Starch metabolism in tubers of transgenic potato (*Solanum tuberosum*) with increased ADPglucose pyrophosphorylase*

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The aim of this work was to use tubers from transgenic lines of potato (*Solanum tuberosum*) containing increased amounts of ADPglucose pyrophosphorylase to study the role of this enzyme in the control of starch synthesis. A 4–5-fold increase in activity of the enzyme, achieved by transformation with the *Escherichia coli* ADPglucose pyrophosphorylase gene *glgC-16*, had no detectable effect on the starch content of developing or mature tubers. No significant effects were found on the contents of ADPglucose, UDPglucose, glucose 1-phosphate, glucose 6-phosphate, PP_i, ATP and ADP. Flux from [U-^{14}C]sucrose, supplied to tubers still attached to the plant, to starch increased roughly in proportion to the increase in ADPglucose pyrophosphorylase activity. These measurements of flux gave a response coefficient close to 1 for the activity of the pyrophosphorylase in respect of starch synthesis. Pulse–chase experiments with [U-^{14}C]sucrose showed that the increased flux into starch in the transformed tubers was accompanied by an increased rate of starch turnover. Further experiments suggested that the increased turnover was associated with an increase in the capacity of the tubers to degrade starch.

INTRODUCTION

We have characterized tubers from potatoes transformed with the *Escherichia coli* ADPglucose pyrophosphorylase (EC 2.7.7.27) gene, *glgC-16*, under the control of a patatin promoter [1]. This gene encodes a mutant form of the enzyme that shows a diminished response to allosteric effectors. Tubers of the transformed lines show a 4-fold increase in the maximum catalytic activity of the pyrophosphorylase, but no significant sign of pleiotropic changes in the activities of other enzymes of starch metabolism. The increased activity of the pyrophosphorylase in the transgenic tubers has the same intracellular location as the enzyme in control tubers [1].

The aim of the work described in the present paper was to use these transgenic tubers to study the role of ADPglucose pyrophosphorylase in the control of starch synthesis in potatoes. The behaviour of the transgenic tubers has been compared with that of tubers transformed in the same way with the gene for β-glucuronidase (GUS-control tubers). We have paid particular attention to any effects on the total starch content of the tubers, for two reasons: first, because of the obvious economic importance of this feature, and secondly, because of the published evidence that increasing the ADPglucose pyrophosphorylase activity does increase the starch content of potatoes [2].

EXPERIMENTAL

Materials

The work was carried out with tubers from GUS-control and *glgC-16*-transformed plants of *Solanum tuberosum* L. var. Prairie. The transformation of the plants, their growth and the harvesting of the tubers are described in the previous paper [1], which also lists the sources of materials used.

Enzyme assays

Tubers were sliced, freeze-clamped and homogenized in liquid N_2 to give a powder that was extracted, centrifuged, desalted and assayed for ADPglucose pyrophosphorylase as described in [1]. The extract was assayed for other enzymes at 25 °C in the following reaction mixtures and according to the accompanying references. α-Glucan phosphorylase (EC 2.4.1.1): 50 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 0.25 mM NAD^+, 0.024 mM glucose 1,6-bisphosphate, 0.0025 % (w/v) potato amylopectin, 4.5 mM Na_2HPO_4, 2 units of phosphoglucomutase and 1.4 units of glucose-6-phosphate dehydrogenase (NAD^+-specific, from *Leuconostoc mesenteroides*) in 1.0 ml [3]. α-Amylase (EC 3.2.1.1): 100 mM Hepes, pH 7.5, 3 mM $CaCl_2$, 100 μl of 2 % (w/v) starch azure suspension and 1000 units of β-amylase in 200 μl [4]. Debranching enzyme (EC 3.2.1.41): 200 mM Hepes, pH 7.5, 10 % (w/v) pullulan from *Aureobasidium pullulans* and 10 mM EDTA in 200 μl [5]. β-Amylase (EC 3.2.1.2) was assayed by incubating a 10 μl extract in 0.2 M Hepes, pH 7.1, 10 mM EDTA, 10 % (w/v) amylopectin and 1 unit of α-glucosidase in 200 μl for 30 min at 25 °C. Then the reaction mixture was kept at 100 °C for 3 min and the amount of glucose present was assayed as described below ('Measurement of substrates'). Total amylolytic activity was measured in a 500 μl reaction mixture that contained 100 mM Hepes, pH 7.5, 10 mM $CaCl_2$, 2 % (w/v) amylopectin and 100 μl of extract. After incubation at 25 °C for 30 min, reducing equivalents were determined as described by Bernfeld [6].

