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# Antisense Repression of Hexokinase 1 Leads to an Overaccumulation of Starch in Leaves of Transgenic Potato Plants But Not to Significant Changes in Tuber Carbohydrate Metabolism[1]

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**Potato (*Solanum tuberosum* L.) plants transformed with sense and antisense constructs of a cDNA encoding the potato hexokinase 1 (StHK1) exhibited altered enzyme activities and expression of StHK1 mRNA. Measurements of the maximum catalytic activity of hexokinase revealed a 22-fold variation in leaves (from 22% of the wild-type activity in antisense transformants to 485% activity in sense transformants) and a 7-fold variation in developing tubers (from 32% of the wild-type activity in antisense transformants to 222% activity in sense transformants). Despite the wide range of hexokinase activities, no change was found in the fresh weight yield, starch, sugar, or metabolite levels of transgenic tubers. However, there was a 3-fold increase in the starch content of leaves from the antisense transformants after the dark period. Starch accumulation at the end of the night period was correlated with a 2-fold increase of glucose and a decrease of sucrose content. These results provide strong support for the hypothesis that glucose is a primary product of transitory starch degradation and is the sugar that is exported to the cytosol at night to support sucrose biosynthesis.**

The route by which carbon is exported from the chloroplast to the cytosol to support Suc biosynthesis remains under critical discussion (Trethewey and Smith, 1999). The presence of a triose-P translocator in the envelope of chloroplasts and the role of this translocator in the support of Suc biosynthesis during the day has long been established (Stitt, 1990). However, there is increasing evidence that Glc, and not triose-P, is the predominant form in which carbon is exported from the chloroplast at night.

First, the presence of a Glc transporter in the chloroplast envelope has been demonstrated in spinach (Schäfer et al., 1977), Arabidopsis (Trethewey and ap Rees, 1994a, 1994b), and tobacco (Häusler et al., 1998). Second, a mutant line of Arabidopsis defective in chloroplast Glc transport is characterized by an excess-starch phenotype; the amount of transient starch in leaves at the end of the light period was around five times that found in wild-type leaves (TC265; Caspar et al., 1991; Trethewey and ap Rees, 1994b). It is conceivable that an inability to export Glc leads to a feedback inhibition of the pathway of starch degradation and the observed excess-starch phenotype. This hypothesis was supported by experiments with wild-type Arabidopsis leaves indicating that there is a net glycolytic flux at night, which would be an impossibility if the primary export of carbon was at the triose-P level. Third, Schleucher et al. (1998) have recently reported the use of NMR to distinguish between Glc synthesized from hexose export and that derived from triose-P; starch degradation was allowed to occur in the presence of $^2$H-enriched water, and the ratio of labeling in Glc at the different carbon atoms was used to determine the relative contribution of the two routes of export. These authors concluded that in tomato and bean leaves, more than 75% of the carbon exported from chloroplasts at night is in the form of hexose.

If the predominant route of carbon export from the chloroplast at night is at the level of Glc, then a cytosolic hexokinase (HK) (EC 2.7.1.1) is required to phosphorylate Glc to Glc-6-P, thus activating the hexose unit for Suc biosynthesis. In plants there have been several different reports about glucokinase (GLK) (Glc phosphorylating) or HK activities. The difference between GLK and HK is a functional classification: the former phosphorylates strictly Glc, while the latter is capable of phosphorylating a range of hexoses (e.g. Fru and Man). Glc-phosphorylating activities have thus far been reported in the following plant organs: tomato fruit, GLK (Martinez-Barajas and Randall, 1998); maize endosperm, GLK (Doehlert, 1989); maize roots, HK (Galina et al., 1995); maize leaves, HK (Schnarrenberger, 1990); castor bean endosperm, HK (Miernyk and Dennis, 1983); pea seeds, HK and GLK (Turner et al., 1977; Turner and Copeland, 1981); spinach leaves, HK (Baldus et al., 1981; Schnarrenberger, 1990); Arabidopsis leaves, HK (Jang et al., 1997); pea leaves, HK (Schnarrenberger, 1990); tobacco leaves, HK (Sindelar et al., 1998); soybean nodules, HK (Copeland and Morell, 1985); and avocado, HK (Copeland and Tanner, 1988). Glc-phosphorylating activities have been reported to be associated with the mitochondrial envelope in avocado (Copeland and Tanner, 1988), spinach (Schnarrenberger, 1990), and maize roots (Galina et al., 1995), and to be soluble in the cytosol of soybean nodules

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(Copeland and Morell, 1985) and spinach (Schnarrenberger, 1990). There has been no convincing report of HK/GLK activity located within the stroma of plastids.

The most thorough characterization of the kinetic properties of HK isoforms has been undertaken in potato (*Solanum tuberosum* L.) (Renz and Stitt, 1993; Renz et al., 1993). These authors reported the presence of three isoforms in potato tubers, all of which had a high affinity for Glc and Man but a significantly lower affinity for Fru. All three HK activities had affinities for ATP that were around 10-fold higher than for other nucleoside triphosphates. One of the HK activities (denoted HK1) was inhibited by Glc-6-P with a physiologically relevant $K_i$ of 4.1 mM; the inhibition was non-competitive with respect to Glc. The balance of the three activities was subject to change according to the developmental status of the tubers. Glc-phosphorylating activity was very low in growing tubers but increased during storage and sprouting, with HK1 activity becoming much more dominant in relation to the activity of HK2; HK3 was always negligible (Renz et al., 1993). No information was provided about the compartmentation of these three isoforms or their relative activities within leaf tissue.

HK has recently been implicated as a sensor in the sugar-dependent regulation of gene expression in plants (Koch, 1996; Jang and Sheen, 1997; Jang et al., 1997; Zhou et al., 1998; Smeekens, 1998). The phenomenon of carbon catabolite repression has been well described in microorganisms, especially in *Saccharomyces cerevisiae*, in which a large number of genes are regulated by HK in accordance with the Glc levels in the cell (Ma and Botstein, 1986; Rose et al., 1991; Ronne, 1995). In mammals, GLK plays a key role in Glc sensing by the insulin-secreting pancreatic $\beta$-cells. This has been shown by analysis of transgenic mice with altered GLK activity (Grupe et al., 1995) and by a high correlation between a form of type II diabetes and the occurrence of GLK mutations in humans (Bell et al., 1996).

