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RESEARCH PAPER

The influence of cytosolic phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPC) on potato tuber metabolism

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Abstract

The aim of this work was to investigate the importance of cytosolic phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPC) in potato carbohydrate metabolism. For this purpose, the cytosolic isoform of phosphorylating GAPC was cloned and used for an antisense approach to generate transgenic potato plants that exhibited constitutively decreased GAPDH activity. Potato lines with decreased activities of phosphorylating GAPC exhibited no major changes in either whole-plant or tuber morphology. However, the levels of 3-phosphoglycerate were decreased in leaves of the transformants. A broad metabolic phenotyping of tubers from the transformants revealed an increase in sucrose and UDPglucose content, a decrease in the glycolytic intermediates 3-phosphoglycerate and phosphoenolpyruvate but little change in the levels of other metabolites. Moreover, the transformants displayed no differences in cold sweetening with respect to the wild type. Taken together these data suggest that phosphorylating GAPC plays only a minor role in the regulation of potato metabolism. The results presented here are discussed in relation to current models regarding primary metabolism in the potato tuber parenchyma.

Key words: Antisense, carbohydrate metabolism, GAPDH, potato tuber.

Introduction

Higher plants possess three distinct isoforms of glyceraldehyde 3-phosphate dehydrogenases (GAPDH); (i) a phosphorylating NADP-specific GAPDH involved in photosynthetic CO_2 fixation, (ii) a phosphorylating NAD-specific GAPDH catalysing the conversion of glyceraldehyde 3-phosphate to 1,3-*bis*phosphoglycerate in the cytosol, and (iii) a cytosolic NADP-dependent non-phosphorylating GAPDH which catalyses the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate in the cytosol (Cerff and Chambers, 1979). Much is known concerning the gene structure, evolution, and enzymic properties of GAPDHs in algal systems (Koksharova *et al*., 1998; Perusse and Schoen, 2004; Valverde *et al*., 2005) which have also been subjected to extensive functional studies via the use of reverse genetics (Koksharova *et al*., 1998; Fillinger *et al*., 2000). However, although the structural and biochemical properties of all GAPDH isoforms have been extensively studied in higher plants (Cerff and Chambers, 1979; Iglesias and Losada, 1988; Mateos and Serrano, 1992) less research effort has focused on their *in vivo* function.

Historically, the majority of work in higher plants has concentrated on evaluation of the photosynthetically important chloroplast isoforms (Melandri *et al*., 1970; Cerff, 1979; Petersen *et al*., 2003; Sparla *et al*., 2005). However, in recent years detailed studies have been carried out on structure–function relationships (Habenicht *et al*., 1994; Michels *et al*., 1994) and kinetic properties (Bustos and Iglesias, 2002; Iglesias *et al*., 2002) of the cytosolic

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isoforms. Although the physicochemical and kinetic properties of the two cytosolic GAPDH enzymes have been investigated in detail in photosynthetic organs, little attention has been devoted to their function within glycolysis in plant heterotrophic tissues (Givan, 1999; Fernie *et al*., 2004). Moreover, cytosolic GAPDH has been demonstrated to be cytoskeletally associated in a wide variety of species including plants (Chuong *et al*., 2004). It has additionally been demonstrated to be a constituent enzyme of the functional association of glycolysis, both to the cytoskeleton and to the *Arabidopsis* mitochondria (Giege *et al*., 2003; Holtgrawe *et al*., 2005), and to be subjected to translational control and multiple post-translational modifications when subjected to stress (Chang *et al*., 2000; Laukens *et al*., 2001; Bustos and Iglesias, 2003). These observations, when taken together suggest that cytosolic GAPDH could well be a key regulator of glycolysis.

The generation of transgenic plants exhibiting a strong reduction in phosphorylating GAPC activity is described here. These plants were subjected to detailed biochemical analysis under normal growth conditions. Furthermore, cold sweetening was investigated in transgenic tubers to evaluate whether a decrease in glycolysis results in enhanced accumulation of soluble sugars. The results are discussed in the context of current understanding of heterotrophic metabolism and the cold sweetening process.

Materials and methods

Plants, bacterial strains and media

Potato plants (*Solanum tuberosum* cv. Solara) were obtained from Bioplant, Ebstorf, Germany. Plants in tissue culture were grown under a 16/8 h light/dark period on MS medium (Murashige and Skoog, 1962) containing 2% sucrose. Plants used for biochemical analysis were grown in individual pots (diameter 20 cm, depth 13 cm) in the greenhouse. *Escherichia coli* strain XL1-blue (Stratagene, La Jolla) was cultivated using standard techniques (Sambrook *et al*., 1989). *Agrobacterium tumefaciens* strain C58C1 containing pGV2260 (Deblaere *et al*., 1985) was cultivated in YEB medium (Verveliet *et al*., 1975).

Enzymes and reagents

Enzymes and biochemicals were purchased from Boehringer (Mannheim, FRG) or Sigma (Deisenhofen, FRG).

Plasmid construction and potato transformation

A 1200 bp Asp718/*Bam*HI fragment of the phosphorylating GAPC encoding cDNA (Acc. No. AF527779) was cloned in the antisense orientation under the transcriptional control of the CaMV 35S promoter. Direct transformation of *Agrobacterium tumefaciens* strain C58C1:pGV2260 was performed as described by Hoefgen and Willmitzer (1988). Potato transformation using *Agrobacterium*-mediated gene transfer was performed as described by Rocha-Sosa *et al*. (1989).

Extraction of mRNA and northern blot analysis

Total RNA was isolated from 1 g leaf or tuber tissue as described by Logemann *et al*. (1987). Standard conditions were used for the transfer of RNA to membranes and for the subsequent hybridization (Sambrook *et al*., 1989). Loading was standardized relative to total RNA levels.

Preparation and analysis of samples for enzyme activities

To measure enzyme activities, 100–200 mg potato tuber slices were homogenized in 0.5 ml 100 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES)-KOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM mercaptoethanol, 15% glycerine, and 0.1 mM Pefabloc phosphatase inhibitor. After centrifugation for 10 min, 13 000 rpm at 4 °C, the supernatant was frozen immediately for further analysis. Invertase, sucrose synthase, and phosphofructokinase activities were determined as described by Hajirezaei *et al*. (1994), glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase activities were determined as detailed in Biemelt *et al*. (1999), phosphoglucomutase activity as defined by Tauberger *et al*. (2000), amylase activities were determined using the megazyme kit (Megazyme, Ireland; as detailed in Hajirezaei *et al*., 2003), whilst starch phosphorylase was assayed using the modifications to the assay of Steup (1990), that were previously described in Hajirezaei *et al*. (2003).

Metabolite determination

Metabolites were extracted essentially as described in Jelitto *et al*. (1992). 50–300 mg of tissue material was immediately frozen in liquid nitrogen. After homogenizing, the frozen material was ground to a fine powder, 1.5 ml of 16% (w/v) trichloroacetic acid (TCA) in diethylether (4 °C) was added and the tissue further homogenized. After incubating the extract on dry ice for 15 min, 0.8 ml of 16% TCA (w/v) in water containing 5 mM EGTA (4 °C) was added to the homogenate, which was then left for an additional 3 h at 4 °C. Following centrifugation for 5 min at 15 000 rpm, the water phase was washed 3–4-fold with 600 µl water-saturated ether each time and thereafter neutralized with 5 M KOH/1 M triethanolamine. The levels of metabolites and ATP/ADP were determined photometrically as in Stitt *et al*. (1989) using a dual wavelength spectral photometer (Sigma-ZWS II, Biochem.). The recovery of small, representative amounts of each metabolite through the extraction has already been documented (Hajirezaei *et al*., 1994).

Determination of soluble sugars and starch

Soluble sugars and starch were quantified in tuber samples extracted with 80% ethanol, 20 mM HEPES-KOH, pH 7.5 as described in Hajirezaei *et al*. (2000).

