Production of high-starch, low-glucose potatoes through over-expression of the metabolic regulator SnRK1

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Introduction

High starch and low glucose levels are desirable traits in commercial potato tubers. More than two million tonnes of starch are produced annually from potato for industrial uses, and an increase in starch content also reduces the processing costs of potato food products such as crisps and French fries. Low glucose improves processing properties because glucose causes blackening during frying. The aim of this study was to modify the carbohydrate content of potato tubers by manipulating the metabolic regulator sucrose non-fermenting-1-related protein kinase-1 (SnRK1).

SnRK1 is a serine/threonine protein kinase that takes its name from sucrose non-fermenting-1 (SNF1), its homologue in yeast (Saccharomyces cerevisiae) (Celenza and Carlson, 1986). There is also a member of the family in the animal kingdom, AMP-activated protein kinase (AMPK). SnRK1, SNF1 and AMPK are heterotrimeric complexes. In animals, these comprise an α subunit containing the protein kinase catalytic domain, a β subunit and a γ subunit that interacts with a regulatory domain in the α subunit (Woods et al., 1996). The yeast complex comprises a catalytic subunit encoded by the SNF1 gene itself, a second subunit that can be any one of a class of proteins that includes SIP1, SIP2 and GAL83, which are homologues of the animal β subunit, and a regulatory subunit called SNF4 (Celenza et al., 1989) that is homologous to AMPKγ.

Summary

Transgenic potato (Solanum tuberosum cv. Prairie) lines were produced over-expressing a sucrose non-fermenting-1-related protein kinase-1 gene (SnRK1) under the control of a patatin (tuber-specific) promoter. SnRK1 activity in the tubers of three independent transgenic lines was increased by 55%–167% compared with that in the wild-type. Glucose levels were decreased, at 17%–56% of the levels of the wild-type, and the starch content showed an increase of 23%–30%. Sucrose and fructose levels in the tubers of the transgenic plants did not show a significant change. Northern analyses of genes encoding sucrose synthase and ADP-glucose pyrophosphorylase, two key enzymes involved in the biosynthetic pathway from sucrose to starch, showed that the expression of both was increased in tubers of the transgenic lines compared with the wild-type. In contrast, the expression of genes encoding sucrose synthase and ADP-glucose pyrophosphorylase was also increased, by approximately 20%–60% and three- to five-fold, respectively, whereas the activity of hexokinase was unchanged. The results are consistent with a role for SnRK1 in regulating carbon flux through the storage pathway to starch biosynthesis. They emphasize the importance of SnRK1 in the regulation of carbohydrate metabolism and resource partitioning, and indicate a specific role for SnRK1 in the control of starch accumulation in potato tubers.

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The catalytic subunit in plants is encoded by the SnRK1 gene (or gene family). The first SnRK1 gene to be characterized was cloned from rye (Alderson et al., 1991), and homologues have since been identified in many plant species (reviewed by Halford and Hardie, 1998; Halford et al., 2003). SNF4/AMPK homologues have been cloned from Arabidopsis (AtSNF4) and maize (AKINβ) (Kleinow et al., 2000; Lumberas et al., 2001), although both contain domains that resemble part of the β subunit as well. Plants contain at least two other families of proteins that show some similarity to SNF4/AMPK, the function of which is not clear; they are not present in fungal or animal systems (Slocombe et al., 2002). Genes related to the SIP1/SIP2/GAL83/AMPKγ family have since been identified in many plant species (reviewed by Halford and Paul, 2003). For example, SnRK1 phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGG-CoA reductase), and expression of an HMG-CoA reductase lacking an SnRK1 target site has been shown to increase the levels of phytosterols in tobacco seeds (Hey et al., 2006). Sucrose phosphate synthase and nitrate reductase are also substrates for SnRK1 in vitro (reviewed by Halford and Hardie, 1998), as is a small heat shock protein (Slocombe et al., 2004). In addition, SnRK1 is required for redox modulation of ADP-glucose pyrophosphorylase activity in response to sucrose (Tiessen et al., 2003) and, like its fungal and animal counterparts, is involved in regulating the expression of genes involved in carbon metabolism: antisense expression of SnRK1 has been shown to reduce sucrose synthase gene expression in potato tubers (Purcell et al., 1998) and α-amylase gene expression in cultured wheat embryos (Laurie et al., 2003).