Measurement of substrates

All substrates were measured in samples of frozen powder prepared from freeze-clamped tissue as described for the assay of enzymes [1]. Starch was measured as described by Hovenkemp-Hermelink et al. [7]. About 100 mg of frozen powdered tissue

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.

was added to 1 ml of 5.5 M $HClO_4$. The suspension was left at 4 °C for 5 min, diluted with 9 ml of water and centrifuged at 10000 ***g*** for 5 min. An aliquot (50 μl) of the supernatant was incubated for 16 h at 37 °C in 0.2 M sodium acetate, pH 5.5, containing 20 units of α-amylase and 2 units of amyloglucosidase in a final volume of 1 ml. The glucose released was determined according to Kunst et al. [8] in a 1.0 ml reaction mixture of 100 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 0.25 mM $NADP^+$, 1 mM ATP and 0.5 unit of glucose-6-phosphate dehydrogenase.

For the assay of sugars and sugar nucleotides, 0.1–0.5 g of the frozen powdered tissue was resuspended in 1 ml of 1.41 M $HClO_4$ and left at 4 °C for 2 h. The suspension was then centrifuged at 10000 ***g*** for 5 min and the sediment was washed by resuspension and centrifugation in two 1.0 ml portions of 1.41 M $HClO_4$. The supernatant fractions were combined, neutralized with K_2CO_3 and centrifuged at 10000 ***g*** for 10 min to give a supernatant that was assayed for glucose as described above. Fructose was then measured by adding 0.7 unit of phosphoglucose isomerase to the reaction mixture. To measure sucrose, the neutralized $HClO_4$ extract was incubated at 37 °C for 1 h with 30 units of yeast invertase in 0.2 M sodium acetate, pH 5.0, and the amounts of hexose released were assayed as above. Sugar nucleotides were measured by HPLC [9]. The neutralized $HClO_4$ extract was filtered through a membrane of 0.45 μm pore size and 200 μl of the filtrate was loaded on to a column (25 mm × 4.6 mm) of Whatman Partisil 10 SAX. ADPglucose and UDPglucose were eluted as follows. Buffer A was 10 mM $NH_4H_2PO_4$, pH 3.0, buffer B was 450 mM $NH_4H_2PO_4$, pH 4.3, and a constant flow rate of 1.6 ml/min was used: 0–10 min, 100 % A; 10–30 min, 92 % A/8 % B; 30–40 min, 100 % B. ADPglucose and UDPglucose were eluted as adjacent but separate peaks and were detected by measuring A_{254}, identified by their retention times and quantified by comparison with standards.

For the assay of all other substrates, powdered freeze-clamped tissue was extracted by a modification of the method of Weiner et al. [10]. Samples (500 mg) of the powder were resuspended in 1 ml of 12 % (w/v) trichloroacetic acid and 5 mM EGTA. After 2 h at 0 °C, the suspension was centrifuged at 10000 ***g*** for 5 min. The supernatant was washed by extraction with three successive 1.0 ml lots of water-saturated diethyl ether and then neutralized with 5 M KOH/1 M triethanolamine, and the ether was removed *in vacuo* at 25 °C. Substrates were then assayed according to the following references: glucose 1-phosphate, glucose 6-phosphate [11]; ATP, ADP [12]; PP_i [13]; 3-phosphoglyceric acid, Method 1 in [14].

Metabolism of [^{14}C]sucrose

Tubers were labelled by making a small feeding well in the side of the tuber, half way between the point of attachment of the stolon and the apical end of the tuber. A hypodermic needle (external diameter 1.5 mm) was inserted to a depth of 15 mm, rotated and withdrawn slowly to remove a core of tissue. The well in the tuber was filled with 20 μl of 0.357 mM [U-^{14}C]sucrose (20.7 MBq/μmol) and sealed with petroleum jelly. At the end of the incubation the [^{14}C]sucrose was removed from the feeding well, which was then rinsed with three 20 μl portions of 0.5 mM sucrose. The tuber was either sampled at once or left as a chase sample under the conditions described above for the pulse.