Gas chromatography–mass spectrometry (GC–MS) analysis

The levels of other metabolites were determined in derivatized methanol extracts by GC-MS using the protocol defined in Roessner *et al*. (2001*a*) with the exception that the machine parameters and time reference standards were set as described in Roessner-Tunali *et al*. (2003). The values of each metabolite were normalized to those determined in the wild type as detailed in Roessner *et al*. (2001*b*). Recoveries of these metabolites through extraction has been documented (Roessner *et al*., 2000).

Measurement of respiration rate in whole potato tubers

Gas exchange measurements were carried out using infrared gas analysis in an open system (Compact minicuvette system CMS-400, Walz GmbH, Effeltrich, Germany). Whole tubers were enclosed in a standard chamber MK-022/A and the release of CO_2 was monitored continuously. Chamber temperature and dew point temperature of the air entering the chamber were adjusted to 20 °C and 13 °C, respectively. Measurements were done at a gas flow rate of 1500 cm^{-3} s^{-1} and an ambient CO_2 concentration of about 100 µmol mol^{-1}. CO_2 evolution rate referred to tuber fresh weight is given as nmol CO_2 g^{-1} FW s^{-1}.

Labelling experiments with tuber slices

Tuber discs (diameter 10 mm, thickness 1 mm) were cut directly from a core removed using a cork borer from the centre of growing tubers (65-d-old plants, *c*. 12 g FW) attached to the fully photosynthesizing mother plant. The discs were washed quickly with 10 mM 2-(*N*-morpholino) ethane sulphonic acid (MES) (pH 6.5; KOH) three times, and preincubated for 20 min in a buffer containing 20 mM sucrose using 50 ml glass vessels at room temperature. U-[^{14}C]glucose (final specific activity 1.11 MBq mmol^{-1}) and U-[^{14}C]sucrose (final specific activity 1.4 MBq mmol^{-1}) (NEN, DuPont, Boston, USA) were added and incubation continued for another 3 h. During the whole incubation and preincubation time, discs were aerated by a continuous shaking at 150 rpm. After 3 h, discs were immediately washed three times with buffer to remove external radioactivity, and then frozen in liquid nitrogen to analyse label distribution.

Fractionation of ^{14}C-labelled tissue extracts

Discs were extracted with 80% (v/v) ethanol at 80 °C (1 ml per two discs), re-extracted in two subsequent steps with 50% (v/v) ethanol (1 ml per two discs for each step), the combined supernatants dried in a speed vac (SC110, Savant, Germany), taken up in 1 ml H_2O ('soluble fraction'), and separated into neutral, anionic, and basic fractions by ion exchange chromatography; the neutral fraction (3.5 ml) was freeze-dried, and taken up in 0.1 ml water. The insoluble material left after ethanol extraction was washed twice with water, homogenized, taken up in 1 ml water, and counted for starch.

Results

Generation and selection of transgenic plants

In order to reduce expression of phosphorylating GAPC, a 1200 bp fragment of the potato coding sequence for this gene (Acc. no. AF 527779) was cloned between the CaMV 35S promoter and the OCS terminator of plasmid pBinAR (Hoefgen and Willmitzer, 1990). Potato leaves were transformed using *Agrobacterium*-mediated gene transfer (Rocha-Sosa *et al*., 1989) and approximately 75 independent kanamycin-resistant transgenic plants were regenerated. From these primary transformants three were selected (97, 56, and 7), that displayed a strong reduction in phosphorylating GAPC, activity (data not shown), and amplified in tissue culture. A second screen, a northern blot analysis, was performed on various tissues harvested from potato plants which were grown for 12 weeks in the greenhouse under ambient growth conditions (see Materials and methods). As depicted in Fig. 1, phosphorylating GAPC gene expression was strongly inhibited in sink leaves, stolons, and growing tubers (tuber size *c*. 10 g) as well as being markedly decreased in source leaves and stem tissue. The reduction of gene expression strongly correlated with the maximum catalytic activity of phosphorylating GAPC determined in the same samples. The phosphorylating GAPC activity was reduced strongly in sink leaves (up to 85%), in stolons (up to 93%), in potato tubers (up to 92%), and in the roots (up to 80%) with respect to the activity observed in the wild-type control (Fig. 2). By contrast, there was a lower decrease of GAPDH activity in source leaves (around 20% in line 56 and 65% in line 7). Similarly, in

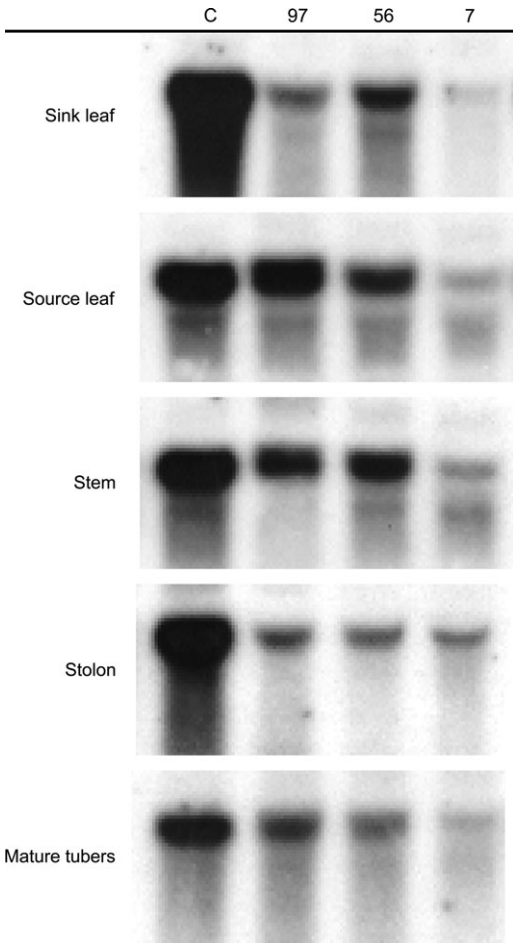


Fig. 1. Northern blot analysis of the expression of phosphorylating GAPC. RNA was extracted from various tissues of wild-type and transgenic plants. C, wild type control; 7, 56, and 97, independent transgenic potato lines.

stem tissue, the phosphorylating GAPC activity was diminished slightly in the lines 97 and 56 (by around 14%) and dramatically in the case of line 7 (by around 60%).

Impact of reduced phosphorylating GAPC activity on growth, phenotype and tuber yield

No significant changes in development or aerial plant growth were observed in the transgenics. In order to estimate the tuber yield 20 potato plants of each individual line were grown for 65 d in the greenhouse at ambient conditions. Plants were harvested and analysed for tuber fresh weight and tuber number. As shown in Table 1, neither total tuber fresh weight nor tuber number was affected by the reduction in phosphorylating GAPC activity.

Metabolite analysis in the leaves

To investigate whether the reduction in phosphorylating GAPC activity has an influence on leaf metabolism, leaf material from 10-week-old plants were harvested and the

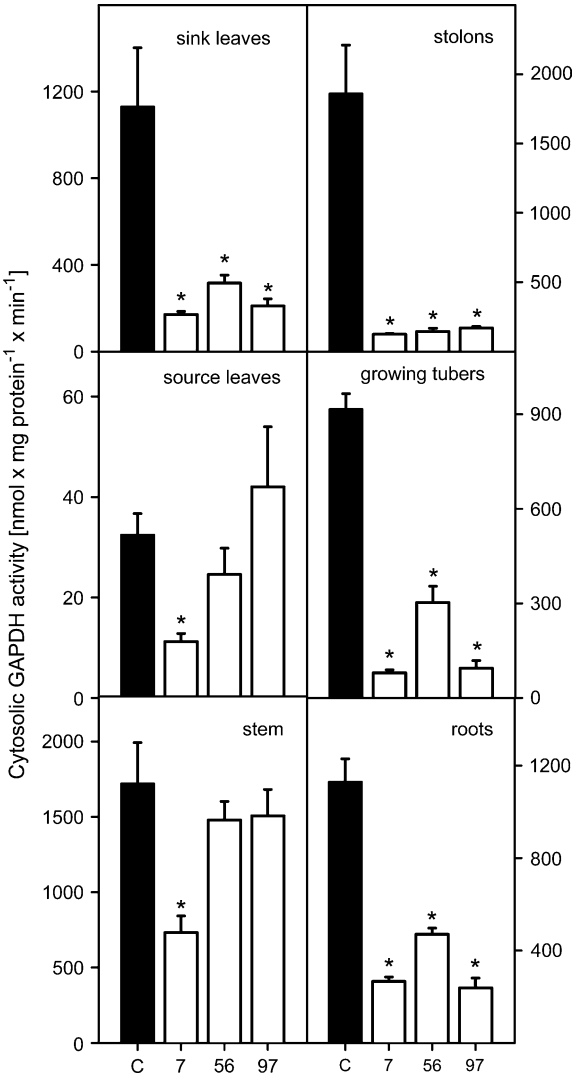


Fig. 2. Measurement of phosphorylating GAPC activity. GAPDH activity was measured in extracts from various tissues of wild-type and transgenic plants. Plant material was harvested and homogenized in buffer as described in the Materials and methods. The untransformed control is indicated as the filled bar and transgenic lines as blank bars. 97, 56, and 7 represent independent transgenic lines. Results are given in nmol mg^{-1} protein min^{-1} and represent the mean ±SE of four independent replicates. Asterisk denotes values that were determined to be highly significant by the *t* test (P <0.02).