Here, we show that over-expression of SnRK1 in potato tubers causes a significant increase in starch content and a decrease in glucose levels, resulting from a dramatic increase in the level of expression and activity of two key enzymes involved in the starch biosynthetic pathway: sucrose synthase and ADP-glucose pyrophosphorylase.

Results

Production of transgenic potato plants over-expressing SnRK1

A chimaeric gene, 02-SnRK1-nos, was constructed containing an SnRK1 polymerase chain reaction (PCR) product from potato cv. Prairie between a 1383-bp patatin gene promoter and nopaline synthase (Nos) gene terminator. 02-SnRK1-nos contained the entire SnRK1 coding region (1515 bp) in the same orientation as the promoter, it was engineered into potato cv. Prairie by Agrobacterium-mediated transformation. Thirty putative transformants were screened by PCR amplification of the transgene using a combination of primers designed to hybridize with the promoter and the SnRK1 sequence. The transgene was detectable in 27 of the putative transformants (not shown). The phenotype of the transgenic plants, including tuber development, sprouting and tuber yield (typically 300–400 g per plant), was not distinguishable from that of control plants.

SnRK1 transcripts were detected in the tubers of the transgenic lines by Northern analysis of total RNA prepared from tubers harvested 12 weeks after planting. Five transgenic lines, 8318, 8326, 8335, 8363 and 8396, were identified in which SnRK1 transcript levels were clearly higher than in non-transgenic controls (Figure 1a). The results of these and subsequent expression analyses were repeatable using RNA from different wild-type plants as the control and using RNA from different tubers and plants from each transgenic line (not shown). They were also repeatable using RNA from tubers harvested from plants produced from subsequent rounds of planting and growth.

Three transgenic lines, 8318, 8363 and 8396, were taken for further analysis. SnRK1 activity in the tubers of these lines was compared with that in the wild-type (Figure 1b). All three showed an increase, and the overall differences between the lines were significant (P = 0.009, using an analysis of variance (ANOVA) on natural log-transformed data, the transformation correcting for variance heterogeneity). The raw means, expressed in nmol/min/mg, and transformed data means (in parentheses) of activity were 0.2 (5.298), 0.310 (5.737), 0.534 (6.280) and 0.426 (6.055) for the wild-type, 8318, 8363 and 8396, respectively; the standard error of the differences (on the transform scale) was 0.2401 on 11 degrees of freedom; t-tests were performed to compare each transgenic line with the control and, for 8363 and 8396, the difference was found to be significant (P = 0.002 and P = 0.009, respectively). The difference for 8318 was significant at the 10% rather than 5% level (P = 0.094).

Levels of sucrose, glucose, fructose and starch in the tubers of the transgenic lines

The levels of sucrose, glucose, fructose and starch in the tubers of the transgenic lines were measured and compared with those of the controls (Figure 2). In each case, four tubers were sampled and the measurements were performed three
times on material from the central part of each tuber. Sucrose and fructose levels in all three transgenic lines (8396, 8318 and 8363) were not significantly different from those of the controls (overall $P$ values were 0.468 for fructose and 0.138 for sucrose). In contrast, significant differences between the plants were found for glucose (overall $P$ value of 0.006). The means of the measurements of glucose levels were 3.319, 1.132, 0.567 and 1.793 $\mu$mol/g fresh weight for the control, 8318, 8363 and 8396, respectively; the standard error of the differences was 0.6406 on 12 degrees of freedom; $t$-tests were performed to compare each transgenic line with the control and, in each case, the difference was found to be significant ($P = 0.005$, $P = 0.001$ and $P = 0.035$ for 8318, 8363 and 8396, respectively). In the case of 8363, glucose was present at 17% of the level in the controls.

The overall $P$ value for the differences between the lines for the starch measurements was 0.122, suggestive of real differences. The means of the measurements were 113.9, 151.0, 154.2 and 144.1 $\mu$mol/g fresh weight for the control, 8318, 8363 and 8396, respectively; the standard error of the differences was 16.80 on 11 degrees of freedom; $t$-tests were performed to compare each transgenic line with the
control, and the differences for 8318 and 8363 were found to be significant ($P = 0.049$ and $P = 0.035$, respectively). The difference for 8396 was significant ($P = 0.098$) at the 10% rather than 5% level. The lower significance (higher $P$ values) here is a result of the variability observed in the measurements (the standard error of within-tuber observations, given the ANOVA, was 7.09 µmol/g fresh weight). Nevertheless, the increase in starch in the transgenic lines was large, the level in the tubers of 8363, for example, being approximately 30% higher than that in the controls.