To determine the distribution of ^{14}C in a tuber, a core of tuber tissue, of diameter 7 mm and concentric with the feeding well, was removed with a cork borer and immediately killed and extracted with boiling 80 % (v/v) ethanol. The insoluble material was homogenized and extracted exhaustively with more 80 % ethanol. The extracts were evaporated to 2–3 ml at 25 °C and made up to 10 ml with water to give the soluble fraction. The insoluble material was digested as described for the assay of starch and the ^{14}C that was released was measured. ^{14}C in the undigested insoluble fraction was determined after solubilization with Scintran tissue solubilizer. ^{14}C was measured by liquid scintillation counting with optiphase Hisafe 3 scintillation fluid.

RESULTS

Starch content

We checked the effectiveness of our assay of starch. First, samples of 100 mg of pure starch from potatoes, roughly 10 times the amount found in our samples of tuber tissue, were assayed by our method. The expected amounts of glucose were found. Secondly, we added measured amounts of starch to samples of GUS-control and *glgC-16*-transformed tubers and measured recoveries in the normal starch assay. Recoveries were 96 ± 4 % and 97 ± 3 % for GUS-control and transgenic tubers respectively (means ± S.E.M., n = 6).

We grew 20 GUS-control and 30 *glgC-16* transgenic plants in the greenhouse for 3 months, harvested the tubers and determined the starch content of tubers of different sizes (Figure 1). We emphasize that in all instances the sample size was the complete

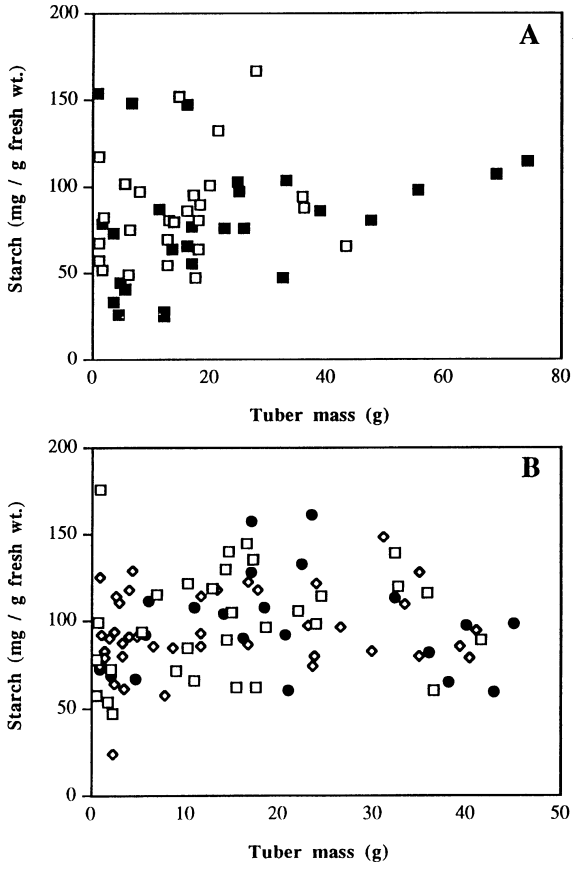

Figure 1 Starch content of tubers of GUS-control and *glgC-16*-transformed plants

Plants were grown for 3 months in a greenhouse. The GUS-control population (**A**) consisted of 10 plants each of lines 5 (□) and 24 (■); the *glgC-16* transgenic population (**B**) consisted of 10 plants each of lines 139 (●), 82 (□) and 123 (◇). Each value is the starch content of a separate tuber.

Table 1 Starch content and ADPglucose pyrophosphorylase activity in tubers from GUS-control and *glgC-16* transgenic plants

Tubers were from four different populations of plants, each of which consisted of 3–10 plants of each line of GUS-control and *glgC-16* transgenic tubers. Each population was grown separately. Developing tubers were analysed immediately after harvesting from 7–10-week-old plants grown in greenhouses. Mature tubers were from field-grown plants, and were harvested at the end of the growing season and stored at 4 °C for 4 weeks before analysis. Tubers of 10 g fresh wt. or heavier were chosen at random. Data are means ± S.E.M. for the numbers of tubers shown in parentheses.