content of some metabolites directly involved in sugar metabolism was measured. The UDPGlucose content was largely unaltered in the genotypes with the exception of line 97 wherein it was significantly decreased (52±4, 57±1.4, 49±6, 37±2 nmol g^{-1} FW in the control and transgenic lines 7, 56, and 97, respectively). The 3-phosphoglycerate content was significantly reduced in all three lines (440±8, 308±15, 228±22, 308±39 nmol g^{-1} FW in the control and transgenic lines 7, 56, and 97, respectively). By contrast, no significant differences in the contents of ATP and ADP between control and transgenic lines could be detected (data not shown).

Table 1. *Influence of antisense expression of phosphorylating GAPC on potato tuber development*

Tubers were harvested from plants which had been growing in the greenhouse for 65 d. Results are mean ±SE of 20 independent replicates. No significant differences could be observed in the measured parameters.

Parameter	Genotype			
	Control	GAP-7	GAP-56	GAP-97
Total fresh weight (g)	133±3.7	123±4.7	130±3.9	129±4.5
Number of tubers	5–13	5–15	5–10	5–11
Mean of tuber number	7.5±0.5	8.5±0.7	6.2±0.4	7.9±0.6

Influence of decreased phosphorylating GAPC activity on tuber soluble sugar and starch content

As a first experiment to investigate tuber metabolism, the content of soluble and insoluble polysaccharides in growing potato tubers (tuber size *c*. 15–20 g) harvested from control and transgenic plants was measured. As illustrated in Fig. 3 a decrease of glucose and fructose contents between 29–44% and 29–50% was observed, respectively, in transgenic tubers. Sucrose content was higher in transgenic tubers, being between 1.25-fold and 1.4-fold increased while the content of starch in the same tubers was not significantly different from that observed in the wild-type control (Fig. 3).

Changes in metabolite contents in tubers with reduced phosphorylating GAPC activity

To evaluate whether reducing the tuber phosphorylating GAPC activity resulted in changes in the levels of intermediates of the glycolytic or sucrose to starch pathways, a broad analysis of the levels of these compounds was performed. As can be seen in Fig. 4, the content of UDPGlc increased, up to 1.4-fold, in tubers from all three transgenic lines whereas the content of hexose phosphates remained largely unchanged (with the exception of a 1.3-fold increase observed in line 56). The level of 3-PGA, the direct product of the reaction catalysed by phosphorylating GAPC, decreased significantly in transgenic lines down to 40% of the wild-type values. The level of PEP was only decreased in line 56, while no significant differences were obtained for pyruvate (Fig. 4). The ratio of ATP and ADP remained unaffected in transgenic tubers compared with control tubers (Fig. 4). In addition, an analysis of a wide range of other metabolites of primary metabolism in control and transgenic tubers by means of an established gas chromatography mass-spectrometry (GC-MS) method was carried out (Roessner *et al*., 2001*a*, *b*). No major differences in the levels of primary metabolites of sugar, amino and organic acid metabolism were observed (Table 2). That said, there were tendential increases in the levels of asparagine (significant in line 97) and shikimate (significant in line 56) and decreases in the levels of leucine (significant in line 97), methionine, glycerate (significant

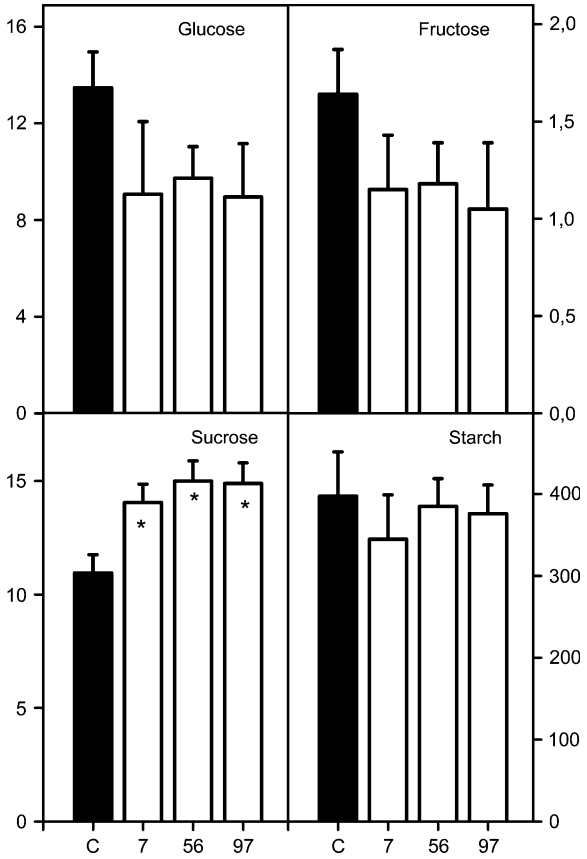


Fig. 3. Impact of reduced expression of phosphorylating GAPC on tuber carbohydrate content. Plant material was harvested from tubers of growing plants and homogenized in buffer as described in the Materials and methods The untransformed control is indicated as the filled bar and transgenic lines as the blank bars. 97, 56, and 7 represent independent transgenic lines. Results are given in μmol g^{-1} FW (in the case of starch as hexose equivalents) and represent the mean ±SE of four independent replicates. Asterisk denotes values that were determined to be highly significant by the *t* test (P <0.02).

in line 7), and spermidine (significant in line 56). In addition, there were significantly elevated levels of arabinose and tyramine (line 7), and quinate (line 97) and significantly decreased levels of saccharate (line 7), although the biological relevance of these isolated changes remains unclear from the present study.

Respiration rate in intact tubers with reduced phosphorylating GAPC activity

Given the reduction in the levels of some glycolytic intermediates in the transgenic tubers, it would seem reasonable to assume that these plants may be compromised in their respiration. In order to evaluate this hypothesis the respiration rate in intact mature tubers and in transgenic tubers stored for 6 weeks and 20 weeks at room temperature was measured. Since the tubers investigated had various sizes and, most probably therefore, different metabolic activity, the variation in respiration rates recorded was enormous, between 0.1 and 0.4 nmol CO_2 g^{-1} FW s^{-1} in