Expression analyses of genes encoding sucrose synthase, ADP-glucose pyrophosphorylase, $\alpha$-amylase and sucrose phosphate synthase

Northern analyses were performed of genes encoding two key enzymes involved in the starch biosynthetic pathway, sucrose synthase and ADP-glucose pyrophosphorylase, and, for comparison, genes encoding sucrose phosphate synthase (sucrose synthesis) and $\alpha$-amylase (starch breakdown). Although the expression of genes encoding sucrose phosphate synthase and $\alpha$-amylase showed no change from the wild-type, there was a dramatic increase in the transcript levels of sucrose synthase and ADP-glucose pyrophosphorylase in tubers from all three independent transgenic lines (Figure 3).

Measurements of sucrose synthase, ADP-glucose pyrophosphorylase and hexokinase activity

The maximum catalytic activity ($V_{\text{max}}$) of sucrose synthase and ADP-glucose pyrophosphorylase was measured in tubers from the transgenic plants harvested 12 weeks after planting. In each case, four different tubers were harvested from each plant and the assay was performed three times on material from the central part of each tuber. The activity of sucrose synthase and ADP-glucose pyrophosphorylase was increased in the tubers of all three transgenic lines (Figure 4). In the case of 8396, the sucrose synthase activity was approximately 80% higher than that of the wild-type, whereas the ADP-glucose pyrophosphorylase activity was approximately five times higher than that of the wild-type.

ANOVA of the data showed that there were significant differences between the plants for both enzymes ($P < 0.001$ for sucrose synthase and $P = 0.018$ for ADP-glucose pyrophosphorylase). The means of the activity measurements for sucrose synthase were 289.8, 350.5, 457.8 and 479.0 nmol/min/g fresh weight for the wild-type control, 8318, 8363 and 8396, respectively; the standard error of the differences between means was 12.33 on 12 degrees of freedom; $t$-tests were performed to compare each transgenic plant with the control and, in each case, the difference was found to be significant ($P < 0.001$).

A loge transformation was applied to correct for variance heterogeneity in the ANOVA of ADP-glucose pyrophosphorylase activity data. The raw means of activity, expressed in µmol/min/g fresh weight, and transformed data means (in parentheses) were 0.075 (−2.88), 0.253 (−1.48), 0.255 (−1.45) and 0.405 (−1.11) for the wild-type, 8318, 8363 and 8396, respectively; the standard error of the differences, on the loge scale, was 0.495 on 12 degrees of freedom; $t$-tests were performed to compare each transgenic plant with the control and, in each case, the difference was found to be significant ($P = 0.015$, $P = 0.013$ and $P = 0.004$ for 8318, 8363 and 8396, respectively).
Measurements of ADP-glucose pyrophosphorylase activity without reduction ($V_{sel}$) were less conclusive. The means were 0.0385, 0.0635, 0.0410 and 0.1158 $\mu$mol/min/g fresh weight for controls, 8318, 8363 and 8396, respectively. Although the activity in the transgenic lines was higher than that in the controls, particularly in the case of line 8396, the variability in the measurements meant that we could not demonstrate a statistically significant difference ($P = 0.471$).

We also measured the activity of hexokinase. Although this enzyme is not involved in the pathway for starch biosynthesis (Figure 5) (indeed increasing flux through this pathway by over-expressing an invertase and a glucokinase in transgenic potato tubers has been shown to reduce starch accumulation dramatically; Trethewey et al., 1998), it has been implicated in glucose signalling (Jang et al., 1997; Moore et al., 2003). As the mechanism by which hexokinase signals is not known, it is only possible to measure its catalytic activity. This showed no overall significant difference between the transgenic lines and controls ($P = 0.220$), the means being 0.0911, 0.0950, 0.0709 and 0.1186 $\mu$mol/min/g fresh weight for controls, 8318, 8363 and 8396, respectively.