	ADPglucose pyrophosphorylase activity (nmol/min per g fresh wt.)		Starch content (mg/g fresh wt.)						
				glgC-16					
Population	GUS-control	*glgC-16*	GUS-control	Line 12	Line 84	Line 85	Line 139	Line 82	Line 123
A (developing)	105 ± 21 (21)	437 ± 44 (24)	78 ± 7 (21)	104 ± 11 (4)	64 ± 15 (4)	74 ± 16 (4)	51 ± 17 (4)	85 ± 11 (4)	77 ± 12 (4)
B (developing)	62 ± 11 (18)	303 ± 37 (18)	164 ± 7 (18)	–	–	–	160 ± 11 (6)	140 ± 9 (6)	145 ± 15 (6)
C (developing)	–	–	89 ± 6 (57)	–	–	–	98 ± 6 (21)	99 ± 6 (31)	93 ± 4 (42)
D (mature)	127 ± 19 (12)	262 ± 16 (15)	112 ± 10 (12)	–	–	–	129 ± 19 (5)	135 ± 10 (5)	101 ± 7 (5)

Table 2 Tuber yield and number from GUS-control and *glgC-16* transgenic potato plants

Tubers were harvested after 10 weeks' growth in a greenhouse. Values are means ± S.E.M. for the numbers of plants shown in parentheses.

Plants	Tubers per plant	Total weight of tubers per plant (g)
GUS-control	3.4 ± 0.4 (17)	64.5 ± 8.0 (17)
glgC-16 line 139	3.5 ± 0.7 (6)	72.4 ± 13.2 (6)
glgC-16 line 82	3.9 ± 0.5 (8)	61.3 ± 13.7 (8)
glgC-16 line 123	4.7 ± 0.7 (9)	68.9 ± 10.8 (9)

Table 3 Metabolite content of developing tubers from GUS-control and *glgC-16*-transformed plants

Tubers were from 8-week-old plants and were freeze-clamped, ground to a powder in liquid N_2 and extracted with 1.41 M $HClO_4$. The extract was neutralized with K_2CO_3 and centrifuged to give a supernatant that was assayed for metabolites. Values are means ± S.E.M. of estimates from 20 tubers, each of 10–20 g fresh wt. The GUS-control tubers were from the harvests of five plants each of lines 5, 19 and 24, and the transgenic tubers from the harvests of five plants each of lines 139, 82 and 123.

	Content (nmol/g fresh wt.)	
Metabolite	GUS-control tubers	*glgC-16*-transformed tubers
Glucose 6-phosphate	167 ± 14	187 ± 13
Glucose 1-phosphate	6.6 ± 1.5	8.1 ± 1.6
ADPglucose	3.1 ± 0.4	3.1 ± 0.5
UDPglucose	224 ± 14	225 ± 20
PP_i	112 ± 2	121 ± 6
ATP	208 ± 10	238 ± 13
ADP	27 ± 3	44 ± 10
3-Phosphoglycerate	54 ± 6	61 ± 6
Sucrose	12305 ± 1347	9721 ± 708
Glucose	25271 ± 4658	17263 ± 2783
Fructose	2622 ± 357	2305 ± 218

tuber. We found wide variation in starch content, but no clear evidence of any difference between the two types of tuber. For a more precise comparison we concentrated on tubers of 10–50 g fresh weight. We sampled three separate, greenhouse-grown, populations of plants for developing tubers, and one field-grown population for mature tubers (Table 1). No significant ($P > 0.05$) differences in the amount of starch per g fresh weight could be demonstrated between tubers of the GUS-control plants and those of the *glgC-16*-transformed plants. We checked that the harvested tubers showed the difference in ADPglucose pyrophosphorylase activity expected from our characterization of the tubers (Table 1). We also compared the yields of GUS-control and *glgC-16* transgenic plants (Table 2). No differences ($P > 0.05$) were detected either in the number of tubers per plant or in the total yield of tubers. Staining of complete cross-sections of tubers revealed no differences in the distribution of starch between GUS-control and *glgC-16* tubers.

Metabolite content

Our failure to detect any differences in the starch content of tubers from GUS-control and *glgC-16* transgenic plants led us to compare their contents of metabolites related to starch metabolism. For each metabolite we checked for losses during extraction and analysis by carrying out recovery experiments (six with GUS-control and six with *glgC-16* tubers) comparable with those described for our enzyme assays [1]. No differences in recovery were found between the two groups of plants. Our estimates of recovery did not differ by more than 10 % from 100 %, except for glucose 1-phosphate, glucose 6-phosphate and PP_i, where the values were 83 %, 86 % and 82 % respectively. We detected no significant ($P > 0.05$) differences between the contents of any of the metabolites assayed between GUS-control and *glgC-16*-transformed tubers (Table 3).