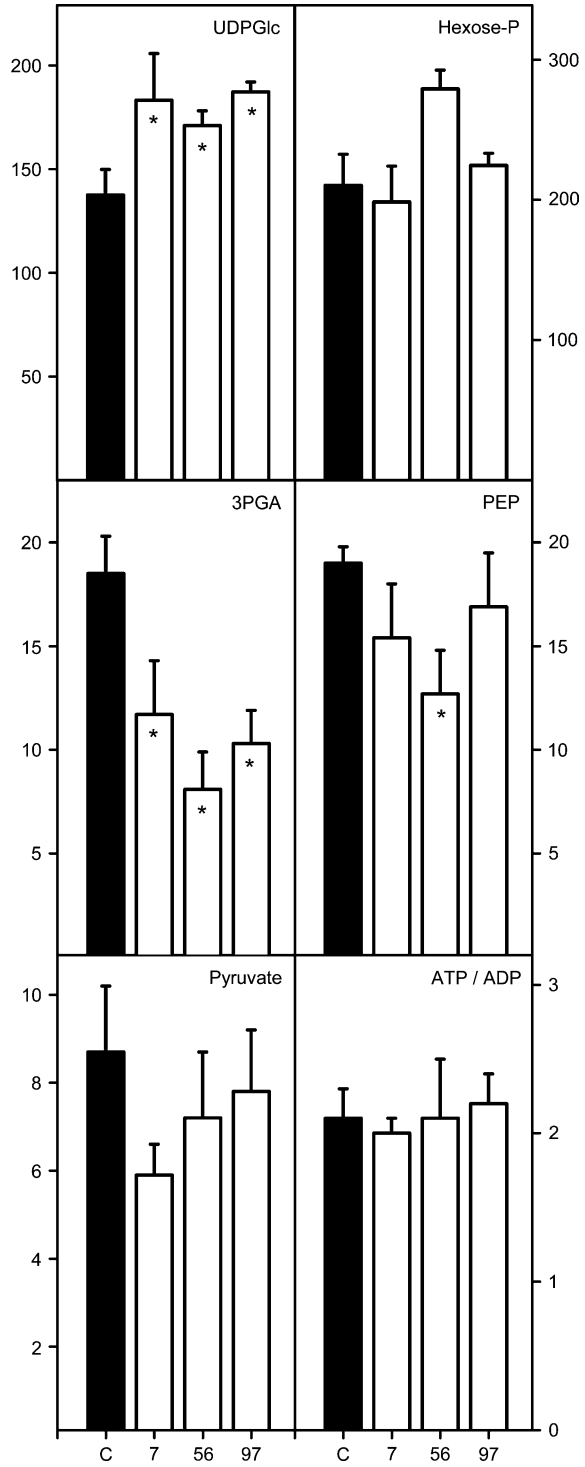


Fig. 4. Influence of reduced expression of phosphorylating GAPC on tuber metabolite levels. Plant material was harvested from tubers of growing plants and homogenized in buffer as described in the Materials and methods. The untransformed control is indicated as the filled bar and transgenic lines as blank bars. 97, 56 and 7 represent independent transgenic lines. Results are given in nmol g^{-1} FW and represent the mean ±SE of four independent replicates. Asterisk denotes values that were determined to be significantly different by the *t* test (P <0.05).

Table 2. *Metabolite levels in developing tubers of wild-type and transgenic potato plants*

Metabolites were determined in samples harvested from 10-week-old plants. Data are normalized to the mean response calculated for the wild type (individual wild-type values were normalized in the same way). Values presented are mean ±SE of six replicates. Values in bold denote significant differences that were determined by the *t* test ($P < 0.05$).

	Wild type	GAPDH-7	GAPDH-56	GAPDH-97
Amino acids				
Alanine	1.00±0.11	0.78±0.15	1.09±0.12	0.83±0.14
Arginine	1.00±0.17	0.75±0.21	1.03±0.19	0.97±0.23
Asparagine	1.00±0.27	0.94±0.23	1.71±0.22	**2.11±0.26**
Aspartate	1.00±0.04	1.04±0.05	1.11±0.08	1.21±0.05
β-alanine	1.00±0.13	0.99±0.07	1.51±0.16	1.23±0.16
Cysteine	1.00±0.20	1.44±0.11	1.23±0.19	1.06±0.20
γ-aminobutyrate	1.00±0.13	1.06±0.04	1.31±0.13	1.15±0.12
Glutamate	1.00±0.14	1.16±0.13	1.03±0.09	1.15±0.08
Glutamine	1.00±0.21	1.22±0.23	1.43±0.12	1.31±0.17
Glycine	1.00±0.10	0.83±0.10	1.02±0.11	0.87±0.09
Isoleucine	1.00±0.11	0.88±0.22	0.96±0.23	**0.60±0.22**
Leucine	1.00±0.26	0.36±0.22	0.34±0.19	0.35±0.26
Lysine	1.00±0.11	0.94±0.10	1.09±0.18	0.82±0.10
Methionine	1.00±0.14	**0.64±0.14**	0.89±0.15	0.78±0.17
Ornithine	1.00±0.14	0.91±0.16	1.38±0.19	1.04±0.20
Phenylalanine	1.00±0.14	1.00±0.15	1.46±0.15	1.08±0.21
Proline	1.00±0.22	**0.40±0.24**	0.82±0.23	1.20±0.24
Pyroglutamate	1.00±0.04	**0.81±0.06**	0.98±0.13	1.12±0.06
Serine	1.00±0.09	0.82±0.10	1.06±0.13	0.85±0.12
Threonine	1.00±0.11	0.93±0.12	1.02±0.14	0.89±0.14
Tryptophan	1.00±0.21	1.18±0.31	1.77±0.26	0.95±0.29
Tyrosine	1.00±0.15	1.11±0.15	1.35±0.20	0.68±0.25
Valine	1.00±0.03	**0.88±0.04**	0.92±0.05	0.98±0.04
Organic acids				
Aconitate	1.00±0.21	0.72±0.15	0.69±0.19	0.90±0.12
Ascorbate	1.00±0.09	0.78±0.08	0.80±0.14	0.98±0.13
Benzoate	1.00±0.14	0.80±0.13	0.82±0.08	0.89±0.10
Citrate	1.00±0.06	0.97±0.05	0.98±0.06	0.97±0.05
Dehydroascorbate	1.00±0.11	1.04±0.10	0.86±0.10	0.91±0.11
Fumarate	1.00±0.13	1.02±0.06	1.45±0.24	1.08±0.10
Gluconate	1.00±0.12	0.88±0.18	1.18±0.12	1.07±0.07
Glycerate	1.00±0.10	**0.64±0.04**	0.98±0.18	0.81±0.10
Isocitrate	1.00±0.13	0.79±0.08	1.07±0.18	1.12±0.07
Malate	1.00±0.17	1.45±0.21	0.99±0.21	0.78±0.04
Quinate	1.00±0.10	0.90±0.22	1.15±0.06	**1.47±0.10**
Saccharate	1.00±0.08	**0.80±0.04**	1.40±0.24	1.45±0.19
Shikimate	1.00±0.12	1.25±0.24	**1.47±0.09**	1.39±0.12
Succinate	1.00±0.22	0.85±0.24	1.02±0.17	0.73±0.16
Threonate	1.00±0.06	0.91±0.11	0.92±0.11	0.87±0.08
Sugars and sugar alcohols				
Arabinose	1.00±0.33	**2.14±0.18**	0.96±0.27	1.02±0.21
Fructose	1.00±0.35	0.89±0.21	1.79±0.36	0.53±0.18
Galactose	1.00±0.21	1.19±0.22	0.83±0.08	0.67±0.20
Glucose	1.00±0.16	0.70±0.12	**0.33±0.37**	0.61±0.23
Inositol	1.00±0.12	0.91±0.16	0.86±0.14	0.89±0.17
Maltose	1.00±0.13	0.91±0.14	1.21±0.28	0.79±0.14
Mannitol	1.00±0.06	1.28±0.09	1.11±0.14	1.16±0.07
Mannose	1.00±0.16	1.73±0.18	1.07±0.22	0.88±0.19
Raffinose	1.00±0.12	0.91±0.16	0.74±0.25	0.67±0.19
Ribose	1.00±0.08	1.07±0.08	1.12±0.15	1.04±0.11
Sorbitol	1.00±0.06	0.99±0.08	1.00±0.12	0.95±0.10
Trehalose	1.00±0.23	0.73±0.14	0.76±0.13	0.78±0.09
Xylose	1.00±0.14	1.31±0.19	0.88±0.16	0.64±0.22
Other metabolites				
Fructose-6P	1.00±0.21	1.23±0.11	0.86±0.17	0.74±0.06
Glucose-6P	1.00±0.13	1.22±0.06	1.15±0.16	0.82±0.05
Inositol-1P	1.00±0.09	1.02±0.08	0.94±0.11	1.01±0.07
Phosphoric acid	1.00±0.05	0.78±0.20	0.72±0.22	1.09±0.05
Putrescine	1.00±0.07	1.17±0.12	1.03±0.06	1.12±0.10
Spermidine	1.00±0.06	0.95±0.03	**0.74±0.07**	0.92±0.09
Tyramine	1.00±0.11	**2.16±0.11**	0.72±0.14	0.89±0.21