**Discussion**

We have described the production of transgenic potato plants over-expressing SnRK1, in which starch levels in the tubers were increased by up to 30% and glucose levels were reduced by up to 83%. The gene expression and activity of two key enzymes involved in the starch biosynthesis pathway, sucrose synthase and ADP-glucose pyrophosphorylase, were increased in the transgenic tubers.

Although $V_{max}$ of ADP-glucose pyrophosphorylase activity showed a clear increase, $V_{sel}$ was only increased significantly in one of the lines. This indicates that the increase in $V_{max}$ was a result of the increase in expression of the enzyme rather than its activation state. Antisense repression of SnRK1 has been shown to prevent the sucrose-dependent redox activation of ADP-glucose pyrophosphorylase (Tiessen et al., 2003), but our results provide no evidence that over-expression of SnRK1 changes the activation state of this enzyme. The signalling pathway from SnRK1 in the cytosol to ADP-glucose pyrophosphorylase in the plastid has not been elucidated, and it is possible that another component in the pathway is limiting.

We did not analyse the effect of over-expression of SnRK1 on other components of the SnRK1 complex because, with the exception of StubGal83 (Lakatos et al., 1999), these have not been characterized in potato. However, our results suggest that, in potato tubers at least, the expression of the catalytic subunit encoded by $SnRK1$ is a limiting factor in determining the activity of the protein kinase.

The possibility that over-expression of $SnRK1$ affects sucrose levels in some way, and that this is sensed by another mechanism that regulates sucrose synthase and ADP-glucose pyrophosphorylase gene expression (in other words, a pleiotropic effect), can be ruled out because there was no consistent difference in tuber sucrose levels between the transgenic plants and controls. The fact that sucrose levels were maintained despite the increase in sucrose synthase activity and flux into starch biosynthesis is also consistent with sucrose synthase being a major determinant of sink strength in potato tubers (Zrenner et al., 1995); the increased demand for sucrose must have been met by an increase in supply.

Plants contain another enzyme, invertase, which metabolizes sucrose. However, sucrose synthase is the predominant production of high-starch, low-glucose potatoes

![Figure 4](image-url)  
**Figure 4** Mean sucrose synthase and ADP-glucose pyrophosphorylase (AGPase) activity ($V_{max}$) in wild-type potato (cv. Prairie) tubers and tubers from plants transformed with the sucrose non-fermenting-1-related protein kinase-1 gene ($SnRK1$) over-expression construct 02-SnRK1-nos. The standard error of the differences between the means was 12.33 for sucrose synthase and 0.495 (on the loge scale) for ADP-glucose pyrophosphorylase, on 12 degrees of freedom. gfw, gram fresh weight.
enzyme for sucrose metabolism in potato tubers (Morrell and Ap Rees, 1986). Moreover, over-expression of invertase together with glucokinase results in a dramatic decrease in starch accumulation (Trethewey et al., 1998), not an increase. This confirms that it is the sucrose synthase pathway, rather than the invertase pathway, that provides the glucose-1-phosphate for starch biosynthesis (Figure 5). We did not measure invertase activity in this study.

Another enzyme that has been implicated in sugar signalling in plants is hexokinase (Jang et al., 1997; Moore et al., 2003). As the glucose levels in transgenic tubers were lower than those in controls, it is conceivable that hexokinase signalling was affected. We measured the catalytic activity of hexokinase in the transgenic tubers, and found it to be unchanged from that in the wild-type. It is not possible to measure the signalling activity of hexokinase because the mechanism by which it signals is not known.

Antisense expression of SnRK1 in potato tubers does not result in a decrease in starch levels (Purcell et al., 1998). However, barley pollen expressing antisense SnRK1 contains little or no starch and arrests at the binucleate stage of development (Zhang et al., 2001). Fungi and animals do not make starch, of course, but they make a similar polysaccharide, glycogen. Both SNF1 and AMPK, the fungal and animal homologues of SnRK1, have been implicated in the regulation of glycogen, but the mechanisms involved are quite different from those that we have shown for SnRK1 in the regulation of starch biosynthesis (neither fungi nor animals make sucrose synthase or ADP-glucose pyrophosphorylase; the glucose donor in glycogen synthesis is UDP-glucose). Yeast cells defective in SNF1 fail to accumulate glycogen (Cannon et al., 1994; Hardy et al., 1994; Huang et al., 1996) because they are unable to activate glycogen synthase; glycogen synthase is held in a hyper-phosphorylated state through the action of glycogen synthase kinase, the activity of which is elevated in snf1 mutants. In humans, glycogen synthase itself is a substrate for AMPK, and mutations in AMPK cause glycogen storage disease (Arad et al., 2002).

