extraction medium, and then assayed.

Unless otherwise stated, enzymes were assayed at 25 °C in the following 1.0 ml reaction mixtures as described in the accompanying references. ADPglucose pyrophosphorylase (EC 2.7.7.27): 40 mM Hepes, pH 8.0, 10 mM MgCl_2 , 0.4 mM NAD^+ , 0.024 mM glucose 1,6-bisphosphate, 1.5 mM ADPglucose, 2 mM $\text{Na}_4\text{P}_2\text{O}_7$, 4 units of phosphoglucosmutase and 1.4 units of glucose-6-phosphate dehydrogenase (NAD^+ -specific from *Leuconostoc mesenteroides*) [12]. Soluble starch synthase (EC 2.4.1.21): 150 mM Bicine, pH 8.4, 400 mM sodium citrate, 0.1 mg of potato amylopectin and 1.4 mM ADP[U- ^{14}C]glucose (5.3 kBq/ μmol) in 200 μl at 30 °C [13]. Starch branching enzyme (EC 2.4.1.18): 100 mM sodium citrate, pH 6.0, 1 mM AMP, 50 mM [U- ^{14}C]glucose 1-phosphate (1.5 kBq/ μmol) and 0.02 unit of glycogen phosphorylase from rabbit muscle in 50 μl at 30 °C [14]. Alkaline inorganic pyrophosphatase (EC 3.6.1.1): 50 mM Tris/HCl, pH 8.0, 5 mM MgCl_2 and 1.5 mM $\text{Na}_4\text{P}_2\text{O}_7$ in 200 μl [15]. Sucrose synthase (EC 2.4.1.13): 100 mM Hepes, pH 7.5, 4 mM MgCl_2 , 0.4 mM UDPglucose, 1 mM phosphoenolpyruvate, 10 mM fructose, 0.2 mM NADH, 10 units of pyruvate kinase and 2 units of lactate dehydrogenase [16]. UDPglucose pyrophosphorylase (EC 2.7.7.9): 80 mM glycylglycine, pH 8.0, 1 mM MgCl_2 , 10 μM glucose 1,6-bisphosphate, 0.4 mM NAD^+ , 0.8 mM UDPglucose, 1 mM $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, 4 units of phosphoglucosmutase and 1.4 units of glucose-6-phosphate dehydrogenase (NAD^+ -dependent) [17]. 6-Phosphofructokinase (EC 2.7.1.11): 100 mM Tris/HCl, pH 8.0, 5 mM MgCl_2 , 5 mM fructose 6-phosphate, 0.1 mM NADH, 1 mM ATP, 1 unit of aldolase, 10 units of triosephosphate isomerase and 1.3 units of glycerol-3-phosphate dehydrogenase [18]. Phosphoglucosmutase (EC 5.4.2.2): 50 mM Hepes, pH 7.6, 1 mM MgCl_2 , 0.25 mM glucose 1-phosphate, 0.024 mM glucose 1,6-bisphosphate, 0.4 mM NAD^+ and 1.5 units of glucose-6-phosphate dehydrogenase (NAD^+ -specific) [19]. Fructokinase (EC 2.7.1.4): 100 mM Hepes, pH 8.2, 4 mM MgCl_2 , 0.2 mM fructose, 2.5 mM ATP, 0.3 mM NAD^+ , 1 unit of glucose-6-phosphate dehydrogenase (NAD^+ -dependent) and 5 units of glucose-6-phosphate isomerase [20]. Alcohol dehydrogenase (EC 1.1.1.1): 50 mM Hepes, pH 7.8, 2 mM NAD^+ and 150 mM ethanol [21].

Immunodetection of the *glgC-16* protein

Portions of the desalted extract used for the enzyme assays were heated to 100 °C for 5 min and were then subjected to discontinuous SDS/PAGE [22]. The separated proteins were electroblotted on to poly(vinylidene difluoride) membranes which were incubated first with polyclonal antiserum raised against the *glgC-16* protein, and then with anti-rabbit IgG conjugated to horseradish peroxidase (Amersham International). Bound antibody was detected with the Amersham ECL immunodetection kit.