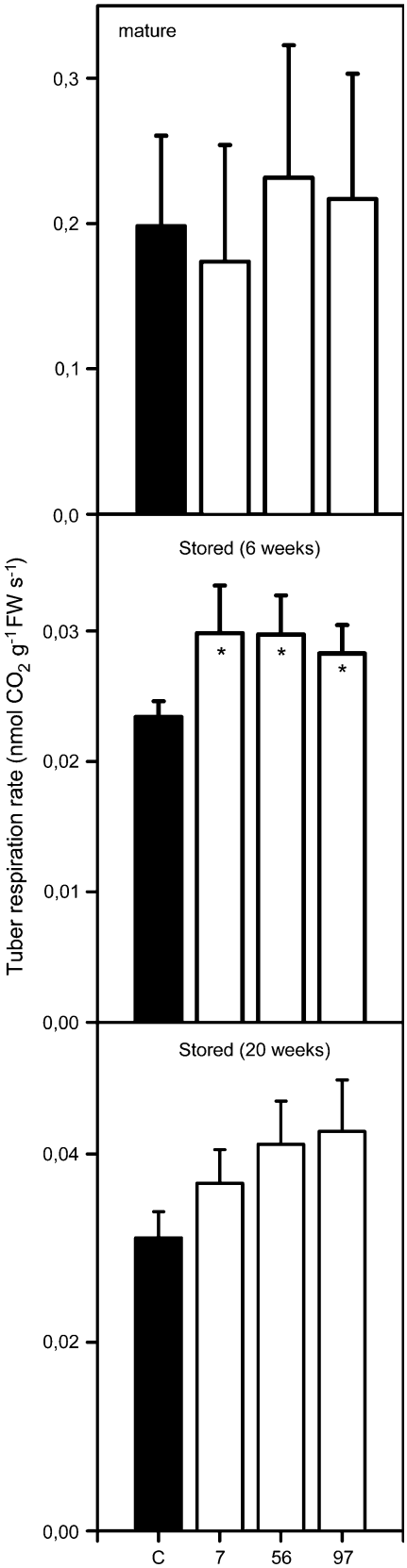


Fig. 5. Influence of reduced expression of phosphorylating GAPC on tuber respiration rate. Whole growing tubers and whole tubers stored for 6 weeks and 20 weeks at room temperature from control (filled bars) and transgenic lines (blank bars) were used for the determination of respiration rate. 97, 56, and 7 represent independent transgenic lines. Data represent mean values and standard errors (n=5) of carbon dioxide evolution. Results are given as nmol g^{-1} FW s^{-1}. Performance of t tests revealed only significant differences between the transformants and the wild type in tubers stored for 6 weeks.

mature tubers and 0.02 and 0.06 nmol CO_2 g^{-1} FW s^{-1} in stored tubers (Fig. 5). Nevertheless, the rate of respiration was unchanged in mature control and transgenic tubers, whilst a slight increase (up to 1.3-fold) in respiration was observed in stored transgenic tubers. However, this was statistically only significant in tubers stored for 6 weeks as confirmed by t test (<0.05; Fig. 5).

Redistribution of radiolabel following incubation of discs from growing tubers of wild-type and transgenic lines

Since the measurement of absolute starch content does not accurately reflect starch turnover, metabolism within the transgenic tubers following incubation in U-[^{14}C]glucose or U-[^{14}C]sucrose was evaluated. Growing tubers comparable to those used for metabolite and enzyme activity measurements were used. There was little consistency in the rate of radiolabel uptake between the different substrates when evaluated as a function of phosphorylating GAPC activity. In the experiment with ^{14}C-glucose as substrate the uptake rate was unrelated to phosphorylating GAPC activity with uptake rates of 1.41±0.1, 2.17±0.2, 1.18±0.1, and 1.34±0.1 μmol g^{-1} FW h^{-1} in wild type and lines 7, 56, and 97, respectively. The extent to which the activity of phosphorylating GAPC influences carbohydrate metabolism was assessed by following the redistribution of ^{14}C into starch, soluble sugar, and anionic and cationic fractions. There was a clear reduction (down to 85% and 65% of wild-type levels), in the percentage of the total label incorporated recovered in soluble sugars in all three transgenic lines. (Fig. 6). This decrease was accompanied by a decrease of radiolabel recovered in anionic (down to 55% of wild-type levels) and cationic (down to 62% of wild-type levels) fractions. By contrast, marked increases of ^{14}C incorporation into insoluble materials such as starch, cell walls, and proteins were observed (values in the transformants being between 1.7-fold and 2.3-fold in excess of the wild type). Similar results were obtained using ^{14}C-sucrose as substrate concerning neutral and insoluble compounds (data not shown). Given that the steady-state levels of these metabolites change contrapuntally in the transformants, the fact that the results were not substrate-dependent strongly implies that these data are not strongly affected by dilution effects and therefore that they give a fairly accurate representation of metabolism within the tuber.

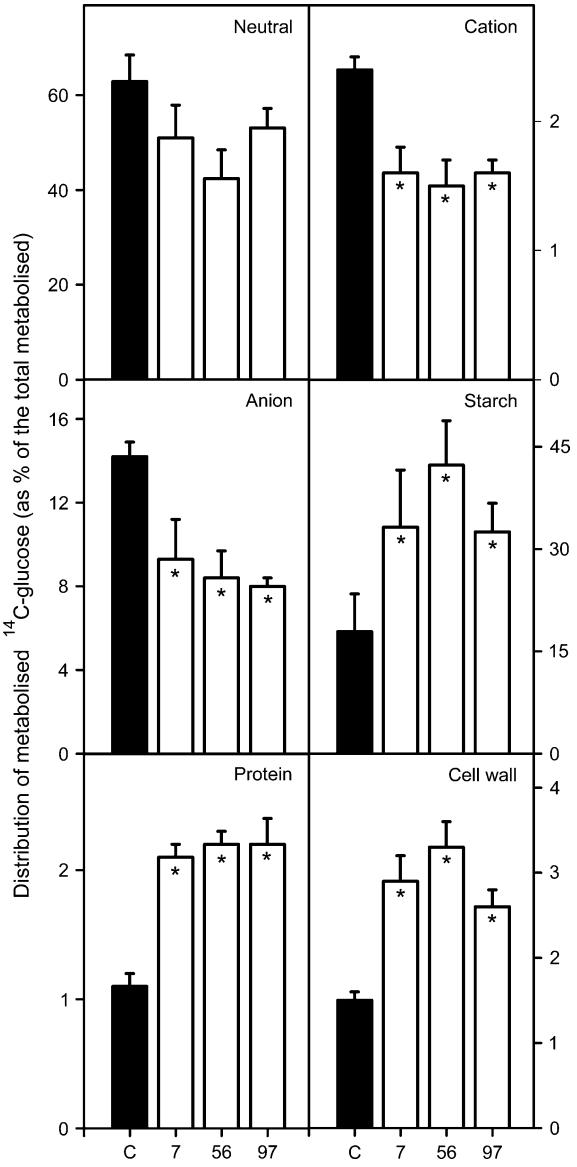


Fig. 6. Metabolism of ^{14}C-glucose by wild-type and transgenic potato tuber slices. Redistribution of radiolabel was calculated as a proportion of the total metabolism for control (filled bars) and transgenic lines (blank bars). The untransformed control is indicated as the filled bar and transgenic lines as blank bars. 97, 56, and 7 represent independent transgenic lines. Data represent mean values and standard errors (n=4). Asterisk denotes values that were determined to be significantly different by the t test (P <0.05).

Analysis of enzymes of sugar metabolism

It was next investigated whether the reduced GAPDH activity in the tubers was accompanied by changes in the activities of other enzymes involved in sucrose breakdown, glycolysis, and starch degradation. As illustrated in Fig. 7 the maximum catalytic activities of sucrose synthase, phosphofructokinase, pyruvate kinase, phosphoglucomutase, starch synthase, and alpha-amylase were unaffected in the transgenic tubers compared with control tubers. However, the maximum activity of β-amylase was significantly higher in all three transgenic lines being between 1.2-fold and 1.3-fold higher than that of control tubers.