Another difference between the plant system and that of fungi and animals is that the plant signalling pathway responds to sucrose, a molecule that is not sensed in fungal or animal systems at all. Genes encoding sucrose synthase and ADP-glucose pyrophosphorylase in potato tubers are inducible by sucrose (Müller-Röber et al., 1990; Fu and Park, 1995). Furthermore, redox activation of ADP-glucose pyrophosphorylase occurs in response to either sucrose or glucose, but it is only the sucrose activation pathway that requires SnRK1 (Tiessen et al., 2003). We propose the hypothesis that SnRK1 in tubers is activated by sucrose arriving from source leaves; if the level of sucrose rises, then SnRK1 increases flux through the starch biosynthesis pathway (Figure 5). Over-expression of SnRK1 causes an increase in flux through the starch biosynthesis pathway without the increase in sucrose supply. This would also explain the decreased levels of glucose in the transgenic tubers.

**Experimental procedures**

**Construction of chimaeric genes**

The full-length potato (Solanum tuberosum) SnRK1 coding region (accession numbers X95997–X96000 and X96372)
was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA prepared from potato cv. Prairie tubers. It was cloned in the sense orientation between a patatin gene promoter (1383 bp) and nopaline synthase (Nos) gene terminator in vector pDV02000 (Advanced Technologies, Cambridge, UK) to make plasmid 02-SnRK1-nos.

**Potato transformation**

We thank Dawn Carter and Jennifer Willing of Advanced Technologies for producing the transgenic potato lines. *S. tuberosum* cv. Prairie was transformed using *Agrobacterium*-mediated transformation. Microtubers were generated on stem explants, excised and placed in individual 2-cm² modules and grown under glasshouse conditions. Plantlets were potted and grown under glass to maturity (12 weeks). The tubers were harvested and frozen immediately in liquid nitrogen for storage at −80 °C, or kept in a cold store at 5 °C.

**Screening of transgenic plant lines**

Leaf tissue from putative transgenic plants was screened using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, Poole, Dorset, UK). The primers used were PAT912F (5′-GGTGCGAGGGAGAGAATC) and pKIN999R (5′-GGAACG-GTGGTCGTACAGCAG). The PCR parameters were 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 40 s, and a final 7 min at 72 °C.

**Northern analyses**

Total RNA was extracted using the RNAqueous Extraction Kit (Ambion, Austin, TX, USA), followed by a lithium chloride precipitation. The RNA was treated with RQ1 RNase-Free DNase (Promega, Southampton, UK) to remove any contaminating genomic DNA. An aliquot (5 μg) was electrophoresed on a 1% MOPS (morpholinopropane sulphonic acid)–formaldehyde gel. Equal loading of each sample was checked by comparing the intensity of ethidium bromide staining of ribosomal RNA bands. The RNA was then transferred to Magnacharge membranes (Micron Separations Inc., Westborough, MA, USA) according to the manufacturer’s instructions. Radiolabelled probes were synthesized using the Prime-it II Random Primer Labelling Kit (Stratagene, Cambridge, UK) and [α-32P]dATP (Amersham, Buckinghamshire, UK). Unincorporated nucleotides were removed using NucTrap Probe Purification Columns (Stratagene). The hybridization buffer contained 50% v/v formamide, 6 × SSPE (standard saline phosphate, EDTA), 5 × Denhardt’s solution, 0.2% w/v sodium dodecylsulphate (SDS), 100 μg/mL denatured herring sperm DNA and 5% w/v dextran sulphate (M₉ = 500 000); hybridization was allowed to proceed overnight at 42 °C. The membranes were washed at increasing stringencies from 2 × standard saline citrate (SSC), 0.1% SDS at 42 °C for 30 min to 0.1 × SSC, 0.1% SDS at 60 °C for 30 min. Hybridization was visualized by autoradiography.