Cell fractionation

Amyloplasts were separated from the cytosol of potato tubers by taking a core (1.4 cm $\times$ 6 cm) of tissue (10 g fresh weight) longitudinally through a tuber. The core was cut into discs 1–2 mm thick with a razor blade into 15 ml of 50 mM Hepes, pH 7.6, 1 M sucrose, 1 mM EDTA, 1 mM MgCl_2 , 1 mM KCl, 0.2% (w/v) BSA and 5 mM dithiothreitol (homogenization medium). This first 15 ml of homogenization medium was discarded, and the discs were washed twice more with 10 ml lots of medium. Next the discs were chopped very finely with razor blades in 10 ml of homogenization medium for no more than 5 min. The resulting suspension was filtered through four layers of muslin that had been soaked in homogenization medium. The filtrate (10 ml) was layered on to a stepped gradient that consisted

of 5 ml of 60 % (w/v) Nycodenz overlaid with 10 ml of 1 % (w/v) Nycodenz. The Nycodenz was dissolved in homogenization medium. The gradient was allowed to stand for 2 h, by which time a white band, referred to as amyloplasts, had formed at the interface between the 1 % and 60 % Nycodenz. The rest of the homogenate remained above the 1 % Nycodenz (the supernatant fraction). These two fractions were completely removed and assayed for the appropriate enzymes. The whole process of homogenization and fractionation was carried out at 4 °C.

Before assay of enzyme activity in the fractions or the unfractionated homogenate, care was taken to rupture any organelles present. For ADPglucose pyrophosphorylase this was done by making the sample 0.1 % (v/v) with respect to Triton X-100. For all other assays the sample was subjected to three cycles of freezing in liquid N₂ and thawing at 37 °C.

Protein was measured with the Bio-Rad assay kit [23], and ¹⁴C was determined by liquid-scintillation counting with Optiphase scintillation fluid.

RESULTS

Increased activity of ADPglucose pyrophosphorylase in transgenic tubers

Initial experiments indicated that tubers from transformed line no. 123 had enhanced ADPglucose pyrophosphorylase activity. We used extracts of mature tubers of this line that had been stored for 8 weeks to authenticate our assay procedure. The concentration of each component in, and the pH of, the assay were optimized to give the values listed in the Experimental section. Activity was shown to be linearly related to the volume of extract used and, after an initial lag, to time. As expected from the work of Merlo et al. [24], we found significant variation in the activity of ADPglucose pyrophosphorylase in extracts of different tubers, we took the complete tuber as our basic sample for analysis. The tuber was sliced and the slices were instantly freeze-clamped and ground to a homogeneous powder in liquid N₂. A subsample of this powder was used for the measurement of enzyme activity. We checked that this procedure did not result in loss of enzyme activity by comparing activity in extracts prepared from the frozen powder and extracts prepared by homogenizing fresh tissue in extraction medium. The native potato ADPglucose pyrophosphorylase and the introduced *E. coli* enzyme respond differently to 3-phosphoglycerate. Thus, in order to compare activities in *glgC-16* transgenic and GUS-control tubers, we assayed the enzyme in the absence of exogenous 3-phosphoglycerate. This explains why the activities that we report are lower than those found by Merlo et al. [24] and by Geigenberger et al. [25].

We completed recovery experiments to check for losses during extraction of the tissue. In each test we took duplicate samples of powdered freeze-clamped tissue. One sample was extracted and assayed in the usual way, and the other was treated similarly except that a measured activity of pure *glgC-16* protein was added to the frozen powder prior to extraction. The added activity was comparable with that present in the untreated sample. Comparison of the activities in the two samples allowed estimation of the recovery of the pure enzyme. Values of 102 ± 10 % and 112 ± 11 % (means ± S.E.M., *n* = 3) were obtained for *glgC-16* transgenic and GUS-control tubers respectively. The optimized assay was used in all subsequent work.

Microtubers from 37 different lines of transformed shoots were assayed for ADPglucose pyrophosphorylase activity. Values ranged from 120 to 560 nmol/min per g fresh weight. For further studies we selected six lines that represented the range of these

Figure 1 ADPglucose pyrophosphorylase activity in developing tubers from *glgC-16* and GUS-control plants

Developing tubers (10–50 g fresh wt.) were freeze-clamped immediately after harvest from 10-week-old plants. Each value is the mean of estimates from at least five tubers; bars represent S.E.M. □, GUS-control lines, ■, *glgC-16* lines.