Direct assessment of flux to and from starch

If there is starch breakdown during net synthesis of starch, then measurements of starch content are not an accurate measurement of the flux into starch. We made direct estimates of this flux by supplying [U-^{14}C]sucrose to developing tubers still attached to the plant and measuring the incorporation of ^{14}C into starch. We placed the [^{14}C]sucrose into a 25 μl well in the side of the tuber. After an appropriate interval we removed a core of tissue, 7 mm in diameter and concentric with the original feeding well, and measured the total ^{14}C present and the percentage of this that was present in starch. This method has been used successfully in other studies of tuber metabolism [15]. The ^{14}C in starch was measured as the label released when the 80 %-ethanol-insoluble fraction of the tissue sample was incubated for 24 h with amyloglucosidase and α-amylase. We checked that increasing the

Table 4 Comparison of labelling of starch by [U-^{14}C]sucrose and activity of ADPglucose pyrophosphorylase in tubers of GUS-control and *glgC-16*-transformed plants

Plants of 10 weeks old were taken from their pots and the tubers were exposed without breaking their stolons. From each selected tuber a core of tissue was removed by inserting a hypodermic needle into the side of the tuber mid-way between the point of attachment to the stolon and the basal end of the tuber. The resulting well was filled with 20 μl of 0.36 mM [U-^{14}C]sucrose (20.7 Bq/μmol) and sealed. After 3 h a core of 7 mm diameter, concentric to the feeding well, was removed, killed and extracted in 80% (v/v) ethanol. The insoluble fraction was incubated with amyloglucosidase and α-amylase, and the ^{14}C released was determined and used as the measure of ^{14}C recovered in starch. A second core of tissue parallel to the first one was removed, extracted and assayed for ADPglucose pyrophosphorylase activity. Values are means $\pm$ S.E.M. of data from the numbers of tubers shown in parentheses. Each tuber was attached to a different plant.

Tuber line	^{14}C incorporated into starch (% of total ^{14}C recovered)	ADPglucose pyrophosphorylase activity (nmol/min per g fresh wt.)
GUS-control	1.59 $\pm$ 0.3 (6)	103 $\pm$ 15 (6)
glgC-16 line 12	2.91 $\pm$ 1.2 (4)	220 $\pm$ 26 (4)
glgC-16 line 82	10.20 $\pm$ 4.2 (4)	433 $\pm$ 62 (4)
glgC-16 line 123	11.40 $\pm$ 3.0 (4)	528 $\pm$ 61 (4)

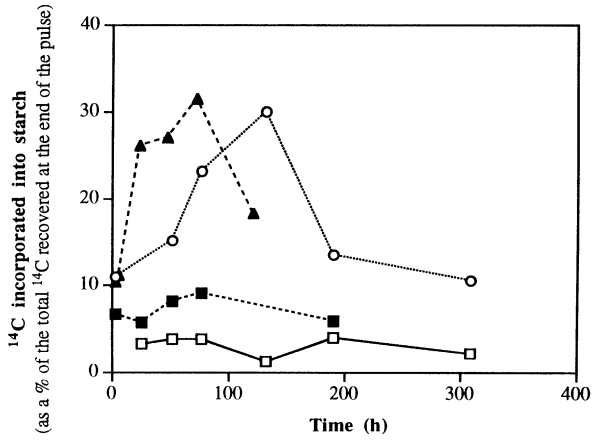

Figure 2 Labelling of starch in tubers of GUS-control and *glgC-16*-transformed plants after a 3 h pulse with [U-^{14}C]sucrose followed by a chase in unlabelled sucrose

[U-^{14}C]Sucrose was supplied to tubers, still attached to 10-week-old plants, as described in the legend to Table 4 (pulse); then the [^{14}C]sucrose was replaced by unlabelled sucrose (chase) and the distribution was analysed after timed intervals. Each value is from a single tuber attached to a different plant. □, GUS-control; ■, *glgC-16* line 12; ○, *glgC-16* line 82; ▲, *glgC-16* line 123.

time of this incubation to 48 h and 72 h did not lead to any detectable increase in the amount of ^{14}C released. We analysed the label released in the normal 24 h incubation by paper chromatography, and showed that 97 $\pm$ 2% (mean $\pm$ S.E.M. for estimates from six different tubers) was present as [^{14}C]glucose. Our labelling method gave appreciable and readily measurable incorporation of ^{14}C into starch. Over the periods that we used, incorporation of ^{14}C into starch was linearly related to the time for which the [^{14}C]sucrose was supplied. For example, when three tubers, each attached to a different plant, were labelled for 1, 2 and 4 h respectively, the percentages of total label per sample that were recovered as starch were 0.9%, 1.7% and 3.7% respectively.