The probe used for SnRK1 was a 514-bp DNA fragment comprising bases 485–999 of the coding region. The sucrose synthase probe was a PCR product comprising bases 4810–5433 of the complete gene sequence of a potato sucrose synthase gene (accession number U24087) (Fu and Park, 1995). The sucrose phosphate synthase probe was a DNA fragment comprising bases 502–1521 of the coding sequence of a potato sucrose phosphate synthase gene (accession number X73477) (Zrenner et al., 1995). The α-amylose probe was a 175-bp PsI restriction fragment from the 3′ untranslated region of wheat α-Amy2 (Lazarus et al., 1985), and the AGPase probe was a 1700-bp EcoRI restriction fragment of the potato ADP-glucose pyrophosphorylase gene AGP B (nucleotide database accession numbers listed in SWISS-PROT P23509).

**SnRK1 activity assays**

SnRK1 activity was measured using the method described by Sugden et al. (1999), with the AMARA peptide (Ala Met Ala Ala Arg Ala Ser Ala Ala Leu Ala Arg Arg Arg) as substrate. Tuber tissue (1 g) was ground in 1 mL of cold extraction buffer [0.1 M tricine, 25 mM NaF, 2 mM sodium pyrophosphate (NaPi), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethyleneglycoltetaacetic acid (EGTA), 1 mM benzamidine pH 8.2, 5 mM dithiothreitol (DTT), 25 mM β-mercaptoethanol, 1 μM pepstatin A, 1 mM phenylmethylsulphonylfuoride (PMSF)]; DTT and β-mercaptoethanol added on day of extraction, pepstatin A and PMSF added immediately before use]. The suspension was transferred to two cold microfuge tubes and clarified by centrifugation for 20 min at 0 °C. The precipitated protein was sedimented by centrifugation (12 000 g, 10 min, 4 °C) and the supernatant was discarded. The pellet was dissolved in 250 μL of resuspension buffer [0.1 M tricine, 25 mM NaF, 2 mM NaPi, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM benzamidine, pH 8.2 (NaOH), 5 mM DTT, 25 mM β-mercaptoethanol, 1 μM pepstatin A, 10% glycerol]. Brief sonication was required to disperse the pellet. Extracts were then clarified by centrifugation (12 000 g, 3 min, 4 °C). The supernatant was transferred to labelled tubes, frozen in liquid N₂ and stored at −80 °C.
The assay was carried out at 30 °C. A master mix of ATP.MgCl₂ containing 12.5 kBq [γ³²P]ATP was made. This mix was divided into two aliquots and AMARA peptide was added to one aliquot and water to the other. A cocktail of tricine–DTT–protease inhibitor (10 µL) was mixed with 10 µL of the ATP.MgCl₂ ± AMARA mix. The tuber extract (5 µL) was then added to start the reaction. A water control (no tuber extract) was included and each assay was performed in duplicate. The reaction was allowed to proceed for 5 min; 15 µL of the reaction mix was then spotted on to a phosphocellulose paper square and placed immediately into 1% (v/v) phosphoric acid to quench the reaction. Four 5-min washes in acid were carried out and the phosphocellulose square was placed in acetone for 5 min. The square was air dried on a paper towel, placed in a pony vial and 4 mL of Ultima Gold LS cocktail (Packard, Meridian, CT) was added. The vial was capped and, after gentle mixing, was placed in a liquid scintillation counter for counting.

**Sucrose synthase activity assay**

Potato tuber tissue was ground under liquid nitrogen with a pestle and mortar, and extracted in 100 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) pH 7.0, 5 mM MgCl₂, 0.1% Triton and 0.1% β-mercaptoethanol. Aliquots of tissue extract were incubated with 100 mM sucrose and 3 mM UDP for 30 min at 30 °C, and UDP-glucose produced over this period was measured as in Dancer et al. (1990).