In higher plants it is generally accepted that sugars regulate the expression of genes involved in many different processes, although whether mechanisms are at work that are similar to those found in microorganisms and mammals is subject to much debate (Koch, 1996; Smeekens, 1998). Jang et al. (1997) have cloned two HKs from Arabidopsis and performed different bioassays with transgenic Arabidopsis lines that overexpressed or contained reduced amounts of HK activity following antisense inhibition. Wild-type seedlings grown on 6% (w/v) Glc plates showed a phenotype characterized by suppression of hypocotyl and root elongation and by the greening and expansion of cotyledons. Transgenics that overexpressed HK showed hypersensitivity to 6% (w/v) Glc (stunted hypocotyls, cotyledons, and roots), while antisense transgenic lines were relatively hyposensitive to Glc. These authors concluded that the HK protein acts as a sensor for Glc levels and that the absence or increased abundance of this sensor led to the observed dramatic phenotypical changes through alterations in gene expression. However, no data were presented on the biochemical characterization of the Arabidopsis lines, leaving open the question of whether the phenotypical changes were an indirect consequence of an altered metabolic system.

In this paper we present a biochemical and physiological analysis of transgenic potato lines in which the activity of an isoform of HK has been modulated using either overexpression or antisense approaches. We chose to work with potato because it is a well-characterized system with a strong sink organ. In particular, we evaluated whether inhibition of HK in leaves would provide data to support the hypothesis that Glc is the primary form in which carbon is exported from the chloroplast at night.

## MATERIALS AND METHODS

### Plant Material

Potato (*Solanum tuberosum* L. cv Desirée) plants were supplied by Saatzucht Lange (Bad Schwartau, Germany). Plants were grown in the greenhouse at 15°C to 22°C under a 16-h light/8-h dark photoperiod (natural daylight was supplemented to give a minimum photon flux of 250 $\mu$mol $m^{-2}$ $s^{-1}$). Developing tubers (10–25 g fresh weight) were harvested from healthy, 2- to 3-month-old plants and used for the determination of enzyme activities, metabolic intermediates, starch, and sugars. Mature tubers were harvested from senescent plants and used for yield analysis. The analysis of carbohydrates in leaves was performed on 8-week old plants grown in the greenhouse from May through June (12 plants/line). For each measurement, three source leaf discs (1 cm in diameter) were harvested from individual plants at the following times: 4:30 AM, 10 AM, 2:30 PM, and 8 PM, immediately frozen in liquid nitrogen, and stored at −20°C prior to analysis.

### mRNA Extraction and Northern Analysis

Total RNA was isolated from 2 g fresh weight of tuber tissue, as described by Logemann et al. (1987). mRNA was extracted from 300 $\mu$g of total RNA using a mRNA purification kit (Dynabeads, Dynal, Germany), and separated on agarose-formaldehyde gels (approximately 4 $\mu$g per sample). Standard conditions were used for the transfer of RNA to membranes and for the subsequent hybridization (Sambrook et al., 1989). A potato ubiquitin cDNA probe was used as a control to standardize loading in each lane. Densitometric analysis was performed using a phosphor imager (BAS-1500, Fuji).

### Yeast Complementation Assays

A full-length cDNA encoding a HK from potato (StHK1; accession no. X94302) was isolated as an EST in the laboratory of Dr. Udo Schmitz (Institut für Genbiologische Forschung, Berlin) and kindly made available for this project. The full-size StHK1 was subcloned as a 1.7-kb *Eco*RI fragment into the p195XE (YEplac195 derivative) yeast expression vector. Two strains of *Saccharomyces cerevisiae* were used for complementation: DBY2219 (*hxk1*, *hxk2*; Ma and Botstein, 1986) and YSH7.4-3C (*glk1*, *hxk1*, *hxk2*; De Winde et al., 1996). The medium for the selection of the transformed colonies contained 0.67% (w/v) yeast nitrogen base (Difco, Detroit, MI) with amino acids and

$(NH_4)_2SO_4$, 2% (w/v) Bacto agar (Difco), and 2% (w/v) Fru (DBY2219 strain) or 2% (w/v) Glc (YSH7.4-3C strain). Crude extracts of the transformed YSH7.4-3C cultures were prepared at 4°C as follows: 100 mL of a fresh culture ($A_{595}$ between 0.7 and 0.9) were centrifuged at 4,000*g* for 10 min, washed with 10 mL of cold water, centrifuged at 4,000*g* for 10 min, and resuspended in 3.5 mL of extraction buffer (Trethewey et al., 1998, without BSA). Following lysis with a French press, the lysate was centrifuged at 12,000*g* for 10 min and the supernatant was desalted using PD-10 columns (Pharmacia). Aliquots of 100 μL were immediately frozen in liquid nitrogen and stored at −80°C until analysis. HK1 activity was assayed using the pyruvate kinase/lactate dehydrogenase test detailed by Renz and Stitt (1993), and the phosphorylation coefficient was also calculated as described by these authors.

### Preparation and Selection of Transgenic Lines

The full-length 1.7-kb StHK1 EST was initially subcloned into the plasmid pSK. For the overexpression construct a *Bam*HI/*Sal*I fragment was introduced in the sense orientation into the vector pBinAR-Kan (Liu et al., 1990) between the CaMV 35S promoter and the *ocs* terminator. For the antisense construction, a 1.3-kb/*Hin*dIII fragment of the StHK1 cDNA was subcloned into pSK in the reverse orientation. An *Asp*718/*Sal*I fragment was then introduced into the vector pBinAR-Kan between the CaMV 35S promoter and the *ocs* terminator (Liu et al., 1990). Both constructs were introduced into potato by *Agrobacterium tumefaciens*-mediated transformation (Rocha-Sosa et al., 1989), and transgenic plantlets were selected on kanamycin-containing medium (Dietze et al., 1995). Initial screening of around 100 lines was performed by determining HK activity in the leaves of plants grown in 6-cm pots in a phytotron. A second activity screen was then performed with six plants per line for seven preselected lines grown in the greenhouse.

