Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves

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Summary

This report describes the analysis of transgenic potato plants stably transformed with chimeric genes comprising either a patatin (tuber-specific) or a ST-LS1 (leaf- and stem-specific) gene promoter and a potato sucrose non-fermenting-1 (SNF1)-related protein kinase gene (PKIN1) sequence in the antisense orientation. Presence of the transgene was confirmed by Southern analysis. The transformants were screened using a peptide kinase activity assay for SNF1-related activity and/or Northern blot analysis, and two independent transgenic lines from each transformation, PAT 1.3, PAT 1.10, LS 1.4 and LS 1.11, were selected. Antisense PKIN1 transcripts were detectable in all four of the selected lines, and measurements made using the specific peptide phosphorylation assay showed that SNF1-related protein kinase activity had decreased in both PAT 1.3 and PAT 1.10 compared with controls. SNF1 regulates the expression of many genes encoding enzymes of carbohydrate metabolism in yeast. In order to investigate an analogous role for PKIN1, the activities of fructokinase, glucokinase, neutral and acid invertase and sucrose synthase in the tubers of PAT 1.3 and PAT 1.10 were compared with those in wild-type controls. Sucrose synthase activity was decreased to 36% of wild-type activity in tubers of PAT 1.10, and sucrose synthase transcript levels were decreased in tubers of both PAT 1.3 and PAT 1.10. Activities of the other enzymes were unaffected. Leaves of lines LS 1.4, LS 1.11 and controls were then excised and cultured on a medium containing 250 mM sucrose. This treatment induced sucrose synthase gene expression in the control leaves but not those of the transgenic lines. This finding is the first demonstration of a role for SNF1-related protein kinases in the regulation of carbohydrate metabolism of higher plants.

Introduction

Regulation of gene expression in response to sugar levels is an important mechanism for the control of carbohydrate metabolism in yeast (Saccharomyces cerevisiae). The most effective sugar for inducing a response is glucose, and many genes are repressed when glucose levels are adequate and derepressed when the yeast is deprived of glucose. Considerable progress has been made in the elucidation of the signal transduction pathway involved in this process (reviewed by Thevelein, 1994), mainly through the use of molecular genetic techniques, and the sucrose non-fermenting-1 (SNF1) protein kinase (Celenza and Carlson, 1986) has been shown to be an integral component of this pathway (Celenza and Carlson, 1989). This role in regulating expression of genes encoding enzymes of carbohydrate metabolism is not the only function of SNF1. It also regulates the activity of a number of metabolic enzymes, including acetyl-CoA carboxylase (ACC) (Woods et al., 1994) and glycogen synthase (Hardy et al., 1994), and is required for sporulation, heat shock response and normal cell cycle control (Thompson-Jaeger et al., 1991).

SNF1-related protein kinases have now also been identified in animals and plants (Hardie et al., 1994). The mammalian member of the family is adenosine monophosphate-activated protein kinase (AMPK) and a cDNA encoding its catalytic subunit has been cloned from rat (Carling et al., 1994). Plant members of the family include RKKIN1 from rye (Alderson et al., 1991), BKKIN2 and BKKIN12 from barley (Halford et al., 1992; Hannappel et al., 1995), PKIN1 from potato (Man et al., 1997), AKIN10 from Arabidopsis (Le Guen et al., 1992) and NPK5 from tobacco (Muranaka et al., 1994). AMPK plays an important role in the regulation of lipid metabolism, inactivating by phosphorylation both acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Clarke and Hardie, 1990; Sim and Hardie, 1988), and there is convincing immunological evidence that HMG-CoA reductase and nitrate reductase are substrates for the plant protein kinases (Ball et al., 1995; Barker et al., 1996; Douglas et al., 1997). A convenient assay for AMPK activity has been developed, using a
synthetic peptide (the SAMS peptide) based on the phosphorylation site of rat acetyl-CoA carboxylase (Davies et al., 1989), and this is also applicable to the plant SNF1-related protein kinases (Ball et al., 1994, 1995; Barker et al., 1996; MacKintosh et al., 1992; Man et al., 1997).

