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 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 pyrophosphorylase. 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...
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, and growing at 22 °C in a 16-h photoperiod of daylight supplemented with artificial light that gave a minimum quantum irradiance of 159 µE/m²/s. Microtubers were propagated clonally by planting cuttings of shoot tips as we have previously described [10]. 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 bisphosphate 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 µE/s per m². 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 ancyimidol, 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 maximum quantum irradiance of 125 µE/s per m². 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 N₂ 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 × 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 MgCl₂, 0.4 mM NAD⁺, 0.024 mM glucose 1,6-bisphosphate, 1.5 mM ADP, 2 mM NaP₂O₄, 4 units of phosphoglucomutase 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-¹⁴C]glucose (5.3 kBq/mmol) 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 µM [U-¹⁴C]glucose 1-phosphate (1.5 kBq/mmol) 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 MgCl₂ and 1.5 mM NaP₂O₄ in 200 µl [15]. Sucrose synthase (EC 2.4.1.13): 100 mM Hepes, pH 7.5, 4 mM MgCl₂, 0.4 mM UDPglucose, 1 mM phosphoenol-pyruvate, 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 glycolliglycerine, pH 8.0, 1 mM MgCl₂, 10 µM glucose 1,6-bisphosphate, 0.4 mM NAD⁺, 0.8 mM UDPglucose, 1 mM NaH₂PO₄, 4 units of phosphoglucomutase 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 MgCl₂, 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 glyceraldehyde-3-phosphate dehydrogenase [18]. Phosphoglucomutase (EC 5.4.2.2): 50 mM Hepes, pH 7.6, 1 mM MgCl₂, 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 MgCl₂, 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 electrophoebotted 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 × 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 MgCl₂, 1 mM KCl, 0.2% (w/v) BSA and 5 mM dithiothreitol (homogenization medium). This first 15 ml of homogenization medium was discarded. The next 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 parts of the same tuber. To combat this heterogeneity within 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 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 transgenic lines.
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 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 ± S.E.M. of results from three tubers of each line.

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 ± S.E.M. of results from three tubers of each line.

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tuber</th>
<th>Activity in homogenate (nmol/min per ml)</th>
<th>Activity recovered in fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amyloplasts</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>GUS-control</td>
<td>70 ± 8</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>glgC-16</td>
<td>75 ± 7</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Alkaline pyrophosphatase</td>
<td>GUS-control</td>
<td>67 ± 6</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>glgC-16</td>
<td>73 ± 2</td>
<td>7.1 ± 1.5</td>
</tr>
<tr>
<td>ADPglucose pyrophosphorylase</td>
<td>GUS-control</td>
<td>4.5 ± 0.5</td>
<td>7.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>glgC-16</td>
<td>17.9 ± 1.2</td>
<td>8.0 ± 0.8</td>
</tr>
</tbody>
</table>

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.

Intracellular location of ADPglucose pyrophosphorylase

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.
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 phosphofructokinase, fructokinase, UDPglucose pyrophosphorylase and inorganic pyrophosphatase, the plastid marker, in GUS-control and glgC-16 tubers 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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