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

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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 two other enzymes of carbohydrate metabolism, α-amylase and sucrose phosphate synthase, showed no change. The activity of 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.

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.

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/AMPKγ homologues have been cloned from *Arabidopsis* (AtSNF4) and maize (AKINβγ) (Kleinow *et al*., 2000; Lumbreras *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/AMPKγ, 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/AMPKβ family have been cloned from *Arabidopsis* (*AKINβ1*, *AKINβ2*) and potato (*StubGAL83*) (Bouly *et al*., 1999; Lakatos *et al*., 1999).

SnRK1, SNF1 and AMPK all play a role in regulating metabolism in response to carbon availability in their respective systems (reviewed by Halford and Paul, 2003). For example, SnRK1 phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-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

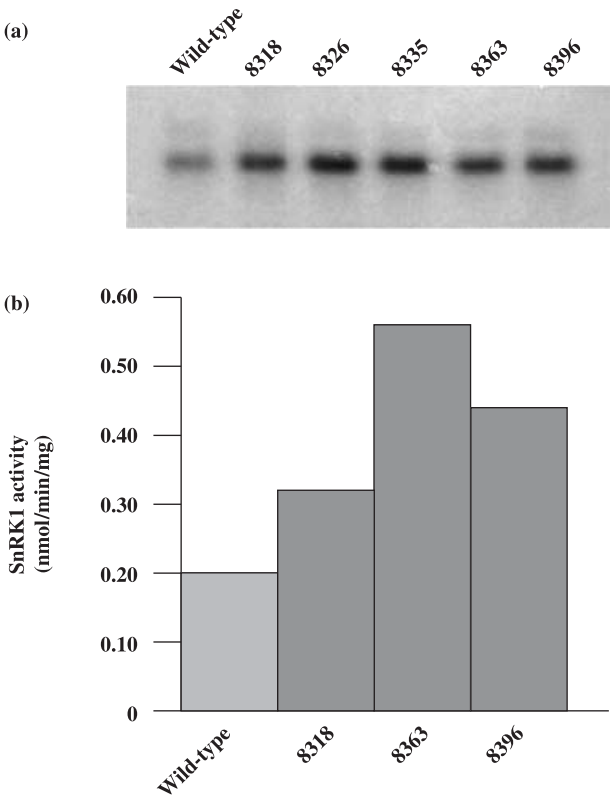

Figure 1 (a) Northern analysis of total RNA (5 μg per lane) from tubers of wild-type potato (cv. Prairie) and five transgenic potato lines transformed with the sucrose non-fermenting-1-related protein kinase-1 gene (*SnRK1*) over-expression construct 02-SnRK1-nos. The RNA was probed with a potato *SnRK1* DNA fragment. Equal loading in each lane was checked by ethidium bromide staining and comparison of the staining intensity of the ribosomal RNA bands (not shown). (b) SnRK1 activity in tubers of wild-type potato (cv. Prairie) and three independent transgenic lines over-expressing *SnRK1*. Activity was measured using the methods described by Sugden *et al*. (1999), with the AMARA peptide (Ala Met Ala Arg Ala Ala Ser Ala Ala Ala Leu Ala Arg Arg Arg) as substrate. The standard error of the differences (on the transform scale) was 0.2401 on 11 degrees of freedom.