### HK Purification from Potato Tubers

The purification procedure was based on that described by Renz et al. (1993). Developing tubers were harvested from non-senescent plants grown in the greenhouse during the spring season and stored at 4°C prior to use. Peeled and sliced potato tuber tissue (approximately 7 g) was homogenized in a blender (Waring) for 1 min in 30 mL of extraction buffer (50 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 mM ϵ-aminocaproic acid, 0.5 mM PMSF, and 2% [w/v] polyvinylpolypirrolidone). The homogenate was filtered, centrifuged, refiltered, and then applied to a DE-52 cellulose column (18 cm long, 1.6 cm in diameter). The column was washed with buffer (50 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$, and 1 mM DTT) and eluted in a linear gradient of 0 to 0.4 M KCl at a flow rate of 1.5 mL $min^{-1}$, and 60 1-mL fractions were collected. Twenty-five microliters of each fraction was checked for HK activity as described below. Two different groups of fractions, corresponding to HK1 and HK2 activities, were further purified by affinity chromatography. HK1 fractions were desalted by passage through PD-10 columns (Pharmacia) prior to loading onto an affinity chromatography column (Matrex Blue A, Amicon, Beverly, MA). HK2 was collected from the flow-through of the chromatography column and concentrated with a Centricon 10 membrane (Amicon). Purified samples of HK1 and HK2 were used to determine the dependence of activity on pH.

### Biochemical Analysis

Metabolites, starch, and sugars were determined as described by Trethewey et al. (1998). The recoveries of metabolites in the TCA extracts were found to be: Glc-6-P, 108% ± 10%; Fru-6-P, 117% ± 10%; Glc-1-P, 119% ± 11%; and 3-phosphoglycerate, 95% ± 7% (mean ± SE; $n = 6$). HK activity was assayed as described by Renz et al. (1993) with the following modifications: NAD was used instead of NADP, the Glc-6-P dehydrogenase was from *Leuconostoc mesenteroides*, the increase in absorbance was measured at 340 nm, and the final volume of the reaction was 300 μL.

### Statistical Analysis

The word "significant" is used in the text only when the change in question has been confirmed to be statistically significant ($P < 0.05$) with the *t* test incorporated into Microsoft Excel 7.0.

## RESULTS

### Molecular Characterization of StHK1

A full-length cDNA encoding StHK1 was isolated as an EST in the group of Dr. Udo Schmitz and kindly made available for this project (accession no. X94302). The cDNA encodes a protein of some 498 amino acids; no evidence was found of an N-terminal targeting peptide. The sequence of StHK1 shows a high similarity to the sequences already reported from Arabidopsis (Jang et al., 1997): 71% nucleotide identity to AtHXK1 and 70% identity to AtHXK2. At the amino acid level, the identity with AtHXK1 and AtHXK2 was 69% and 67%, respectively, while the computed similarities were 81% and 79% using the BESTFIT algorithm incorporated into the Genetics Computer Group package, version 8.0. Comparison with HKs from yeast revealed 35% identity and 55% similarity with both HXK1 and HXK2. Conserved binding domains for sugar and ATP (two regions for phosphate, one for adenosine) could be identified in the amino acid sequence of StHK1 (Bork et al., 1993). Analysis of mRNA northern blots using the StHK1 cDNA as a probe revealed a band of around 1.7 kb. The transcript was present in young and mature leaves, stems, roots, stolons, and developing and mature tubers (data not shown).

### StHK1 Can Complement Two Yeast Deficient Mutants and Phosphorylate Both Glc and Fru

To determine whether the protein product of the potato StHK1 cDNA is enzymatically active, we performed a func-

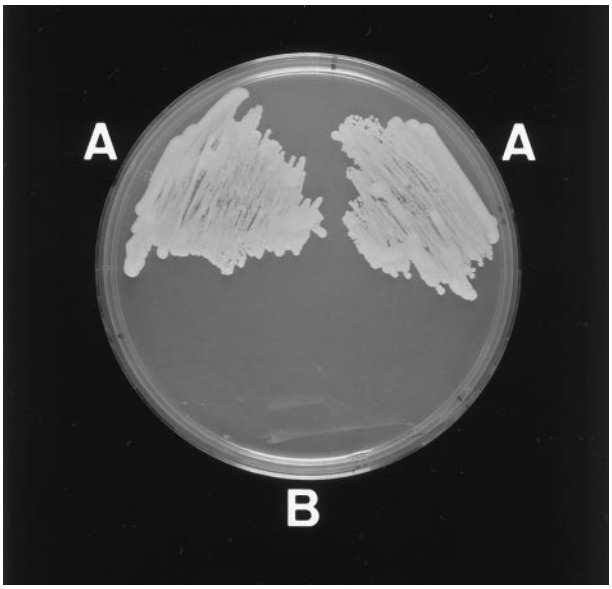


**Figure 1.** Complementation of the yeast YSH7.4-3C *hxk1/hxk2/glk1* triple mutant. Potato HK1 cDNA provided catalytic activity to support the growth of the yeast mutant on the Glc plate. A, Two independent transformants; B, culture transformed with the empty plasmid p195XE.

tional complementation of two different yeast strains: DBY2219 (lacking HK activity) and YSH7.4-3C (lacking HK and GLK activity). Transformed yeast cells were able to grow on a selective medium containing Fru (DBY2219) or Glc (YSH7.4-3C) as the only carbon source. The vector p195XE alone was not able to complement the mutants (Fig. 1). The kinetic properties of HK1 were analyzed in extracts of complemented YSH7.4-3C cultures (Table I). HK1 showed a high affinity for Glc and Man ($K_m$ values were 33 and 29 $\mu$M, respectively). The $K_m$ for Fru was 50 times higher than that for Glc and Man. The $V_{max}$ was found to be higher for Glc and Fru than for Man. The estimated values for the phosphorylation coefficient showed a high selectivity of HK1 for Glc and Man compared with Fru. The $K_m$ and $V_{max}$ of HK1 with respect to ATP was detected to be 103 $\mu$M and 360 nmol $min^{-1}$ $mg^{-1}$ protein, respectively. No HK activity was found in the original yeast strain YSH7.4-3C or following expression of the empty p195XE vector.