We measured the labelling of starch from [U-^{14}C]sucrose in developing tubers on GUS-control and on a range of *glgC-16*-transformed plants (Table 4). The latter were chosen to include a range of ADPglucose pyrophosphorylase activities, which were verified for the actual tubers used in the feeding experiments by removing a second 7 mm core of tissue, parallel to the first, at the end of the feeding experiment and assaying it for pyrophosphorylase activity. Our results show that tubers with increased ADPglucose pyrophosphorylase activity incorporated proportionally more label into starch.

The clear effect of increased ADPglucose pyrophosphorylase activity on the flux from [^{14}C]sucrose to starch contrasted with our failure to find a comparable effect on starch content. Thus we estimated the rate of starch accumulation in developing tubers. We plotted the data in Figure 1 as mg of starch per tuber against the fresh weight of tuber. A linear relationship was found: r was 0.909 for the GUS-control tubers and 0.741, 0.843 and 0.912 for the *glgC-16* lines 139, 82 and 123 respectively. Previous detailed studies of potato tuber development [16], with three different varieties, established that in each variety there was a linear rate of growth that did not differ significantly between varieties. The average rate from all three varieties was 0.153 g fresh weight per day. We have used this value to convert tuber weight, measured in the present work (Figure 1), to tuber age. Then we plotted tuber age against our measurements of starch content. Linear regression analysis of these plots gave the following estimates of the rate of starch accumulation (nmol of anhydrous hexose/min per g fresh weight): 53 $\pm$ 2, 59 $\pm$ 5, 47 $\pm$ 6 and 56 $\pm$ 5 for GUS-control and *glgC-16*-transformed lines 82, 139 and 123 respectively (means $\pm$ S.E.M.). These rates do not differ significantly from each other ($P > 0.05$). On the (admittedly contestable) assumption that there is little turnover of starch in the GUS-control tubers, it is possible to use our measurements of starch accumulation and flux into starch to estimate the rate of starch synthesis in the control tubers. A value close to 0.4 μmol/min per g fresh weight is obtained. Given that in potato tubers about half of the total activity of starch synthase is granule-bound [11], then this rate would be sustainable by the activity of starch synthase reported in the previous paper [1].

The implication from our measurements of starch content and assessments of the rate of starch accumulation is that the increased flux into starch from [^{14}C]sucrose was accompanied by an increased flux of label out of starch. We investigated whether this was the case by extending our feeding experiments with [U-^{14}C]sucrose so that we determined the labelling of starch not only after a brief pulse with [^{14}C]sucrose but also during a prolonged chase in which the [^{14}C]sucrose was replaced with unlabelled sucrose.

For the pulse–chase experiments we chose three lines of *glgC-16*-transformed tubers that differed in their maximum catalytic activities of ADPglucose pyrophosphorylase. The experiments were carried out with developing tubers still attached to the plant (Figure 2). The total ^{14}C recovered in the core of tissue removed from the tuber declined during the chase. For GUS-control tubers this decline was from 320 512 d.p.m. at the end of the pulse to 159 854 d.p.m. at the end of a chase of 310 h. Comparable values for a tuber of *glgC-16* line 82 were 533 170 and 188 896 d.p.m. respectively.

Accordingly, we have expressed the ^{14}C recovered in starch at different times during the chase as a percentage of the total label present in the core at the end of the pulse. The detailed distribution of ^{14}C at the end of the pulse was determined. For cores from GUS-control tubers, the percentages of total ^{14}C recovered that were found in the different fractions were: insoluble material, 12.8%; acidic, basic and neutral components

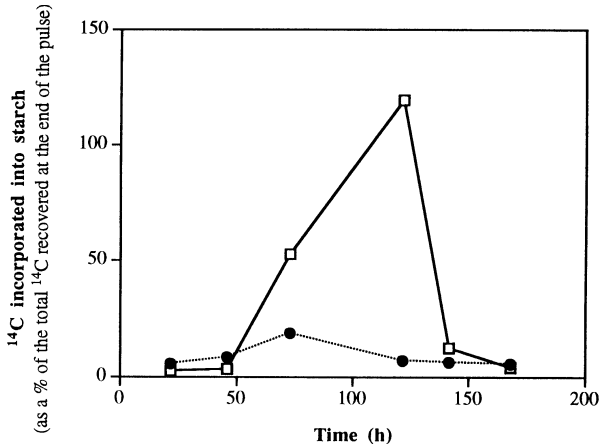

Figure 3 Labelling of starch in harvested tubers of GUS-control and *glgC-16*-transformed plants after a 3 h pulse with [U-^{14}C]sucrose followed by a chase in unlabelled sucrose