Figure 2 Western blot analysis of *glgC-16* protein in extracts of cold-stored tubers from *glgC-16*-transformed and GUS-control plants

Extracts of frozen powders from tubers that had been stored at 4 °C for 3 months were centrifuged and the supernatant fractions desalted prior to analysis by SDS/PAGE and Western blotting. Protein loading was 100 µg per lane. Lanes 1–3, GUS-control line 5; lanes 4–6, *glgC-16* line 12; lanes 7–9, *glgC-16* line 82; lanes 10–12, *glgC-16* line 123. The arrow indicates *glgC-16* protein.

activities. First, we compared the maximum catalytic activity of ADPglucose pyrophosphorylase in tubers from 10-week-old plants of each of these lines with that of comparable tubers from six GUS-control lines (Figure 1). The activity in each of the *glgC-16* lines was higher than the average activity of the GUS-control lines (*P* < 0.05). The activity in each of the *glgC-16* lines, except for no. 12, was greater than that of the highest value found in any GUS-control line (*P* < 0.05).

We used Western blotting to determine whether the increase in ADPglucose pyrophosphorylase activity in the *glgC-16* lines was paralleled by increases in the amount of the *glgC-16* protein. We extracted proteins from tubers that had been stored at 4 °C for 3 months, separated them by SDS/PAGE and treated them with an antibody raised to *glgC-16* protein from *E. coli*. The *glgC-16* protein has a molecular mass of 50 kDa [26], and was clearly present in tubers from the transformed lines 82 and 123 (Figure 2). The *glgC-16* protein could not be detected in tubers of the

Figure 3 Relationship between ADPglucose pyrophosphorylase activity and the amount of glgC-16 protein in extracts of *glgC-16*-transformed tubers

Cold-stored tubers of *glgC-16* transgenic lines 12, 82 and 123 were freeze-clamped. Extracts of the powders were centrifuged and the supernatant fractions desalted, assayed for enzyme activity and analysed by Western blotting. The density of the bands on the blots was measured by scanning densitometry. Values are the means $\pm$ S.E.M. of results from three tubers of each line.

GUS-control line 5, or in tubers of the transformed line 12 which did not have a significantly increased activity of ADPglucose pyrophosphorylase. The antibody also recognized a second protein, of unknown identity, with a molecular mass of approx. 65 kDa. This protein was present in both control and transformed tubers, but is not the tuber ADPglucose pyrophosphorylase, which has a molecular mass of 50 kDa [27].

Qualitative comparison of Figures 1 and 2 suggests that the activity of ADPglucose pyrophosphorylase rose in proportion to the amount of glgC-16 protein present. We prepared extracts from three different *glgC-16* transgenic lines and measured both enzyme activity and the density of the band corresponding to glgC-16 protein on Western blots, by scanning densitometry, for each extract. Figure 3 shows that there is a linear relationship between our estimates of enzyme activity and our estimates of the amount of enzyme protein. The plot intersects the x -axis at a value that is equivalent to the activity of the enzyme in cold-stored GUS-control tubers.

Intracellular location of ADPglucose pyrophosphorylase

For the relatively few non-photosynthetic tissues for which data are available, the evidence strongly suggests that ADPglucose pyrophosphorylase is largely, if not exclusively, confined to the plastid. However, there is some evidence that there is a cytosolic ADPglucose pyrophosphorylase in some cereal endosperms [28]. No data are available for potato tubers. In order to investigate whether the increased activity of the pyrophosphorylase in the *glgC-16*-transformed tubers had the same intracellular location as the pyrophosphorylase in the control tubers, we made preparations of amyloplasts from each type of tuber (Table 1). We used alkaline inorganic pyrophosphatase as a marker for amyloplasts [29] and alcohol dehydrogenase as a marker for the cytosol. Our fractionation procedure yielded only two fractions, a particulate amyloplast fraction and a supernatant. For each enzyme studied the sum of the activities recovered in the amyloplast fraction and the supernatant was similar to the activity found in the unfractionated homogenate. Thus our data are not seriously affected by losses during analysis. The activities of the marker enzymes were comparable in the extracts of the GUS-control and *glgC-16* transgenic tubers. As expected, the latter showed higher activity of ADPglucose pyrophosphorylase. We obtained a modest but significant yield of amyloplasts that were essentially free of cytosolic contamination. The distribution of the marker enzymes in preparations from GUS-control tubers was not significantly different from that in preparations from *glgC-16*-transformed tubers. Of most importance is the fact that the distribution of ADPglucose pyrophosphorylase followed that of the amyloplast marker in the preparations of both the GUS-control and *glgC-16* tubers.