Effect of reduced phosphorylating GAPC activity on sugar metabolism at low temperature

Having established that phosphorylating GAPC plays only a minor role during growing tuber development, attention was next turned to addressing the question whether phosphorylating GAPC plays a crucial role during tuber storage at low temperature. For this reason mature tubers of control and transgenic plants were placed at 4 °C. Slices were harvested after 42 d and 85 d and analysed for carbohydrate content. Prior to the sugar measurements the activity of phosphorylating GAPC and the content of important diagnostic metabolites such as 3PGA were measured to confirm the reduction in enzyme activity and glycolytic intermediates. As shown in Fig. 8, the activity of phosphorylating GAPC was decreased up to 95% and 97% of the activity found in the control in mature tubers and those stored for 42 d at 4 °C, respectively. In transgenic tubers stored for 85 d, a reduction of phosphorylating GAPC activity of up to 95% was maintained (data not shown). The decreased GAPDH activity was accompanied by a reduction of 3PGA content between 38% and 66% in both mature tubers and stored tubers (Fig. 8). The content of hexose phosphates did not change significantly in mature tubers, but there was a slight significant increase of up to 1.3-fold in the transgenic lines 7 and 56 following cold treatment (Fig. 8). The UDPGlc level was significantly higher in mature tubers up to 1.2-fold, whereas there was only an increase of 1.2-fold in transgenic line 56 (Fig. 8).

As expected, the content of soluble sugars, glucose and fructose increased during storage at 4 °C in both control and transgenic tubers. However, only a significant difference of glucose and fructose was detected (which were up to 1.8-fold increased), in line 97 after 42 d and in lines 56 and 97 (which were increased to a similar extent), after 85 d (Fig. 9). The sucrose content was higher (up to 1.4-fold) in mature transgenic tubers compared with the wild-type control, but this significant difference was not conserved after storing the tubers for 42 d and 85 d at 4 °C (Fig. 9). The content of starch was unchanged in mature transgenic tubers and decreased up to 40% of control levels in transgenic tubers stored for 42 d at 4 °C (Fig. 9). In transgenic tubers stored for 85 d, the starch content was similar to that in control tubers or lower about 25% in transgenic line 56 (Fig. 9) suggesting that the kinetics of cold-nduced sweetening may vary, possibly due to the induction of beta-amylase, the manipulation of phosphorylating GAPC activity has little effect on the absolute mobilization of starch.

Discussion

The results of this paper suggest that the phosphorylating GAPC exhibits a relatively minor influence on both leaf

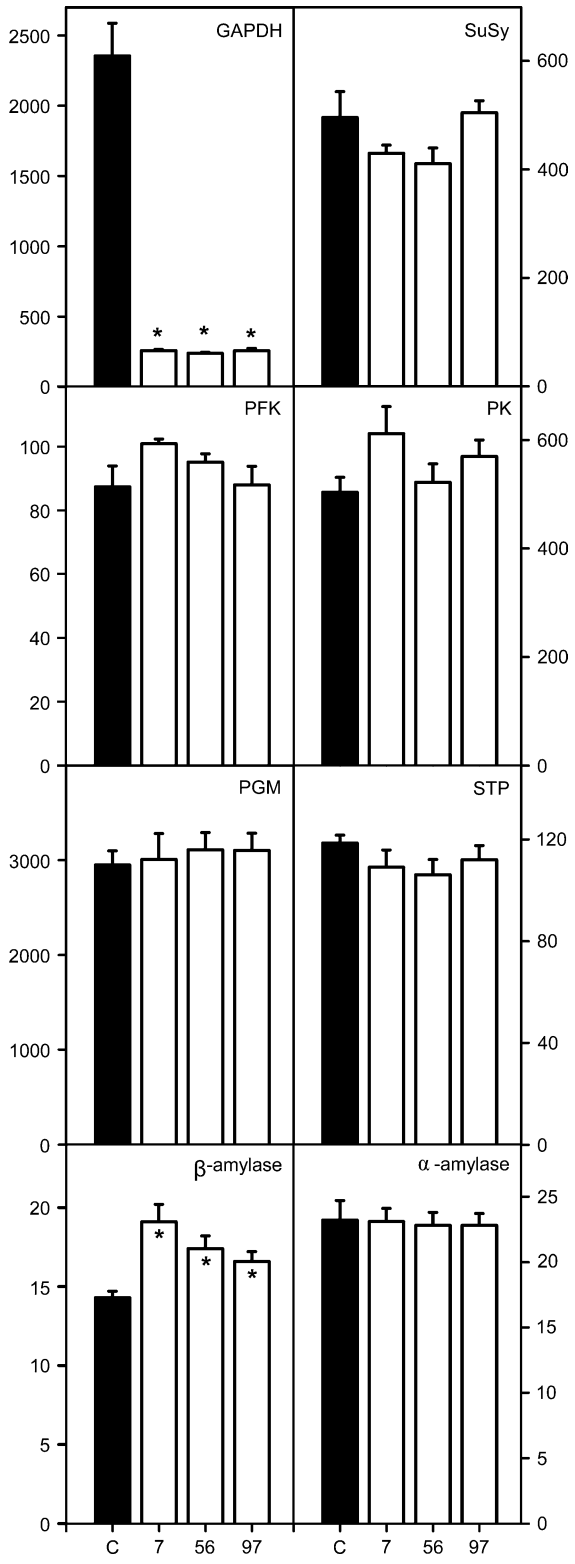
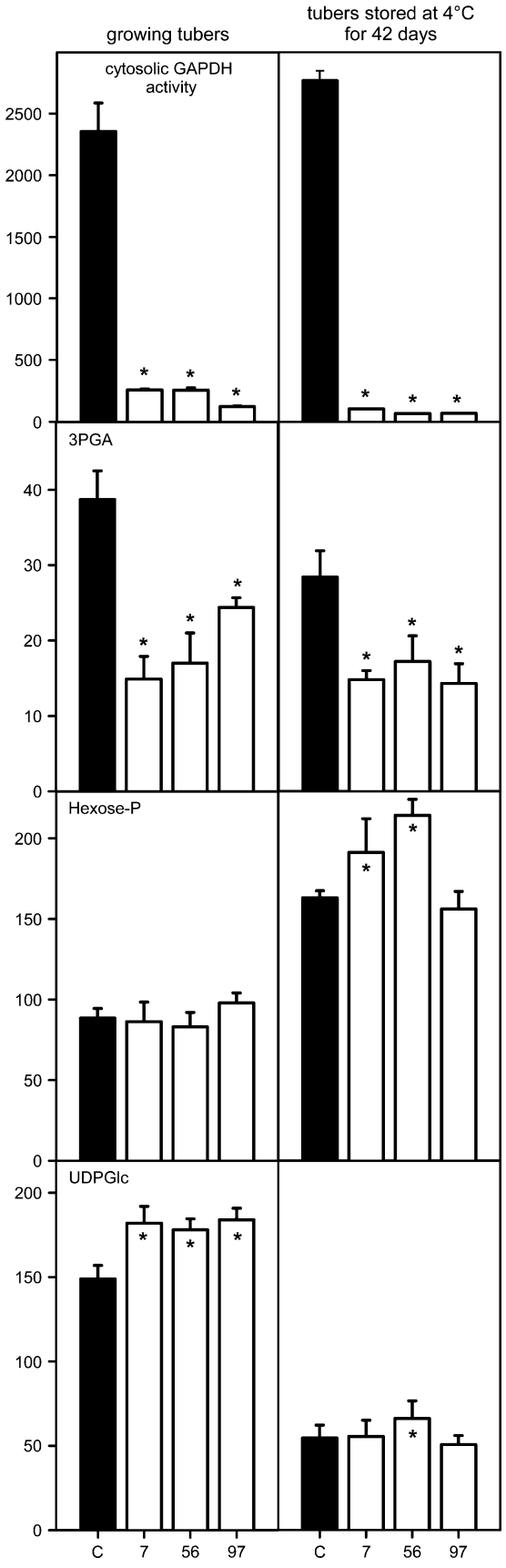