**Table I.** *Kinetic constants of the StHK1 (expressed in the YSH7.4-3C yeast strain) for Glc, Fru, and Man*

The $K_m$ and $V_{max}$ values were calculated from Eadie-Hofstee plots. Each value is the mean of three independent assays (SE was 2%–16% of the mean).

| Substrate | $K_m$ | $V_{max}$ | Phosphorylation Coefficient |
|---|---|---|---|
| | *mM* | *nmol $min^{-1}$ $mg^{-1}$ protein* | |
| Glc | 0.033 | 349 | 1.000 |
| Fru | 1.470 | 523 | 0.033 |
| Man | 0.029 | 221 | 0.710 |

**Table II.** *Hexokinase activity in antisense (αHK1) and overexpression (PHK1) transgenic lines*

Plants were grown in the greenhouse in the autumn/winter season in 3.5-L pots. Leaf samples were taken from 8-week-old plants, tuber samples from 12-week-old plants. Data are presented as the means ± SE; $n = 6$.

| Line | HK Activity | |
|---|---|---|
| | Leaf | Tuber |
| | *nmol $min^{-1}$ $g^{-1}$ fresh wt* | |
| Antisense (αHK1) | | |
| Wild type | 61 ± 2 | 101 ± 4 |
| 8 | 25 ± 3 | 33 ± 1 |
| 9 | 29 ± 1 | 42 ± 2 |
| 11 | 19 ± 3 | 49 ± 6 |
| 13 | 13 ± 1 | 44 ± 7 |
| 48 | 26 ± 1 | 36 ± 5 |
| 60 | 23 ± 1 | 47 ± 5 |
| 82 | 19 ± 1 | 33 ± 4 |
| Overexpression (PHK1) | | |
| Wild type | 72 ± 3 | 106 ± 5 |
| 5 | 214 ± 14 | 166 ± 4 |
| 22 | 267 ± 22 | 154 ± 4 |
| 32 | 151 ± 11 | 130 ± 6 |
| 37 | 300 ± 37 | 174 ± 5 |
| 70 | 351 ± 26 | 188 ± 9 |
| 89 | 143 ± 9 | 137 ± 4 |
| 95 | 265 ± 15 | 222 ± 11 |

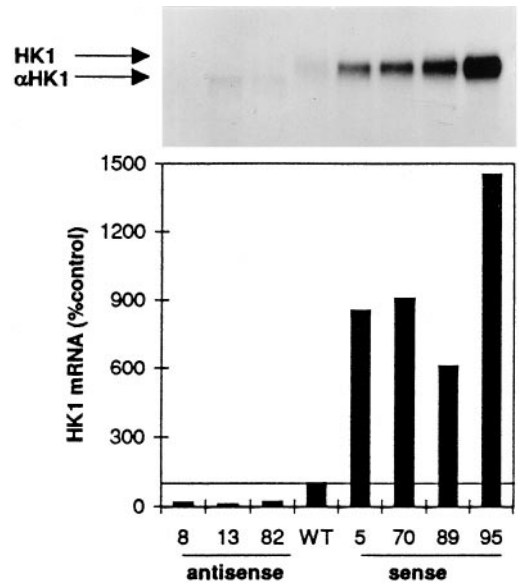


**Figure 2.** Northern-blot analysis of transgenic plants with altered expression of StHK1. mRNA was extracted from developing tubers of greenhouse-grown plants. The filter was hybridized with a 1.3-kb/*Hin*dIII cDNA fragment derived from the StHK1 cDNA. The histogram shows the ratio of StHK1 mRNA to potato ubiquitin mRNA (used as a control) in each transformed line (antisense lines 8, 13, and 82 and sense lines 5, 70, 89, and 95). WT, Wild type.

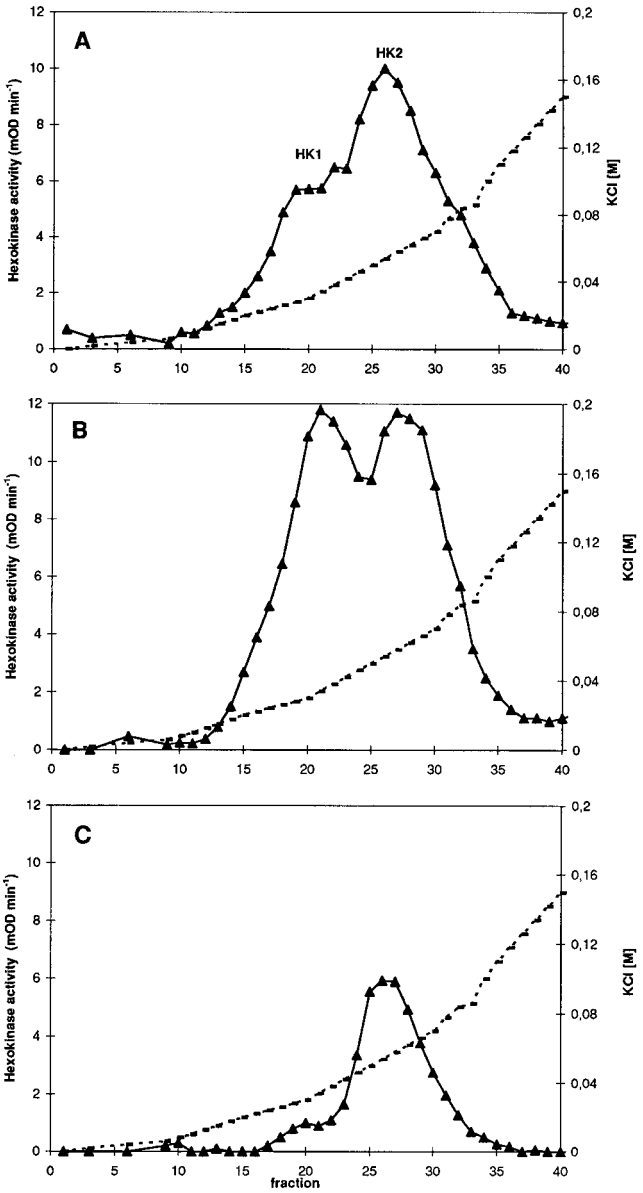


**Figure 3.** Separation of HK activities from developing potato tubers of the wild type (A), and the PHK1-70 (B) and αHK1-13 (C) lines after elution from a cellulose column with a KCl gradient. ▲, HK activity; dashed lines, KCl concentration.