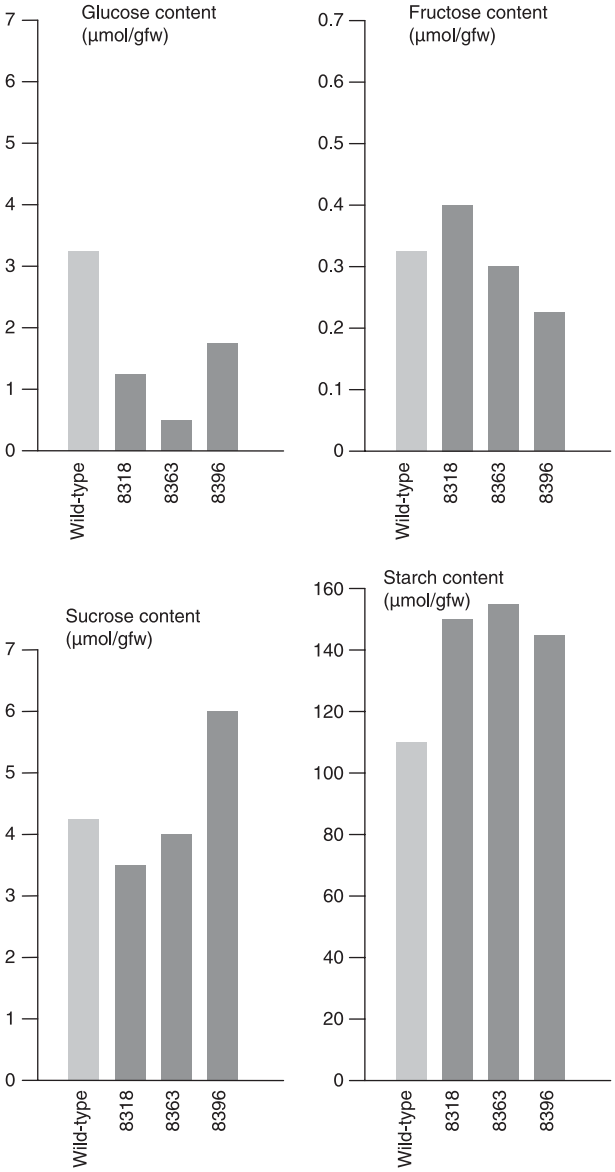

Figure 2 Mean glucose, fructose, sucrose and starch contents of wild-type potato (cv. Prairie) tubers and tubers from plants transformed with the sucrose non-fermenting-1-related protein kinase-1 gene (*SnRK1*) over-expression construct 02-SnRK1-nos. The standard error of the differences between means was 0.6406 for glucose, 0.1185 for fructose, 1.0246 for sucrose, each on 12 degrees of freedom, and 16.80 for starch, on 11 degrees of freedom. The starch content was determined by measuring the amount of glucose produced after degradation by α-amylase and amyloglucosidase (Sonnewald *et al*., 1991). gfw, gram fresh weight.

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 μmol/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 μmol/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, α-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 α-amylase (starch breakdown). Although the expression of genes encoding sucrose phosphate synthase and α-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).

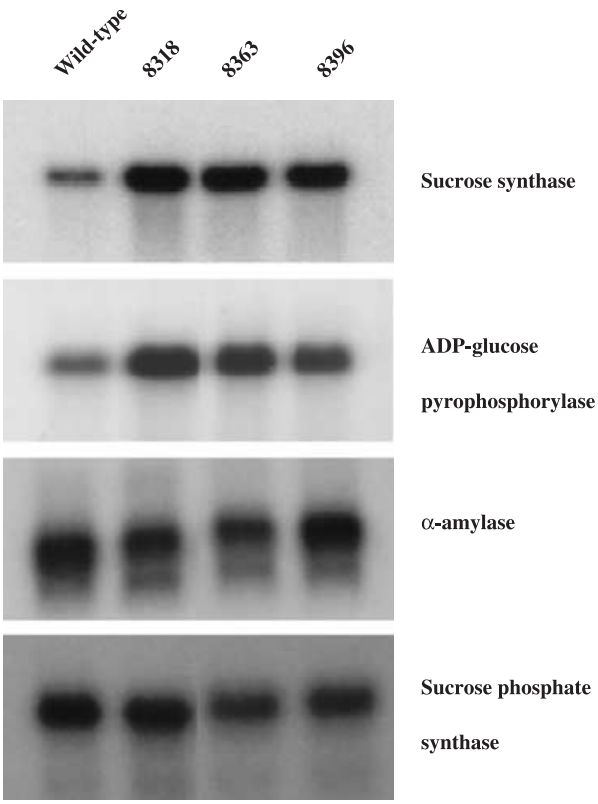

Figure 3 Northern analysis of total RNA (5 μg per lane) from tubers of wild-type potato (cv. Prairie) and three independent transgenic potato lines transformed with the sucrose non-fermenting-1-related protein kinase-1 gene (*SnRK1*) over-expression construct 02-SnRK1-nos. The RNA was probed with a polymerase chain reaction (PCR) product comprising part of the potato sucrose synthase gene *sus4-16* (accession number U24087), an *Eco*RI restriction fragment of the ADP-glucose pyrophosphorylase gene *AGP B* (accession number P23509), a DNA fragment comprising part of a potato sucrose phosphate synthase gene (accession number X73477) or a *Pst*I restriction fragment from the wheat α-amylase gene *α-Amy2* (Lazarus *et al*., 1985), as indicated. Equal loading in each lane was checked by ethidium bromide staining and comparison of the staining intensity of the ribosomal RNA bands (not shown).

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

The maximum catalytic activity (V_{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 $\log_e$ 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 $\log_e$ 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).