The aim of the study described here was to investigate the role of plant SNF1-related protein kinases in the transcriptional regulation of genes encoding enzymes of carbohydrate metabolism, a role analogous to one of the functions of SNF1 in yeast. Expression of the rye SNF1-related CDNA, RKIN1, in a yeast snf1 mutant restored SNF1 function to the extent that it could utilize non-fermentable carbon sources such as ethanol and glycerol, indicating that RKIN1 could substitute for SNF1 in the sugar sensing signalling pathway (Alderson et al., 1991). This suggested that a similar pathway could exist in plant cells. The role of SNF1 in controlling expression of the invertase gene, SUC2, has been demonstrated clearly in yeast (Neigeborn and Carlson, 1984) and an obvious starting point to look for a role in the regulation of gene expression in plants is enzymes that catalyse the conversion of sucrose to hexose and hexose phosphate, i.e. invertases, hexokinases and sucrose synthase.

Potato was chosen for the investigation because carbohydrate metabolism in the tuber has been studied extensively. We have shown previously that, although there is a small family of SNF1-related genes in potato, the individual members are almost identical (> 98% nucleotide sequence identity) (Man et al., 1997). Here we describe the molecular and biochemical analyses of transgenic potato plants expressing an antisense potato SNF1-related sequence under the control of tuber- and leaf/stem-specific promoters.

Results

Southern blot analysis of transgenic plants

Two chimeric genes were constructed and used to transform potato cv. Desiree (Halford et al., 1994). One consisted of 1.5 kb of the promoter of a patatin gene (Rocha-Sosa et al., 1989), an antisense 503 bp PCR product derived from the potato SNF1-related gene PKIN1 (Man et al., 1997), and an octopine synthase gene polyadenylation signal (Gielen et al., 1984). The other was identical except that the patatin promoter was replaced with 1.6 kb of the promoter of ST-LS1, the gene which encodes the 10 000 molecular mass protein of photosystem II. The patatin promoter is expressed only in the tubers, while the ST-LS1 promoter gives highest levels of expression in the leaves and is active to a lesser extent in stems (Eckes et al., 1985, 1986). A schematic diagram of the chimeric gene constructs is shown in Figure 1. Transgenic lines transformed with the construct containing the patatin promoter were given the prefix PAT, those with the construct containing the ST-LS1 promoter were given the prefix LS.

A Southern analysis was performed to confirm that the transformations had been successful. Genomic DNA from 10 PAT lines (PAT 1.1–PAT 1.10) and five LS plants (LS 1.4, 1.11, 1.12, 1.14 and 2.6) and a wild-type control was restricted with EcoRI to produce a fragment of ~ 2 kb comprising the promoter and the antisense PKIN1 fragment (Figure 1). Autoradiographs were made from the Southern blots after hybridization with a radiolabelled probe prepared from PKIN503, a PCR product derived from the potato SNF1-related gene, PKIN1 (Man et al., 1997), and the octopine synthase gene termination sequence (Gielen et al., 1984).

Figure 1. Schematic diagram of chimeric genes comprising a patatin (Rocha-Sosa et al., 1989) or ST-LS1 (Eckes et al., 1986) promoter, an antisense PCR fragment (PKIN503) derived from the potato SNF1-related gene, PKIN1 (Man et al., 1997), and the octopine synthase gene termination sequence (Gielen et al., 1984).

Figure 2. Southern blot analysis of EcoRI-cut genomic DNA extracted from PAT (a) and LS (b) transgenic potato lines and wild-type controls hybridized with a radiolabelled probe prepared from PKIN503, a PCR product derived from the potato SNF1-related gene, PKIN1 (Man et al., 1997). The positions of size markers are shown on the left of each panel and the transgenes are indicated with arrows.
the endogenous PKIN1 gene family. The DNA samples from the transgenic plants also contained a hybridizing fragment of the size expected for the transgene. Line PAT 1.10 contained an additional, faint hybridization signal that may have resulted from the presence of a disrupted fragment of the transgene.

**SAMS peptide kinase activity in the transgenic tubers and leaves**

The SAMS peptide kinase assay (Davies et al., 1989) uses a peptide substrate (His Met Arg Ser Ala Met Ser Gly Leu His Leu Val Lys Arg Arg), the sequence of which is derived from the phosphorylation site of rat acetyl-CoA carboxylase, one of the substrates of the mammalian SNF1 homologue, AMPK. The SAMS peptide is phosphorylated by partially purified SNF1-related protein kinases from cauliflower (Ball et al., 1995) and barley (Barker et al., 1996). Use of the assay to measure PKIN1 activity in potato has been described previously (Man et al., 1997) and preliminary measurements made in some putative transgenic plants transformed with the PAT construct had suggested that it would prove to be suitable for screening the transgenic lines (Halford et al., 1994).