## Overexpression and Antisense Transgenic Potato Lines Showed a Broad Range of HK Activities

Antisense (αHK1) and overexpression (PHK1) transgenic potato plants were selected following screening at the level of HK activity. Three lines of αHK1 were found (8, 13, and 82), all of which showed a reduction in activity compared with wild type of up to 78% in source leaves and 76% in developing tubers (Table II). Four lines of PHK1 were selected (5, 70, 89, and 95), all of which demonstrated up to a 4-fold increase in HK activity in leaves and up to a 2-fold increase in tubers (Table II). In the wild-type plants, HK activity was always higher in tubers than in leaves; in the PHK1 lines this relationship was inverted. Northern analysis of mRNA preparations from the selected lines corroborated the enzyme activity data (Fig. 2). Densitometric analysis of the blots indicated that there was up to a 10-fold reduction in transcript levels in the αHK1 lines and up to a 15-fold increase in the PHK1 lines.

## StHK1 cDNA Corresponds to the Partially Purified HK1 Described Previously

To determine whether the StHK1 cDNA corresponded to any of the three HK activities previously described by Renz et al. (1993), we chose an antisense (αHK1-13) and an overexpression line (PHK1-70) for a partial purification experiment using the same method described by Renz et al. (1993). Separation of wild-type tuber extracts after elution from the DE-52 cellulose column showed a pattern similar to that described by Renz et al. (1993). Two different activities (named HK1 and HK2 by Renz et al., 1993) could be separated (Fig. 3). The PHK1-70 sample showed a large increase in the first peak; in this line both HK peaks were of similar size (Fig. 3B). In the antisense line αHK1-13, the first peak almost disappeared (Fig. 3C).

One of the differences between HK1 and HK2 is the dependence of their activities on pH (Renz and Stitt, 1993). Therefore, we performed a pH experiment with completely separated HK1 and HK2 preparations from the wild type and from both transgenics. We used an affinity chromatog-

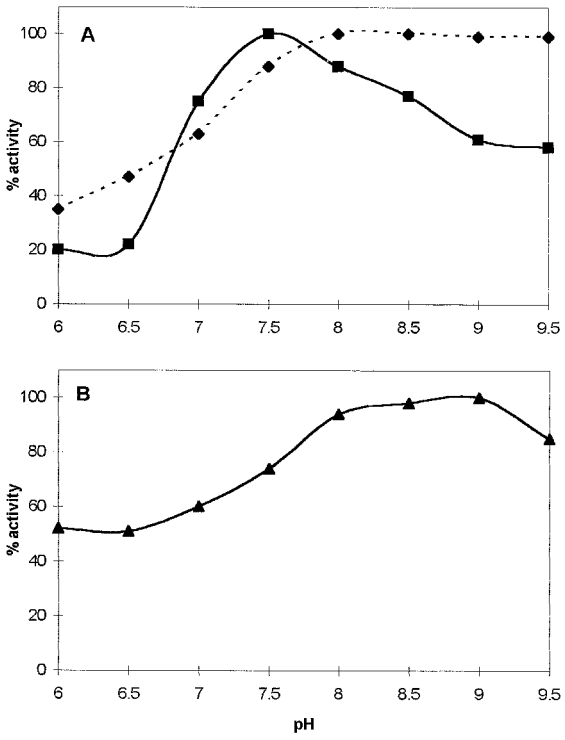


**Figure 4.** Dependence on pH of HK1 and HK2 activities purified from tubers of the HK1-70 line (A) and HK1 activity following expression of the StHK1 cDNA in the YSH7.4-3C yeast strain (B). The assays were carried out with 50 mM MES-KOH (pH 5.0–6.5), 50 mM imidazole (pH 6.0–7.4), or 50 mM Tris-HCl (pH 7.0–9.5). ■, HK1; ◆, HK2; ▲, HK1 (yeast).

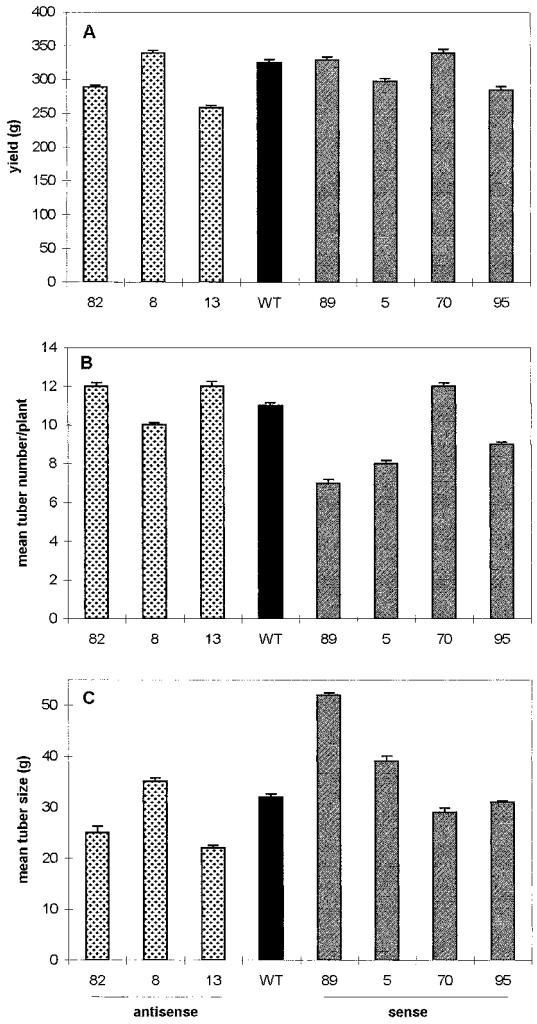


**Figure 5.** Yield (A), average tuber number (B), and average tuber size (C) of transgenic lines altered in HK activity (antisense lines 82, 8, and 13 and sense lines 89, 5, 70, and 95) and the wild type (WT). For each line, 15 plants were grown in 3.5-L pots in the greenhouse during the spring season. Mature tubers were harvested from senescent plants. Data are presented as the means ± SE.