Fig. 7. Enzyme activities in potato tubers exhibiting decreased expression of phosphorylating GAPC. Plant material was harvested from tubers of growing plants and homogenized in buffer as described in the Materials and methods. SuSy, sucrose synthase; PK, pyruvate kinase; PFK, phosphofructokinase; PGM, phosphoglycerate mutase, and STP, starch phosphorylase. The untransformed control is indicated as the filled bar and transgenic lines as blank bars. 97, 56, and 7 represent independent transgenic lines. Results are given in nmol mg^{-1} protein min^{-1} and represent the mean ±SE of four independent replicates. Asterisk denotes values that were determined to be significantly different by the *t* test ($P < 0.05$).

and tuber metabolism with the observed changes being confined to metabolites intimately associated with the reaction. Moreover, despite a strong constitutive inhibition of phosphorylating GAPC, transgenic potato plants were not altered in height, flowering time, leaf number or leaf size compared with control plants, demonstrating that potato plants are able to cope with a reduced GAPDH activity of more than 90% without any growth retardation. Similar results were obtained with potato plants which were inhibited to a similar extent in pyrophosphate-dependent phosphofructokinase, cytosolic fructose-1,6-*bis*phosphatase or either of the two potato isoforms of hexokinase (Hajirezaei *et al*., 1994; Zrenner *et al*., 1996; Veramendi *et al*., 1999, 2002). By contrast, inhibition of other enzymes associated with glycolysis such as the cytosolic isoforms of fructokinase (Davis *et al*., 2005), phosphoglucomutase (Fernie *et al*., 2002*b*), and cytosolic phosphoglycerate mutase (Westram *et al*., 2002) in potato resulted in discernible and dramatic alterations in phenotype, even in instances wherein the documented level of repression was considerably lower than that observed here.

As stated above, the expression of the antisense phosphorylating GAPC construct resulted in dramatically reduced activity of this enzyme, the inhibition being particularly effective in sink leaves, stolons, roots, and tubers but less effective in source leaves and stems. A cursory analysis of leaf metabolism revealed that this was somewhat altered with clear and notable changes in 3-phosphoglycerate content. However, given that the effects of altering glycolysis in potato leaves has been relatively well documented (Knowles *et al*., 1998; Grodzinski *et al*., 1999; Veramendi *et al*., 1999; Geigenberger *et al*., 2004*a*), and the fact that the plastidial isoforms of GAPDH are probably more important during photosynthetic metabolism (Petersen *et al*., 2003; Sparla *et al*., 2005), the major aim of this study was to characterize the role of phosphorylating GAPC in tuber metabolism and function. Measurement of the levels of tuber phosphorylated intermediates of the transformants revealed an increase in UDPglucose and decreases in the levels of 3-phosphoglycerate and phospho*enol*pyruvate that would be anticipated following reduction of the *in vivo* capacity of this reaction step. Surprisingly, there was not a corresponding increase in hexose phosphate levels which are generally maintained at equilibrium with UDPglucose (Tauberger *et al*., 2000). The exact reason for this is unclear in the current study. However, it seems likely that one of two mechanisms lie behind this. It is conceivable that the transgenics additionally exhibit an inhibition in the UDPglucose pyrophosphorylase reaction via an, as yet unknown, regulatory



mechanism. Alternatively, it is possible that UDPglucose pyrophosphorylase and the hexose phosphate pools are in equilibrium within the cytosol, but that fewer hexose phosphates are partitioning to the plastid in the transformants. In keeping with this hypothesis the levels of 3PGA, a potent activator of AGPase, are dramatically reduced. Whilst the latter hypothesis is perhaps favourable, further experimentation is required in order fully to comprehend these observations. In addition, a decrease of label incorporation into anionic and cationic compounds in transgenic lines, following incubation of tuber discs in ^{14}C-labelled substrates also indicate that the transformants displayed altered tuber metabolism. Whereas the increased ^{14}C label incorporation into both sucrose and cell wall suggest that an induction of glyconeogenic flux may result in an enhanced cell-wall synthesis. In keeping with this hypothesis, transgenic tobacco calli expressing a modified mammalian 6-phosphofructo 2-kinase/fructose 2,6-*bis*phosphatase gene and displaying elevated triose- to hexose-phosphate cycling (Fernie *et al*., 2001) were shown to display enhanced cell-wall biosynthesis under conditions of phosphate stress (Fernie *et al*., 2002*a*). However, measurement of steady-state cellulose content in the present study revealed no difference between the control and transgenic lines (data not shown). Equally surprisingly, the depletion of specific glycolytic intermediates had no impact on the respiration rate in intact tubers of growing plants (Fig. 5). Two potential explanations can be proposed for these results. First, it is possible that even a reduction of phosphorylating GAPC of up to 95% can be compensated by another isoform of GAPDH. Secondly, it is conceivable that the residual GAPDH activity is sufficient to maintain respiratory activity. The occurrence of a non-phosphorylating GAPDH, which catalyses the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate in a single reaction (Kelly and Gibbs, 1973; Mateos and Serrano, 1992; Habenicht *et al*., 1994), provides correlative support for the first hypothesis. In addition, Bustos and Iglesias (2003) demonstrated that the non-phosphorylating GAPDH in heterotrophic cells of wheat is a target for multi-level regulation. Due to low level expression it was not possible to demonstrate that the non-phosphorylating GAPDH (GAPN) was up-regulated at the transcriptional level (data not shown). When taken together with the fact that the exact role of this enzyme in heterotrophic tissues remains unknown, the second possibility cannot be formally excluded – especially

Fig. 8. Influence of reduced expression of phosphorylating GAPC on tuber primary metabolites during cold storage. Plant material was harvested from tubers of growing plants and homogenized in buffer as described in the Materials and methods. The untransformed control is indicated as the filled bar and transgenic lines as blank bars. 97, 56 and 7 represent independent transgenic lines. Results are given in nmol g^{-1} FW and represent the mean ±SE of 12 and 6 replicates in the wild-type and transgenic lines, respectively. Asterisk denotes values that were determined to differ significantly from control values by the *t* test (P <0.05).