A specific activity of 0.062 ± 0.003 nmol min⁻¹ mg⁻¹ protein (mean ± standard error of measurements on five separate plants) was measured in the tubers of untransformed plants in the present study. Activity in the tubers of transgenic plants grown under identical environmental conditions ranged from approximately the same as in the control tubers to 0.013 ± 0.005 nmol min⁻¹ mg⁻¹. Three lines, PAT 1.1, PAT 1.3 and PAT 1.10, were chosen for further analysis because they showed a range of activities (Figure 3). The activity in PAT 1.1 was not significantly different from the untransformed line, whereas the activity in PAT 1.3 and PAT 1.10 was decreased to 52% and 21% of wild-type, respectively.

The SAMS peptide kinase assay proved less useful in the analysis of the leaves of the LS lines. A previous study of SAMS peptide kinase activity in potato found that it was lower in leaves than in any other tissue, measuring it at 0.033 nmol min⁻¹ mg⁻¹ (Man et al., 1997). We were unable to show reproducibly that the activity in the leaves of any of the LS lines was significantly lower than this, and relied on Northern analyses to select lines for further study.

**Northern blot analysis of PKIN1 transcript levels in the transgenic plants**

The relative level of PKIN1 transcripts in control tubers and those of PAT 1.3 and PAT 1.10 was determined by Northern blot analysis of poly(A)+ RNA, using a probe synthesized from the PCR product PKIN503 (Man et al., 1997). The blots (Figure 4a) showed a single hybridization band in the control tuber lane, corresponding to the endogenous sense transcript. There was a very faint signal at the same...
Each value is the mean ± SE of measurements made on five tuber samples, each from a separate plant.

<table>
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<tr>
<th>Enzyme</th>
<th>Control</th>
<th>1.1</th>
<th>1.3</th>
<th>1.10</th>
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<tr>
<td>Fructokinase</td>
<td>0.218 ± 0.032</td>
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<td>Glucokinase</td>
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<td>Neutral invertase</td>
<td>0.0405 ± 0.0159</td>
<td>0.0502 ± 0.0172</td>
<td>0.0667 ± 0.0147</td>
<td>0.0511 ± 0.0168</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>Sucrose synthase</td>
<td>1.173 ± 0.153</td>
<td>1.029 ± 0.212</td>
<td>1.350 ± 0.239</td>
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Sucrose synthase gene expression in excised leaves of LS 1.4 and LS 1.11 after induction with 250 mM sucrose

Sucrose synthase transcripts can be induced to accumulate in potato leaves that have been detached and incubated with sucrose (Fu and Park, 1995). We performed an induction experiment with leaves of LS 1.4 and LS 1.11, using as controls LS 2.6 (the transgenic line in which PKin1 transcript levels had not been decreased significantly) and a wild-type plant. The detached leaves were incubated for 3 days on MS basal medium supplemented with 250 mM sucrose, then total RNA was prepared and a Northern analysis performed. In two separate experiments, sucrose synthase transcripts accumulated in the wild-type and LS 2.6 leaves in response to the treatment, but not in the LS 1.4 and LS 1.11 leaves (Figure 6a). Control blots of the same RNA samples hybridized with a potato HMG-CoA reductase sequence (Choi et al., 1992) showed that the difference in hybridization signal with the sucrose synthase probe could not have been caused by degradation of the RNA (Figure 5b), and a repeat experiment with a separately prepared RNA sample from PAT 1.10 confirmed that the result was reproducible (data not shown). The fact that a decrease in sucrose synthase transcript levels was observed in independent transgenic lines confirmed that it resulted from expression of the transgene and not from disruption of an endogenous gene.