raphy column to obtain full separation of the two isoforms, and HK1 bound to the column, while HK2 passed through. This step also served as a control to ensure that the HK1 and HK2 enzyme activities assigned here correspond to the HK1 and HK2 previously named by Renz et al. (1993). HK2 had a broad pH response, with only a 20% decrease in activity at pH 9.6 in relation to pH 7.5. HK1 activity, however, decreased quite strongly in the same pH range (up to 60%) (Fig. 4A). Similar patterns were observed with extracts derived from wild-type and transgenic plants. Our results were in full agreement with the previous observations from Renz and Stitt (1993).

In addition, we performed a partial purification of the HK activity produced following expression of the StHK1 cDNA in a yeast strain deficient in Glc-phosphorylating activities (YSH7.4-3C). The crude extract was desalted and loaded onto an affinity chromatography column as previously described. As expected, the HK activity bound to the column and enzyme activity was exclusively present in the eluate, but was not found in the flow-through (data not shown). However, we were surprised to observe that the dependence of this HK activity on pH was strikingly different from that of HK1 from plant extracts. The HK1 activity extracted from yeast showed a broader range of maximal activity and relatively high activity at acidic pH (Fig. 4B). These differences could be explained by post-translational processing of HK1 in yeast that is different from that which occurs in potato tubers.

### Tuber Yield from the Transgenic Lines

We determined the yield of tubers from the antisense and overexpression lines (Fig. 5A). Because yield trials are subjected to considerable variation between individuals for each line, we grew 15 plants in large 20-cm pots, and all plants were transferred simultaneously from tissue culture to the greenhouse. No significant differences were found in the tuber fresh weight per plant of the overexpression lines. In the antisense lines, only line 13 showed a significant yield reduction (21%) relative to the wild type. There were no significant changes in the mean tuber number per plant in the antisense lines, whereas three of the four sense lines showed a reduction (Fig. 5B).

**Table III.** *Carbohydrate contents of antisense transgenic tubers (αHK1)*

The same set of antisense plants described in Table II were also used for starch and sugar measurements.

| Line | Starch | Glc | Fru | Suc |
|---|---|---|---|---|
| | *μmol Glc equivalents $g^{-1}$ fresh wt* | *μmol $g^{-1}$ fresh wt* | | |
| Wild type | 718 ± 48 | 5.2 ± 2.7 | 1.2 ± 0.1 | 8.3 ± 1.1 |
| 13 | 761 ± 81 | 13.3 ± 3.8 | 0.7 ± 0.2 | 11.5 ± 2.3 |
| 8 | 712 ± 99 | 6.3 ± 3.2 | 1.1 ± 0.2 | 8.7 ± 1.0 |
| 82 | 755 ± 23 | 0.7 ± 0.2 | 0.9 ± 0.2 | 8.6 ± 0.6 |

**Table IV.** *Carbohydrate contents of overexpression transgenic tubers (PHK1)*

The same set of overexpression plants described in Table II were also used for starch and sugar measurements.

| Line | Starch | Glc | Fru | Suc |
|---|---|---|---|---|
| | *μmol Glc equivalents $g^{-1}$ fresh wt* | *μmol $g^{-1}$ fresh wt* | | |
| Wild type | 884 ± 33 | 1.5 ± 1.1 | 1.1 ± 0.2 | 28.8 ± 2.4 |
| 89 | 935 ± 34 | 0.6 ± 0.2 | 0.8 ± 0.1 | 41.1 ± 1.4 |
| 5 | 819 ± 20 | 0.5 ± 0.2 | 1.2 ± 0.2 | 26.3 ± 5.0 |
| 70 | 861 ± 21 | 0.2 ± 0.1 | 0.7 ± 0.0 | 29.2 ± 6.7 |
| 95 | 874 ± 28 | 0.7 ± 0.3 | 0.8 ± 0.1 | 22.3 ± 3.5 |

### Transgenic Tubers Did Not Show Changes in Starch and Sugar Contents

The sugar and starch contents of developing tubers are shown in Tables III and IV. The analysis was performed on material from the same set of plants for which the HK activities have been presented in Table II. No significant differences were found in the antisense lines, except for the increase in Glc observed in line 13 (Table III). However, given the large variation in all of the Glc samples measured (a general phenomenon in tuber metabolism) no particular significance was attached to this. The overexpression lines also showed no clear differences; one of the lines (PHK1-89) contained elevated Suc, and there was a tendency for a reduction in the Glc levels, although high variability was again observed (Table IV).

### Glycolytic Metabolites in the Transgenic Tubers Were Essentially Unchanged

The measurements of glycolytic intermediates presented in Tables V and VI were confirmed by recovery experiments and low SEs. The wild-type values were comparable to those previously reported for potato tubers (Burrell et al., 1994; Geigenberger et al., 1998; Trethewey et al., 1998). In the antisense lines there was a tendential decrease of Glc-6-P and Glc-1-P, but these differences were not significant. Hexose-P levels were unchanged in the overexpression lines. In 3-phosphoglycerate there were no significant changes except in line PHK1-70; the tendency was for a lower 3-phosphoglycerate content in the overexpression lines. The PPi content of the antisense lines was also not significantly changed compared with the wild type.