**ADP-glucose pyrophosphorylase assay**

Frozen tuber tissue (300 mg) was homogenized in liquid N₂ and extracted rapidly with 1 mL of extraction buffer (50 mM HEPES-KOH (pH 7.8), 5 mM MgCl₂ and 3 mM DTT) at 4 °C. The fresh extract was mixed vigorously for 20 s and centrifuged for 30 s at 10 000 g at 4 °C. The ADP-glucose pyrophosphorylase assay in the pyrophosphorylase direction was performed as described by Tiessen et al. (2002, 2003) at 30 °C in a total volume of 200 µL containing 50 mM HEPES-KOH, pH 7.8, 5 mM MgCl₂, 10 µM glucose-1,6-bisphosphate, 0.6 mM oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺), 1.3 mM NaPPI, 1 U/mL phosphoglucomutase (from rabbit muscle), 2 U/mL glucose-6-phosphate dehydrogenase (from yeast) and 2.5 mM ADP-glucose. To activate the AGPase, 1 mM of ADP-glucose and 10 mM DTT were added to 30 µL of fresh extract in a final volume of 100 µL, and 30 µL of the activated extract was used for the assay.

**Hexokinase assay**

Tuber material (300 mg) was ground in 1 mL of fresh extraction buffer [50 mM HEPES/KOH (pH 8.0), 1 mM EGTA, 1 mM EDTA, 1 µL/mL β-mercaptoethanol, 1 µL/mL Triton, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM PMSF], transferred to a cold microtube and centrifuged for 5 min at 12 000 g at 4 °C. The supernatant was collected and frozen in liquid nitrogen.

Assay buffer [195 µL; 50 mM HEPES/KOH pH 8.0, 5 mM MgCl₂, 2.5 mM ATP, 0.33 mM NADP⁺, 1 mM glucose, 2 µL glucose-6-phosphate dehydrogenase (7 U)] was transferred to microtitre plate wells. Controls without the plant extract or without glucose were included. The extract (5 µL) was added to the wells and the absorbance change at 340 nm over 30 min was measured.

**Carbohydrates**

Glucose, fructose and sucrose were measured through the reduction of NADP⁺ by glucose-6-phosphate dehydrogenase after the sequential addition of hexokinase, phosphoglucomutase and invertase (Jones et al., 1977). The method was adapted for use on microtitre plates. Frozen tuber tissue (300 mg) was ground in liquid N₂ and placed in a 1.5-mL tube with 1 mL of 80% ethanol. The tube was placed at 70 °C in a heating block for 1 h and agitated intermittently. Following incubation, the tube was centrifuged for 2 min at 10 000 g on a bench top centrifuge and the supernatant was removed and placed at ~20 °C. The pellet containing the starch fraction was also stored at ~20 °C.

Each assay was performed in 200 µL of reaction buffer containing 100 mM imidazole, 10 mM MgCl₂, 1.1 mM ATP, 0.5 mM NADP⁺, 20 µL of tuber extract and 0.14 U of glucose-6-phosphate dehydrogenase. The starting absorbance at 340 nm was recorded. For glucose, 0.12 U of hexokinase was added and the reaction was allowed to progress to completion (approximately 10 min). The final absorbance was recorded at 340 nm. For fructose, 1 U of phosphoglucomutase was added, and for sucrose 4 U of invertase was added.

Starch was measured after breakdown of starch to glucose in the insoluble pellet using α-amylase and amyloglucosidase (Sonnewald et al., 1991). The pellet was washed twice with sterile distilled water and mixed well with 500 µL of 50 mM sodium acetate buffer, pH 4.8, containing 2 U of α-amylase and 0.05 U of amyloglucosidase. The solution was incubated at 37 °C for 72 h and centrifuged. The supernatant was diluted 1:15 and 5 µL of this dilution was used to assay the glucose content.
Statistical analyses

ANOVA was applied to the data sets comprising the measurements of sucrose, fructose, glucose and starch, as well as sucrose synthase and ADP-glucose pyrophosphorylase activity, from the three transgenic lines (8318, 8363 and 8396) and the control line (wild-type Prairie). A natural logarithm transformation (log_e) was used where necessary to account for heterogeneity of variance. It should be noted that, because a common transformation was applied to all observations, the comparative values between the plants were not altered and comparisons between them remained valid. Following each ANOVA, t-tests were performed using the standard error of the difference between the means (on the residual degrees of freedom from the ANOVA) to compare the transgenic lines with the wild-type control line. The Genstat™ statistical system (GenStat Release 8.1 (2005), ©Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK) was used for all analyses. A full explanation of the application of these statistical analyses is given by Gomez and Gomez (1984).

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