Sucrose synthase gene expression in the transgenic tubers of PAT 1.3 and PAT 1.10

To investigate effects of the antisense gene expression on carbohydrate metabolism, the activities of enzymes that catalyse the conversion of sucrose to hexose and hexose phosphate, i.e. fructokinase, glucokinase, neutral and acid invertase and sucrose synthase, were measured in the tubers of PAT 1.1, PAT 1.3 and PAT 1.10 (Table 1). These enzymes were chosen because expression of the invertase gene, SUC2, is used as a marker for SNF1 activity in yeast. Assays were performed on five tubers from separate plants for each line. The activities of fructokinase, glucokinase and neutral invertase in the transgenic tubers were not significantly different from those of untransformed controls, and acid invertase was undetectable. However, sucrose synthase activity was only 36% of that in the controls in tubers of PAT 1.10. The difference in activity between PAT 1.10 and control tubers was significant (P < 0.01%, Student’s t-test). The number and size of tubers from the transgenic lines were not significantly different from those of controls, and preliminary results indicated that there were also no marked differences in the amounts of glucose, fructose and sucrose (data not shown).

Sucrose synthase gene expression in the transgenic tubers of PAT 1.3 and PAT 1.10

We investigated the cause of the decrease in sucrose synthase activity by Northern blot analysis of total RNA from tubers of the control and transgenic lines. Great care was taken to ensure that RNA was prepared from tubers at comparable stages of development and from the same part of each tuber (see the Experimental procedures), and that equal amounts of RNA had been loaded in each lane (this was checked by comparing ethidium bromide staining intensity of the 25S and 18S ribosomal bands after electrophoresis). The Northern blot was hybridized with a potato sucrose synthase PCR product amplified from potato tuber RNA (Zrenner et al., 1995). It showed that, whereas the level of sucrose synthase transcripts in RNA from PAT 1.1 tubers was comparable with that in wild-type, the level in RNA from PAT 1.3 and PAT 1.10 tubers was much lower (Figure 5a). A blot of the same RNA samples hybridized with a potato HMG-CoA reductase sequence (Choi et al., 1992) showed that the difference in hybridization signal with the sucrose synthase probe could not have been caused by degradation of the RNA (Figure 5b), and a repeat experiment with a separately prepared RNA sample from PAT 1.10 confirmed that the result was reproducible (data not shown). The fact that a decrease in sucrose synthase transcript levels was observed in independent transgenic lines confirmed that it resulted from expression of the transgene and not from disruption of an endogenous gene.
Figure 5. (a) Northern analysis of total RNA (10 µg) from mature tubers of a wild-type potato plant and transgenic plants PAT 1.1, PAT 1.3 and PAT 1.10, hybridized with a radiolabelled probe prepared from a potato sucrose synthase PCR product. (b) Control experiment showing the samples used in the blot shown in (a) hybridized with a potato HMG-CoA reductase probe (Choi et al., 1992). The relative amount of each sample was checked by comparing ethidium bromide staining intensity of the 25S and 18S ribosomal bands after electrophoresis, confirming that approximately equal amounts of RNA had been loaded (data not shown).

Discussion

We have described the analysis of transgenic potato lines expressing a portion of the potato SNF1-related gene, PKIN1, in the antisense orientation. Expression of the antisense transcript in tubers, confirmed by Northern analysis, resulted in a decrease of 48% and 79% in SAMS peptide kinase activity in two independent lines (PAT 1.3 and PAT 1.10, respectively), although activity was not affected in a third line (PAT 1.1). The fact that expression of the antisense PKIN1 sequence could result in such a large decrease in SAMS peptide kinase activity indicates that PKIN1 is responsible for most of this activity in potato tubers. In contrast, it was not possible to show reproducibly a significant reduction in SAMS peptide kinase activity in the leaves of plants from two independent transgenic lines (LS 1.4 and LS 1.11) that had been transformed with the antisense PKIN1 sequence under the control of a leaf-specific promoter. However, Northern analyses showed clearly that the antisense sequence was being expressed in these lines.