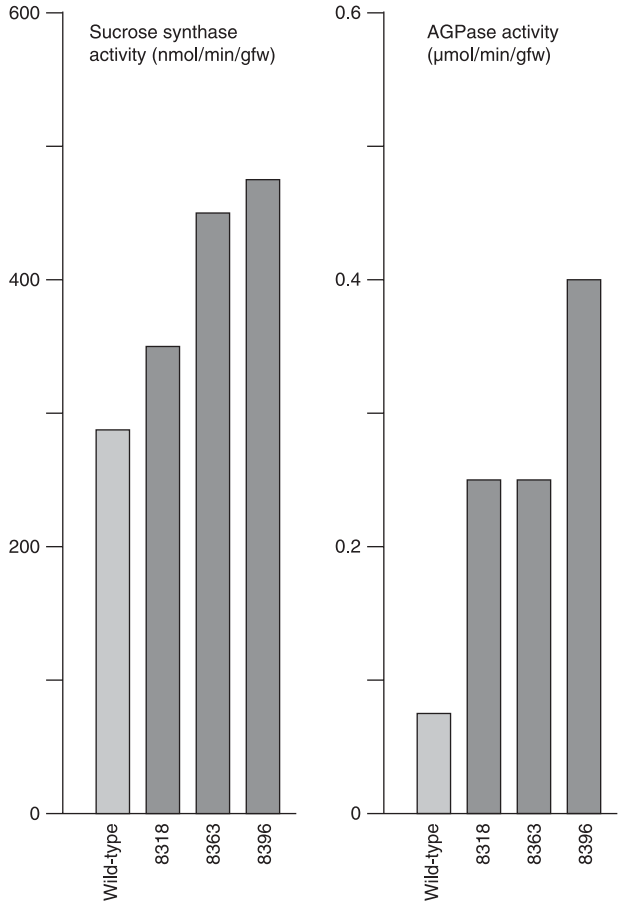

Figure 4 Mean sucrose synthase and ADP-glucose pyrophosphorylase (AGPase) activity (V_{max}) in wild-type potato (cv. Prairie) tubers and tubers from plants transformed with the sucrose non-fermenting-1-related protein kinase-1 gene (*SnRK1*) over-expression construct 02-SnRK1-nos. The standard error of the differences between the means was 12.33 for sucrose synthase and 0.495 (on the $\log_e$ scale) for ADP-glucose pyrophosphorylase, on 12 degrees of freedom. gfw, gram fresh weight.

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 μmol/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 μmol/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

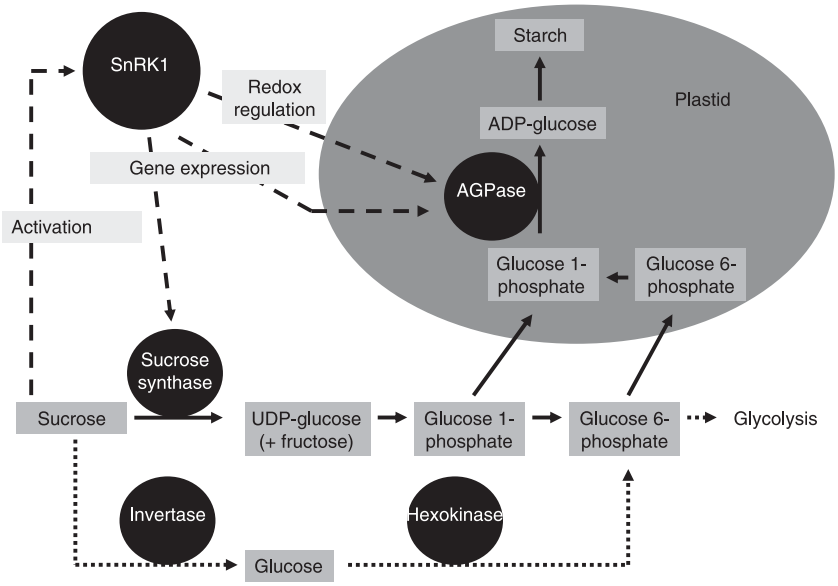

Figure 5 Diagram showing the regulation of the starch biosynthesis pathway by sucrose non-fermenting-1-related protein kinase-1 (SnRK1) in potato tubers. The pathway for carbon destined for conversion to starch is shown with solid arrows, whereas that of carbon destined for glycolysis is shown with dotted arrows. Regulatory interactions are shown with broken arrows. For a review of starch biosynthesis in potato tubers, see Fernie *et al*. (2002).

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 AMPKγ 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-cm^2 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-GTTGTCGTACAGCAG). 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-^{32}P]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 (M_r = 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-amylase probe was a 175-bp *Pst*I restriction fragment from the 3′ untranslated region of wheat α-*Amy2* (Lazarus *et al*., 1985), and the AGPase probe was a 1700-bp *Eco*RI 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 Arg Ala Ala Ser Ala 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 (NaPPi), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM benzamidine pH 8.2, 5 mM dithiothreitol (DTT), 25 mM β-mercaptoethanol, 1 μM pepstatin A, 1 mM phenylmethylsulphonylfluoride (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 5 min at 12 000 ***g*** at 4 °C. The supernatant (~1 mL) was retained and 0.224 vol of 60% (w/v) PEG 6000 was added with gentle mixing over a period of 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 NaPPi, 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 N_2 and stored at –80 °C.

The assay was carried out at 30 °C. A master mix of ATP.$MgCl_2$ containing 12.5 kBq [γ-^{33}P]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.$MgCl_2$ ± 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-ethanesulphonic acid (HEPES) pH 7.0, 5 mM $MgCl_2$, 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 N_2 and extracted rapidly with 1 mL of extraction buffer (50 mM HEPES-KOH (pH 7.8), 5 mM $MgCl_2$ 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 $MgCl_2$, 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/w) polyvinylpolypyrrolidone (PVPP), 1 mM PMSF], transferred to a cold microfuge tube 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 $MgCl_2$, 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, phosphoglucose isomerase and invertase (Jones *et al*., 1977). The method was adapted for use on microtitre plates. Frozen tuber tissue (300 mg) was ground in liquid N_2 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 $MgCl_2$, 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 phosphoglucose isomerase 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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