### αHK1 Plants Accumulated Transitory Starch in Leaves after the Dark Period

In preliminary experiments, we observed a significant increase of starch in leaves of the antisense plants, but no changes in the overexpression plants. We therefore performed a time-course experiment with three antisense lines (12 plants per line) in the greenhouse: we harvested samples from source leaves at four time points during the course of the day (Fig. 6). Plants from the different lines and the controls were placed randomly in the greenhouse to neutralize the consequences of any position effects. At the first time point (at dawn), the antisense plants showed a 2.1- to 3.2-fold increase in starch relative to the wild type (Fig. 6A). The rate of starch accumulation during the morning in wild-type and transgenic plants was similar: The difference between the first and second time points was 73 μmol $g^{-1}$ fresh weight for the wild type and between 57 and 90 73 μmol $g^{-1}$ fresh weight in the αHK1 transgenics. The situation changed in the evening (between the third and fourth time points), with an increase of 78 μmol $g^{-1}$ fresh weight in the wild type, but a significantly reduced accumulation in the αHK1 transgenics (between 16 and 49 μmol $g^{-1}$ fresh weight). At the end of the light period, the amount of starch present in the source leaves of the αHK1 transgenics was only moderately higher (around 25%) than in the wild type.

The higher starch content in the transgenics at dawn was correlated with a significant increase of 1.6- to 2.1-fold in Glc in the αHK1 transgenic lines relative to the wild type (Fig. 6B). Glc levels equilibrated during the course of the day, and no significant differences between wild type and transgenics were observed at the end of the light period.

**Table V.** *Metabolite contents of tubers from selected antisense lines (αHK1)*

Metabolites were determined in the same samples used for carbohydrate analysis presented in Table III. The data are presented as the means ± SE of measurements on six individual plants per line.

| Metabolite | Line | | | |
|---|---|---|---|---|
| | Wild type | 13 | 8 | 82 |
| | *nmol $g^{-1}$ fresh wt* | | | |
| Glc-6-P | 166 ± 26 | 140 ± 8 | 138 ± 8 | 141 ± 8 |
| Glc-1-P | 11 ± 2 | 12 ± 0 | 13 ± 0 | 13 ± 1 |
| Fru-6-P | 42 ± 8 | 36 ± 2 | 36 ± 3 | 36 ± 2 |
| 3-Phosphoglycerate | 79 ± 5 | 72 ± 5 | 68 ± 3 | 70 ± 2 |
| PPi | 1.2 ± 0.3 | 1.4 ± 0.4 | 1.1 ± 0.2 | 0.7 ± 0.0 |

**Table VI.** *Metabolite contents of tubers from selected overexpression lines (PHK1)*

Metabolites were determined in the same samples used for carbohydrate analysis presented in Table IV. The data are presented as the means ± SE of measurements on six individual plants per line.

| Metabolite | Line | | | | |
|---|---|---|---|---|---|
| | Wild type | 89 | 5 | 70 | 95 |
| | *nmol $g^{-1}$ fresh wt* | | | | |
| Glc-6-P | 113 ± 11 | 97 ± 5 | 107 ± 10 | 120 ± 10 | 108 ± 10 |
| Glc-1-P | 9 ± 1 | 9 ± 1 | 10 ± 1 | 12 ± 1 | 9 ± 1 |
| Fru-6-P | 29 ± 3 | 24 ± 2 | 27 ± 2 | 30 ± 3 | 27 ± 3 |
| 3-Phosphoglycerate | 69 ± 5 | 81 ± 5 | 77 ± 6 | 90 ± 4 | 68 ± 3 |

Moreover, the increased starch level at dawn in the transgenics correlated with reduced Suc levels at the start of the day in two of the three αHK1 transgenics (Fig. 6C). Interestingly, at the time points 10 AM and 2:30 PM, source leaves of the transgenics had tendentially higher Suc content than the wild type (significant for line 13 at 2:30 PM).

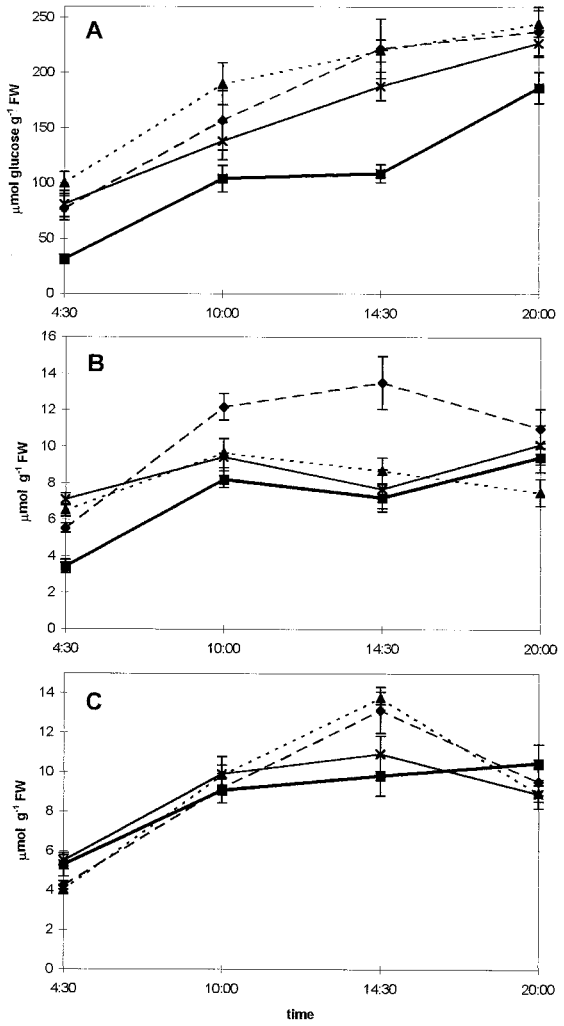


**Figure 6.** Diurnal changes in leaf starch (A), Glc (B), and Suc (C) in wild-type (■) and in the αHK1 lines 8 (◆), 13 (▲), and 82 (×). At each time point, samples were taken from mature source leaves and the data presented represent the means ± SE of measurements on 12 plants per line.