Tubers were harvested at the end of the growing season and stored at 4 °C for 8 weeks. The transgenic tubers were from *glgC-16* line 123; otherwise the experiment was carried out as described in the legend to Figure 2. ●, GUS-control; □, *glgC-16*.

of the soluble fraction, 19.3, 21.6 and 46.3 % respectively. The labelling of starch at the end of the 3 h pulse confirmed the conclusions drawn from the initial feeding experiments (Table 4). As the ADP glucose pyrophosphorylase activity increased, so did the labelling of the starch. During the chase there was little or no loss of ^{14}C from starch in the GUS-control tubers. In the *glgC-16*-transformed tubers the labelling of the starch actually increased during the early part of the chase. The extent of this increase was roughly proportional to the increase in ADPglucose pyrophosphorylase activity. In the transgenic lines with high pyrophosphorylase activities there was a decline in the labelling of the starch during the chase. In no instance could we detect any net breakdown of starch during the chase.

We also carried out pulse–chase experiments with mature tubers that had been stored at 4 °C for 8 weeks (Figure 3). Again there was greater labelling of starch at the end of the 3 h pulse in the *glgC-16* transgenic line than in the GUS-control tubers, and there was clear evidence of increased turnover of starch in the *glgC-16* transgenic line.

Enzymes of starch breakdown

We investigated whether the increased turnover of starch in the *glgC-16* plants with high ADPglucose pyrophosphorylase activity was associated with changes in the maximum catalytic activities of enzymes that might contribute to starch breakdown. We assayed α-amylase, β-amylase, α-glucan phosphorylase and debranching enzyme. As there is an appreciable likelihood that the classical α-amylase and β-amylase may not constitute all of the amylolytic activity in a plant tissue [17], we also measured the ability of extracts to release reducing equivalents from amylopectin. We refer to this activity as total amylolytic activity.

We optimized the assays for each of the enzymes of starch degradation, as described for the enzymes of starch synthesis [1]. For α-amylase, β-amylase and debranching enzyme we carried out recovery experiments with pure enzyme as described previously [1]. Our lowest recovery was 89 %. Table 5 shows the maximum catalytic activities of the enzymes of starch breakdown in developing tubers with different activities of ADPglucose pyrophosphorylase. We detected no changes in the activities of α-glucan phosphorylase, α-amylase and debranching enzyme. However, the *glgC-16* transgenic lines with the highest pyrophosphorylase activity (lines 82 and 123) showed increases in both β-amylase and total amylolytic activity.

Table 5 Estimates of the maximum catalytic activities of enzymes of starch breakdown in developing tubers of GUS-control and *glgC-16*-transformed plants

Developing tubers of 10–60 g fresh wt. were harvested from 10-week-old plants, freeze-clamped and ground in liquid N_2 to give a powder that was resuspended in extraction medium. After centrifugation and desalting (Pharmacia PD-10), the extract was assayed. Values are means ± S.E.M. for the numbers of tubers shown in parentheses. *Significantly different from GUS-controls ($P < 0.05$).

	Enzyme activity (nmol/min per g fresh wt.)			
		glgC-16		
Enzyme	GUS-control	Line 12	Line 82	Line 123
α-Glucan phosphorylase	224 ± 23 (18)	249 ± 35 (6)	211 ± 48 (6)	242 ± 43 (6)
α-Amylase	76 ± 11 (18)	81 ± 2 (6)	81 ± 8 (6)	72 ± 8 (6)
β-Amylase	10.0 ± 4.5 (6)	9.5 ± 3.5 (3)	17.6 ± 6.6 (3)*	28.1 ± 7.1 (3)*
Total amylolytic activity	135 ± 19 (18)	149 ± 20 (6)	172 ± 16 (6)*	199 ± 16 (6)*
Debranching enzyme	82 ± 10 (6)	82 ± 25 (3)	66 ± 26 (3)	72 ± 20 (3)

DISCUSSION

We suggest that our data are adequately replicated and authenticated and that the problem of variation within tubers has been met by making the whole tuber the sample. We emphasize the very close agreement between the behaviour of several independently transformed lines of *glgC-16* tubers.