The reduction in SAMS peptide kinase activity in the tubers of lines PAT 1.3 and PAT 1.10 was associated with decreased sucrose synthase gene expression, to the extent that sucrose synthase transcripts were undetectable by Northern blot analysis. A significant decrease in sucrose synthase enzyme activity was also observed in line PAT 1.10. No marked differences in sugar and starch levels between control and transgenic tubers were observed, and the decrease in sucrose synthase activity in the transgenic tubers was relatively small compared with the reduction in sucrose synthase transcript levels. Tubers of PAT 1.3, in which sucrose synthase transcripts were present at much lower levels than in controls, showed normal sucrose synthase activity. However, this is consistent with results obtained in the study of potato tubers expressing an antisense sucrose synthase sequence (Zrenner et al., 1995), some of which retained ~ 40% of wild-type sucrose synthase activity despite transcript levels being greatly decreased.
reduced. Starch content and yield of these tubers was almost unaffected, although large changes in starch content were found in tubers in which sucrose synthase activity had been decreased further (Zrenner et al., 1995).

Sucrose synthase gene expression was also shown to be affected in excised leaves from lines LS 1.4 and LS 1.11. In accordance with a previous report (Fu and Park, 1995), treatment with 250 mM sucrose of excised leaves from a wild-type plant and from a LS line (LS 2.6) that was not expressing the antisense sequence to detectable levels induced sucrose synthase gene expression. However, this treatment failed to induce sucrose synthase gene expression in leaves of lines LS 1.4 and LS 1.11.

These results, from four independent transgenic lines, provide clear evidence that PKIN1 is involved in the control of sucrose synthase gene expression in potato. This identifies for the first time a role for SNF1-related protein kinases in the control of carbohydrate metabolism in plants through the transcriptional regulation of an important enzyme. This role is distinct from those defined previously in the control of HMG-CoA reductase and nitrate reductase activity, confirming that SNF1-related protein kinases are involved in the regulation of several aspects of carbon metabolism in plants, as they are in yeast and animals.

Sucrose synthase catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose. It is the most important sucrose cleavage enzyme in potato tubers (Morrell and ap Rees, 1986; Sung et al., 1989) and its activity is closely correlated with sink strength and starch accumulation (Zrenner et al., 1995). Its transcriptional regulation is therefore a mechanism by which sink strength and starch accumulation could be regulated. Recently, it has been reported that sucrose synthase activity in maize leaves may be regulated in part by direct phosphorylation of the enzyme (Huber et al., 1996). However, this is highly unlikely to be due to a SNF1-related protein kinase, since the phosphorylation is reported to be Ca$^{2+}$-dependent.

The sucrose synthase gene responsible for most of the activity in potato tubers (Sus4) is not expressed at high levels elsewhere in the plant under normal conditions, but it is inducible by wounding and anaerobiosis (Fu and Park, 1995; Salanoubat and Belliard, 1989). Sus4 is also the gene that is expressed in detached leaves in response to incubation with high concentrations of sucrose (Fu and Park, 1995). There is a second sucrose synthase gene in potato (Sus3), but it is expressed predominantly in stems and roots and is not induced by the sucrose treatment of excised leaves (Fu and Park, 1995).

It has been established that yeast SNF1 is activated in response to low glucose levels, although the mechanism by which cellular glucose levels are perceived and a signal is created is still not understood (Wilson et al., 1996). It is tempting to speculate that PKIN1 is involved in a signal transduction pathway that mediates the response of changing sugar levels on the expression of sucrose synthase, and possibly other enzymes of carbohydrate metabolism. However, as yet, plant SNF1-related protein kinase activity has not been shown to be regulated by glucose or other hexose or hexose phosphate levels.

There are glucose-repressible genes in plants (reviewed by Jang and Sheen, 1997) and it is notable that they include the genes which encode isocitrate lyase and malate synthase (Graham et al., 1994), since the genes encoding these enzymes in yeast are glucose-repressible and require SNF1 activity for their derepression. Sus4 is regarded as being sucrose-inducible rather than glucose-repressible. However, sucrose import into plant cells can have a significant effect on cellular hexose levels, depending on the relative activities of those sucrose-metabolizing enzymes present. This has been demonstrated clearly in developing cotyledons of Vicia faba (Weber et al., 1996). A seed coat-associated invertase cleaves sucrose as it arrives during the early stages of cotyledon development, keeping the cotyledonary hexose levels high and sucrose levels low. This activity disappears as the cotyledon cells begin to differentiate into storage tissue and the cellular hexose levels fall and sucrose levels rise. Intriguingly, this coincides with a steep rise in sucrose synthase activity and the onset of starch biosynthesis in the cotyledons.