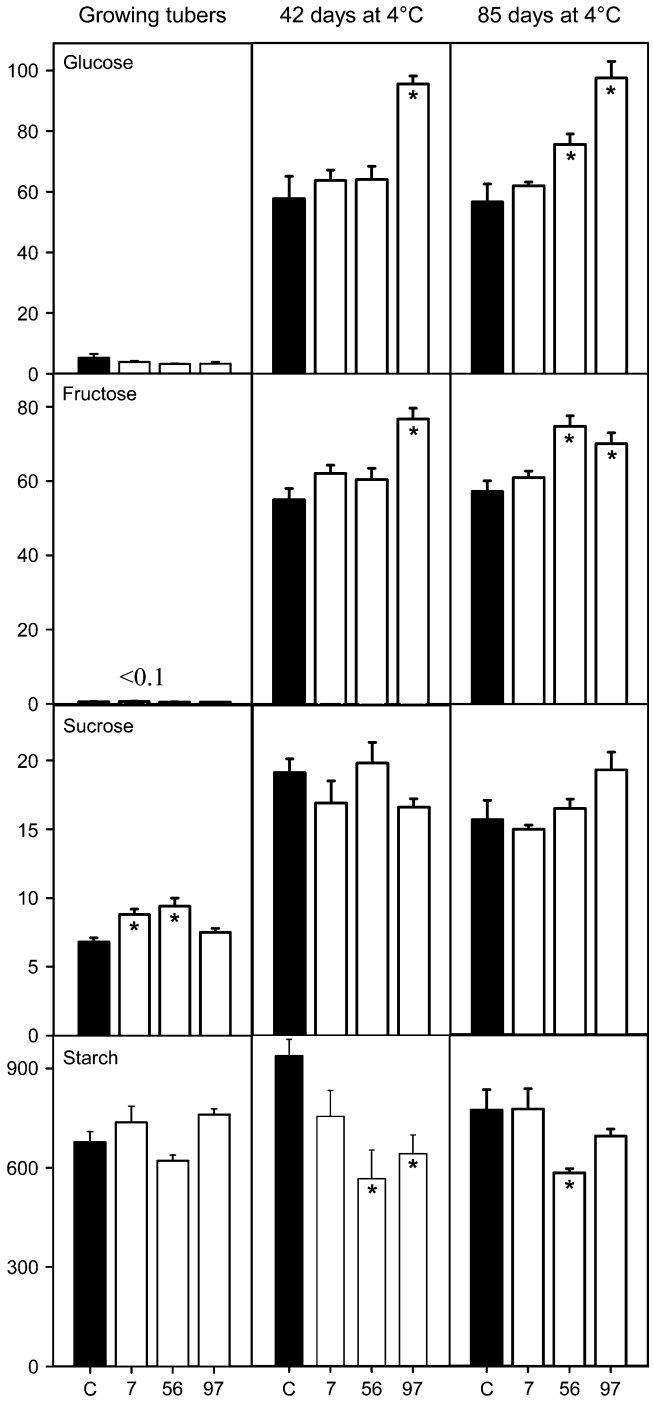


Fig. 9. Influence of reduced expression of phosphorylating GAPC on tuber carbohydrate levels during cold storage. Plant material was harvested from tubers of growing plants and homogenized in buffer as described in the Materials and methods. The untransformed control is indicated as the filled bar and transgenic lines as blank bars. 97, 56, and 7 represent the independent transgenic lines. Results are given in μmol g^{-1} FW and represent the mean ±SE of 12 and 6 replicates in the wild-type and transgenic lines, respectively. Asterisk denotes values that were determined to be significantly different by the *t* test (P <0.05).

given that reduction of the pyrophosphate-dependent phosphofructokinase to a similar extent also displayed little change in the rate of respiration (Hajirezaei *et al*., 1994). Thus, whilst it is conceivable that glycolytic metabolism could be maintained by a non-phosphorylating GAPDH in the transgenic tubers, these results, alongside the metabolic profiles of the transformants, suggest that the reaction step catalysed by GAPDH exerts very little control over the rate of respiration. This finding is largely in keeping with theoretical approaches which suggest that the control of respiration is vested almost entirely in the reactions subsequent to the formation of phosph*enol*pyruvate (Thomas *et al*., 1997), but reveals little concerning the *in vivo* function of the phosphorylating GAPC.

Given that the inhibition of phosphorylating GAPC had little effect on either the levels of glycolytic intermediates or the rate of respiration, the metabolism of ^{14}C-labelled glucose was evaluated in order to assess its general impact on metabolism. Since 3PGA is an activator of the enzyme ADPGlc pyrophosphorylase in potato tubers involved in starch synthesis (Sowokinos and Preiss 1982; Tiessen *et al*., 2002) and a decrease of 3PGA was monitored in transgenic tubers with reduced phosphorylating GAPC activity, they might be expected to have reduced starch content. However, recent studies have revealed that several enzymes that catalyse reactions external or peripheral to the sucrose-to-starch transition are characterized by negative control coefficients for starch synthesis (Tjaden *et al*., 1998; Regierer *et al*., 2002; Geigenberger *et al*., 2004*b*, 2005). Interestingly, whilst in discs isolated from wild- type tubers, about 18% of the label metabolized was incorporated in starch, an incorporation of between 30% and 40% was observed in the transformants (Fig. 6). Although these data are strongly indicative of an enhanced rate of starch synthesis, the steady-state level of starch content remained unchanged in the transformants (Fig. 3). This result is, however, consistent with earlier literature reports since previous studies in which an unregulated bacterial AGPase was expressed in potato was demonstrated to elevate both starch synthesis and degradation (Sweetlove *et al*., 1996*a*) and a recent study revealed that starch turnover is also apparent in wild-type tubers (Roessner-Tunali *et al*., 2004). Our survey of the maximum catalytic activities of starch-degrading enzymes only revealed the induction of β-amylase activity (Fig. 7). However, since β-amylase is thought to be one of the most crucial starch-degrading enzymes in potato tubers (Nielsen *et al*., 1997), transgenic tubers with diminished GAPDH activity appear to trigger starch breakdown rather than to increase the starch content. Intriguingly, a similar picture also emerged on the over-expression of the bacterial AGPase (Sweetlove *et al*., 1996*b*), although the exact mechanism underlying this induction remains unclear from studies to date.

When potato tubers are harvested and stored at low temperatures they accumulate huge amounts of soluble

sugars, primarily glucose, fructose, and sucrose (Sowokinos, 1990). This process, called cold sweetening, is based on starch mobilization in amyloplasts shown by an activation of amylolytic enzymes such as β-amylase (Cochrane *et al*., 1991; Nielsen *et al*., 1997). While cold stress reduces the glycolytic capacity, gluconeogenesis is stimulated resulting in a resynthesis of sucrose. It was previously demonstrated that metabolism of ^{14}C-glucose is appreciably diminished in tubers stored at low temperatures leading to increased levels of hexose-P and an elevated incorporation of label in sucrose (ap Rees, 1988), most likely due to a specific up-regulation of sucrose-P-synthase (Hill *et al*., 1996). This sucrose is subsequently hydrolysed to its constituent hexoses following the induction of vacuolar invertase. Ectopic expression of a proteinous invertase inhibitor targeted to the vacuole can overcome this process (Greiner *et al*., 1999). However, repression of glycolysis is also known to be involved in cold sweetening since the activities of key enzymes such as phosphofructokinase, PFP and pyruvate kinase are reduced during low temperature storage (ap Rees, 1988; ap Rees and Morrell, 1990). To find out whether the metabolic shift imposed by the inhibition of phosphorylating GAPC would further stimulate the accumulation of soluble sugars in potato tubers at low temperature, transgenic tubers with reduced GAPDH activity (up to 97% compared with wild-type controls) were stored at 4 °C and sugar contents were monitored after 42 d and 85 d (Fig. 9). However, there was little difference in sugar accumulation between transgenic and control tubers, suggesting that phosphorylating GAPC also does not play an important role in the accumulation of soluble sugars during cold storage.

In summary, on the basis of the results presented here, it is concluded that an inhibition of phosphorylating GAPC does not greatly affect sugar metabolism in leaves or tubers and has little influence on plant morphology despite clear changes being observed in the levels of several glycolytic metabolites. This suggests that the lack of this phosphorylating GAPC can be compensated by the existence of other isoforms such as the non-phosphorylating GAPDH or even by the plastidial isoform of the enzyme. The results suggest that phosphorylating GAPC exerts little influence on respiratory or starch biosynthetic flux, at least under the conditions tested here. Analysis of the expression of genes encoding phosphorylating GAPC in publicly accessible microarray depositories found at www.genevestigator.ethz.ch (Zimmermann *et al*., 2004), suggests that they are somewhat responsive to stress. However, detailed analysis reveals that most glycolytic enzymes are induced under similar conditions so this may merely reflect the previously documented transcriptional coregulation of this pathway (Urbanczyk-Wochniak *et al*., 2003*a*, *b*). That said, it is quite possible that a specific role for this isoform could be elucidated under sub-optimal growth conditions.

Despite the fact that the changes described here were generally of minor magnitude they allow several important conclusions to be drawn concerning the regulation of tuber metabolism. The lack of change in the steady-state level of starch is intriguing, since, whilst this could perhaps be anticipated from the contrapuntal changes in the levels of hexose phosphates and 3-phosphoglycerate, increased levels of sucrose (such as those observed here), have recently been postulated to promote starch synthesis via redox-related activation of the AGPase reaction (Tiessen *et al*., 2002). Whilst this model is a highly attractive explanation for the co-ordination of starch synthesis the results here suggest that the role of starch degradative enzymes in the regulation of starch accumulation has been very much overlooked in most studies aimed at elevating starch content by stimulating its synthesis (Stark *et al*., 1991; Sonnewald *et al*., 1997; Trethewey *et al*., 2001). The fact that both this and a previous study (Nielsen *et al*., 1997), revealed a coincidence between up-regulation of starch degradation and transcriptional activation of β-amylase suggests that further research effort should be expended on this process.

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