Although the activity of ADPglucose pyrophosphorylase in the *glgC-16*-transformed tubers was increased 4-fold, we were unable to detect any significant effect on starch content. We conclude that increasing the maximum catalytic activity of this enzyme does not necessarily give tubers with an increased content of starch. Stark et al. [2] presented evidence that tubers of potato variety Russett Burbank, transformed essentially in the same way as those used in our work, showed a 30 % increase in starch. There is no obvious explanation for this difference in response. At present it is impossible to compare our work with that of Stark et al. [2]. The latter do not report any measurements of ADPglucose pyrophosphorylase activity, nor do they provide any evidence that their transformation of Russett Burbank potatoes was not accompanied by significant pleiotropic changes in other enzymes involved in starch synthesis.

Although increasing the activity of ADPglucose pyrophosphorylase did not increase the starch content of the tubers in our work, direct assessment of the movement of ^{14}C from [^{14}C]sucrose into starch very strongly suggests that the rate of starch synthesis was increased. We argue that, despite evidence of starch turnover, our estimates of flux are accurate because the pulse of ^{14}C was of sufficiently short duration for there to have been no appreciable loss of label from starch during the feeding period. We used our estimates of flux to calculate the response coefficient for the activity of ADPglucose pyrophosphorylase with respect to starch synthesis. As the number of *glgC-16* transgenic lines for which we have full information is limited to three, and as the changes in ADPglucose pyrophosphorylase activity are relatively large, we have calculated the response coefficient using the deviation index described by Small and

Kacser [18]. By comparing each of the *glgC-16* transgenic lines with the GUS control line, we obtained the following values ($\pm$S.D.) for the response coefficient: 0.9 ± 0.9, 1.1 ± 0.2 and 1.1 ± 0.2. The calculation of the deviation index and hence the response coefficient assumes that the kinetics of the reaction catalysed by ADPglucose pyrophosphorylase are linear. The fact that three independent estimates of the response coefficient are not significantly different demonstrates that the assumption is valid in this case. Examination of the S.D.s of the response coefficients indicates that the value of 1.1 is the most reliable. Thus a small change in the activity of ADPglucose pyrophosphorylase can change the rate of starch synthesis by the same relative magnitude. We emphasize, however, that the response coefficient is valid only for a change in activity generated by the expression of the *glgC-16* gene. We suggest that small changes in the activity of ADPglucose pyrophosphorylase can exert considerable control over the rate of starch synthesis in potato tubers. It is not clear, however, whether such changes do occur *in vivo*. It is difficult to compare our results with published work, as the latter does not take into account the possibility of starch turnover and estimates of synthesis depend solely on measurements of starch content. Our data on starch content and ADPglucose pyrophosphorylase activity are comparable with those of Müller-Röber et al. [19], who found that enzyme activity had to be decreased by more than half before the starch content was decreased. Our suggestion that the activity of ADPglucose pyrophosphorylase can exert considerable control over the rate of starch synthesis bears comparison with an estimated control coefficient of 0.64 found in leaves of *Arabidopsis* [20], but differs from the work of Denyer et al. [21], who estimated a value of 0.1 for pea embryo, the only other storage tissue examined.

Perhaps our most important conclusion is that a major increase in the rate of starch synthesis does not necessarily lead to an increase in starch content. We attribute this to the occurrence of appreciable turnover of starch in the tubers with increased ADPglucose pyrophosphorylase activity. The extent of this turnover was roughly proportional to the increase in pyrophosphorylase activity. Thus as flux into starch increased, so did turnover, so the amount accumulated did not alter. This conclusion has considerable implications for programmes aimed at increasing the yield of plant products.

The mechanism whereby increased flux into starch is accompanied by increased flux out of starch is not apparent. Our evidence of increased amylolytic activity suggests that coarse control, in the shape of increased synthesis of degradative enzymes, may play a part. This view is supported by the fact that no enzyme thought to be directly involved in starch breakdown in higher plants has been shown to possess extensive regulatory properties. The possibility occurs to us that changes in gene expression can increase flux into starch through changing the maximum catalytic activities of enzymes of starch synthesis, and that the resulting increase in flux can then alter the expression of genes encoding enzymes involved in starch breakdown, in a way that negates the original increase in flux.

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