**Experimental procedures**

**Plant material**

Wild-type and transgenic potato (Solanum tuberosum L., cv. Desiree) plants were grown in soil-based compost under greenhouse conditions with supplementary lighting as required and at a minimum temperature of 12°C. Tubers were harvested from plants 15 weeks after planting and used immediately. Plants of this age were rapidly growing and non-senescent. Material for Northern analyses and enzyme activity assays was prepared by taking sections (0.1–0.3 g fresh weight) from representative slices at the approximate middle of the potato tuber and discarding the outer 2–3 mm.

Sucrose induction experiments in detached leaves were performed as described by Fu and Park (1995), using a sucrose concentration of 250 mM.

**Southern analyses of transgenic plants**

Potato genomic DNA for Southern blotting was extracted as described by Chen and Dellaporta (1994) and 10 μg was used for each experiment. Prehybridization was carried out at 65°C in 3 × SSC, 1% (w/v) SDS, 1% (w/v) N4PO4, 0.1% denatured herring sperm DNA, and hybridization took place overnight under the same conditions with the inclusion of 10% (w/v) dextran sulphate and the radiolabelled probe. The membrane was washed twice at 65°C in 3 × SSC (0.45 M NaCl, 0.045 M sodium citrate), 1% (w/v) SDS, 1% (w/v) N4PO4, and once at 65°C in 1 × SSC, 1% (w/v) SDS, 1% (w/v) N4PO4, for 30 min.

Northern analyses

Total RNA was extracted from potato tissues by the method of Logemann et al. (1987). Polyadenylated [poly(A)+] RNA was isolated from tuber tissue as described by Man et al. (1997). Northern blotting of total RNA (10 µg) or poly(A)+ RNA (3 µg) was performed as described by Man et al. (1997) except that 20 × SSPE (3.6 M NaCl, 200 mM NaH2PO4, 20 mM EDTA, pH 7.4) was used as transfer buffer. Prehybridization was carried out at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt’s solution (Denhardt, 1966), 0.1% SDS, 100 mg ml–1 denatured herring sperm DNA for at least 1 h. Hybridization took place overnight in the same conditions after addition of the radiolabelled probe. The membrane was washed twice at 42°C for 15 min in 5 × SSPE and once at 42°C for 30 min in 1 × SSPE, 0.1% (w/v) SDS.

Synthesis of radiolabelled DNA probes

Radiolabelled probes were prepared by the random-primed extension method (Feinberg and Vogelstein, 1984) using the Prime-It II Random Primer Labelling Kit (Stratagene). The templates used for probe synthesis were the PCR product (PKIN503) of the potato SNF1-related gene, PKIN1 (Man et al., 1997), a Scal/Ncol fragment from the potato HMG-CoA reductase cDNA HMG 1 (Choi et al., 1992), kindly provided by Professor Richard Bostock, University of California, Davis, and a 2.44 kb potato sucrose synthase PCR product amplified from potato tuber RNA using the method described by Zrenner et al. (1995).

SAMS peptide kinase assays

Protein extraction, ammonium sulphate (40% saturation) precipitation and SAMS peptide kinase activity measurements were performed using the methods described by Man et al. (1997), the method for SAMS peptide kinase activity measurements being based on the standard assay described by Davies et al. (1989). Specific activity was expressed as nmol phosphate incorporated into peptide per minute per mg of protein.

Enzyme assays and measurements of soluble sugars and starch

For enzyme assays, tuber material was frozen in liquid nitrogen and ground to a fine powder in the presence of polyvinylpyrrolidone and then suspended in 1 ml of extraction buffer (50 mM MOPS, pH 7.4, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 10% ethandiol, 0.1% BSA, 0.5 µg ml–1 leupeptin, 0.7 µg ml–1 pepstatin A). The samples were vortexed to ensure complete mixing and then centrifuged at 10 000 g, 4°C for 5 min. The supernatant is referred to as the soluble extract. Sucrose synthase activity was assayed in the soluble extract as described by Dancer et al. (1990). Fructokinase, glucokinase and invertase assays were performed as described by Zrenner et al. (1995).

For measurement of soluble sugars and starch, tissue was frozen in liquid nitrogen immediately after harvest and extracted by grinding in 1 ml perchloric acid. Sugars were measured in neutralized extracts and starch in the washed sediment from the extract, according to Stitt et al. (1989).

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