Search for pleiotropic effects

At no stage during development could we detect any phenotypic difference between the *glgC-16*-transformed plants and the GUS-control plants.

We investigated whether the increased activity of ADPglucose pyrophosphorylase in the *glgC-16* plants was accompanied by changes in the maximum catalytic activities of other enzymes connected with starch synthesis. We checked the assays for each enzyme as described for the assay of ADPglucose pyrophosphorylase. Each assay was optimized. For each enzyme we showed that freeze-clamping the tuber tissue and then extracting the frozen powder gave a similar activity to that found when the tuber tissue was homogenized directly in extraction medium.

Table 1 Activities of ADPglucose pyrophosphorylase in amyloplast preparations from GUS-control and *glgC-16* transgenic tubers

Tubers were from 8-week-old plants and had been stored at 4 °C for 6 weeks. Cores of tuber were chopped with razor blades into extraction medium to give a suspension that was filtered through muslin to produce the unfractionated homogenate. The latter was fractionated on a stepped gradient of Nycodenz to give an amyloplast fraction and a supernatant fraction, and activities in each fraction are expressed as percentages of that in the unfractionated homogenate. Values are means $\pm$ S.E.M. of data from fractionations of three different tubers of each type.

Enzyme	Tuber	Activity in homogenate (nmol/min per ml)	Activity recovered in fraction (%)	
			Amyloplasts	Supernatant
Alcohol dehydrogenase	GUS-control	70 $\pm$ 8	0.7 $\pm$ 0.2	97 $\pm$ 1
	<i>glgC-16</i>	75 $\pm$ 7	0.9 $\pm$ 0.2	102 $\pm$ 3
Alkaline pyrophosphatase	GUS-control	67 $\pm$ 6	6.9 $\pm$ 1.5	100 $\pm$ 7
	<i>glgC-16</i>	73 $\pm$ 2	7.1 $\pm$ 1.5	104 $\pm$ 7
ADPglucose pyrophosphorylase	GUS-control	4.5 $\pm$ 0.5	7.3 $\pm$ 1.1	92 $\pm$ 4
	<i>glgC-16</i>	17.9 $\pm$ 1.2	8.0 $\pm$ 0.8	95 $\pm$ 5

Table 2 Estimates of the maximum catalytic activities of enzymes of starch metabolism in GUS-control and *glgC-16* transgenic tubers after harvest from 10-week-old plants

Frozen powder from freeze-clamped tubers was suspended in extraction medium at 4 °C for 5 min and then centrifuged at 10 000 *g* for 5 min. The supernatant was assayed for enzyme activities (nmol/min per g fresh wt., except where indicated otherwise). Values are means $\pm$ S.E.M. of results from the numbers of tubers shown in parentheses.

	Activity (nmol/min per g fresh wt.)						
	GUS-control	<i>glgC-16</i> transgenic					
		Line 12	Line 84	Line 85	Line 139	Line 82	Line 123
ADPglucose pyrophosphorylase	100 $\pm$ 12 (12)	212 $\pm$ 33 (5)	273 $\pm$ 24 (5)	345 $\pm$ 45 (5)	386 $\pm$ 51 (5)	398 $\pm$ 53 (5)	493 $\pm$ 34 (5)
Alkaline inorganic pyrophosphatase	408 $\pm$ 40 (12)	365 $\pm$ 27 (3)	397 $\pm$ 19 (3)	370 $\pm$ 37 (3)	405 $\pm$ 48 (3)	444 $\pm$ 51 (3)	406 $\pm$ 42 (3)
Soluble starch synthase	119 $\pm$ 7 (12)	118 $\pm$ 36 (4)	164 $\pm$ 50 (4)	135 $\pm$ 48 (4)	173 $\pm$ 56 (4)	142 $\pm$ 33 (4)	150 $\pm$ 48 (4)
Branching enzyme*	4.1 $\pm$ 0.4 (12)	5.1 $\pm$ 1.8 (4)	4.5 $\pm$ 1.5 (4)	5.4 $\pm$ 1.3 (4)	4.0 $\pm$ 1.6 (4)	4.9 $\pm$ 1.4 (4)	3.7 $\pm$ 1.9 (4)
Phosphoglucomutase†	9.2 $\pm$ 0.4 (6)	11.4 $\pm$ 0.5 (4)	—	—	—	9.4 $\pm$ 0.6 (4)	10.2 $\pm$ 0.8 (4)
Sucrose synthase	959 $\pm$ 129 (18)	674 $\pm$ 297 (3)	705 $\pm$ 18.3 (3)	1034 $\pm$ 282 (3)	956 $\pm$ 100 (3)	640 $\pm$ 292 (3)	788 $\pm$ 371 (3)
Fructokinase	242 $\pm$ 17 (18)	214 $\pm$ 60 (3)	278 $\pm$ 15 (3)	272 $\pm$ 23 (3)	208 $\pm$ 26 (3)	280 $\pm$ 72 (3)	230 $\pm$ 33 (3)
Phosphofructokinase	164 $\pm$ 10 (12)	161 $\pm$ 10 (4)	—	—	—	195 $\pm$ 21 (4)	192 $\pm$ 18 (4)
UDPglucose pyrophosphorylase†	7.0 $\pm$ 0.7 (18)	6.5 $\pm$ 1.2 (3)	7.6 $\pm$ 1.0 (3)	8.1 $\pm$ 0.1 (3)	7.6 $\pm$ 1.2 (3)	8.0 $\pm$ 1.4 (3)	8.3 $\pm$ 0.4 (3)

* Values are $10^{-3} \times$ fold stimulation of the activity of glycogen phosphorylase/g fresh wt.

† Values are μ mol/min per g fresh wt.

Recovery experiments were carried out with both *glgC-16*-transformed tubers (line 123) and GUS-control tubers for phosphofructokinase, fructokinase, UDPglucose pyrophosphorylase and phosphoglucomutase. Recoveries of phosphoglucomutase were 79% and 73% respectively for the GUS-control and *glgC-16* tubers. For the remaining enzymes, recoveries were not significantly different from 100%. For soluble starch synthase, branching enzyme and sucrose synthase we carried out recombination experiments, where we measured activity in a sample composed of frozen tuber powder and material from the spadix of *Arum maculatum*, and compared the value obtained with that predicted from measurements made on the separate components of the mixture. The observed activities of the mixtures as percentages of the predicted values were: soluble starch synthase, $69 \pm 10\%$; branching enzyme, $86 \pm 3\%$; sucrose synthase, $113 \pm 8\%$ (means $\pm$ S.E.M., $n = 3$).

Our estimates of the maximum catalytic activities of enzymes related to starch synthesis in GUS-control and six different *glgC-16*-transformed lines are given in Table 2. The activity of ADPglucose pyrophosphorylase in each of the *glgC-16* lines was confirmed as being significantly higher ($P < 0.05$) than that in the GUS-control tubers. For starch synthase, analysis of variance shows that the activity in *glgC-16* line 139 was significantly greater ($P < 0.05$) than that in the GUS-control. However, if the values for starch synthase from all six *glgC-16* lines are treated as a single population and compared with the GUS-control lines, then no significant difference is found. For each of the other enzymes, no statistically significant difference was found between any one *glgC-16* line and the GUS-control.

DISCUSSION

We argue that our estimates of enzyme activity are adequately authenticated and reflect the maximum catalytic activities in the tubers. Thus we conclude that the transformation procedure was effective in that it produced a range of tubers with increased activity of ADPglucose pyrophosphorylase. That this increase was due to the presence of the *glgC-16* protein is shown by our Western blot analysis and the demonstration of a linear relationship between enzyme activity and amount of protein (Figures 2 and 3).

Our cell fractionation studies failed to reveal any difference in the distribution of ADPglucose pyrophosphorylase between GUS-control and *glgC-16* transgenic tubers. The close correlation between the distribution of ADPglucose pyrophosphorylase and inorganic pyrophosphatase, the plastid marker, in GUS-control and *glgC-16* tuber extracts strongly suggests that ADPglucose pyrophosphorylase is located in the plastids in both types of tuber.

The *glgC-16*-transformed lines did not show any pleiotropic effect of the increased activity of ADPglucose pyrophosphorylase. We conclude that the *glgC-16*-transformed tubers described in this paper show enhanced activity of ADPglucose pyrophosphorylase due to the presence of the *glgC-16* protein. We also suggest that the *glgC-16* protein is correctly located in the cell and that no serious pleiotropic changes have occurred. Thus the *glgC-16* tubers may be used to analyse the role of the pyrophosphorylase in starch synthesis, and this is investigated further in the accompanying paper [30].

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