There were no differences in Fru levels between the wild type and the transgenics (data not shown).

## DISCUSSION

Antisense inhibition of HK in potato leaves leads to an excess-starch phenotype. We report a 30% increase in leaf starch at the end of the light period and a 3-fold increase in starch accumulation at the end of the night period. This is exactly what would be expected if Glc were the predominant route by which carbon is exported from chloroplasts at night. The phenotype of the antisense potato plants was comparable to that of the TC265 Arabidopsis mutant line. TC265 is inhibited in the export of Glc from the chloroplasts, and this deficiency leads to a 5-fold accumulation of starch in leaves (Trethewey and ap Rees, 1994a, 1994b). These two mutant/transgenic lines are defective at adjacent steps in the proposed pathway of Suc biosynthesis at night and together provide convincing evidence that export of Glc is the predominant route by which carbon leaves the chloroplast at night. The fact that both lines have an excess-starch phenotype at the end of the night strongly indicates that there are feedback mechanisms that restrict the degradation of transitory starch.

However, the data also indicate that in both the potato antisense lines and the Arabidopsis mutant, significant turnover of starch still occurs. In both cases, it is impossible at the level of experimental observation to determine if the amount of turnover is identical to the wild type or if there is a modest reduction. We favor the latter proposal, because a small reduction in turnover of starch could lead to the observed large accumulation in leaf starch over the lifetime of the plant. Significant turnover of starch may still be possible in the antisense lines because inhibition of the HK activity is incomplete; the most severe lines had an 78% reduction in activity in the leaves. The occurrence of degradation in the mutant Arabidopsis line indicates that there is some export of carbon via the triose-P translocator at night (Trethewey and ap Rees, 1994a, 1994b).

The uncertainties in the measurement of starch are of the same order of magnitude as the measured levels of soluble sugars in leaves; therefore, the results of the Suc and Glc determinations must be evaluated independently of whether starch turnover is altered in the transgenic potato lines. Measurements of sugar levels in the leaves of transgenic potato plants indicated that Glc is significantly elevated in all three lines at the end of the night period, and

in two of the three lines for one-half of the day period. This is a clear indication that there is a buildup of Glc following starch degradation in the leaves. We propose that the residual HK activity is not strong enough to bring all of this Glc rapidly into metabolism. The accumulation of Glc is a further indication of the involvement of HK in the pathway of interconversion of starch to Suc at night.

The argument that the αHK1 potato transgenics are a phenocopy of the Arabidopsis TC265 lines requires that HK be located in the cytosol. We found no evidence of a chloroplast-targeting peptide in the sequence of the StHK1 cDNA, and there was no lengthy N-terminal extension found in similarity comparisons with the HKs from yeast. We also performed chloroplast import experiments with the in vitro translation product of the StHK1 cDNA but found no evidence of any processing of the translation product (controls were performed; data not shown). We therefore conclude that HK1 is located outside of the chloroplast.

Several experiments with transgenic lines have demonstrated a considerable flexibility in the overall day/night rhythms of carbohydrate allocation and export in potato. For example, in potato plants in which the triose-P translocator was inhibited through antisense repression, a 3-fold increase in starch in the leaves was observed during the light period (Riesmeier et al., 1993) and there was a subsequent elevated rate of export of carbohydrate from the leaves during the night period (Heineke et al., 1994; Häusler et al., 1998). Conversely, leaf-specific inhibition of ADP-Glc pyrophosphorylase in potato led to a 60% reduction in the transitory starch content of leaves (Leidreiter et al., 1995); there was a compensating higher mobilization of photoassimilates from the leaf during the day. Thus, there is a considerable potential for flexibility in the timing and partitioning of carbohydrate allocation. Based upon the previous results with other mutants and transgenics, it may be expected that the disturbance in transitory starch metabolism in the αHK1 potato lines would lead to an altered partitioning of carbohydrate within leaves and between sink and source organs. This could be indicated by the tendentially higher Suc levels in the leaves (significant in line 13 at the 2:30 PM time point), however, more experimentation is required to adequately resolve this question.

Our choice of potato for this study provided the opportunity to examine the role of HK in a heterotrophic tissue. We found no significant differences in the metabolism of the potato tubers from either the antisense or the overexpression lines. This, coupled with the observation that there was no great change in the yield of tubers per plant, indicates that HK1 does not play a significant role in the normal function of tuber induction and development. We were able to assign the StHK1 cDNA to the HK1 activity previously described in tubers by Renz et al. (1993) following analysis of the antisense and overexpression transgenic lines. In these studies we found that the HK1 activity peak was enhanced or reduced in the overexpression and antisense lines, respectively, and that the HK1 activity when overexpressed in either potato or yeast demonstrated the same behavior on an affinity chromatography column as that previously described for HK1 by Renz et al. (1993). However, we were concerned to note that the pH curve for the HK1 activity purified from potato was different from that obtained following expression of the StHK1 cDNA in yeast. This indicates that some posttranslational modifications may occur in one or more of the systems, and demonstrates that caution is required in interpreting and comparing kinetic data from heterologous systems.

Our observations that the yield of tubers, a strong sink tissue, was largely unchanged in the antisense and overexpression lines indicates that the overall physiology of the transgenic plants was not greatly affected. More significant changes might have been expected if HK were an important molecular sensor providing a direct link between carbon status, gene expression, and photosynthesis. However, our demonstration that inhibition of HK leads to significant effects on the biochemistry of leaves indicates the need for caution in the assignment of a function for this enzyme as a molecular signal of Glc status in plant leaves (Jang et al., 1997). There are a myriad of regulatory factors that can link metabolic status to gene expression, enzyme activity, and flux in leaf tissue, and once the metabolic network is disrupted it becomes extremely difficult to deconvolute cause and effect. Much more detailed work will be required before the mechanisms behind the effects described by Jang et al. (1997) are truly understood. At this stage, however, we do not identify HK as a target in the regulatory networks of plant metabolism for the manipulation of sink strength in crop plants.

Previous analysis of lines overexpressing a yeast invertase in the cytosol of tubers (Sonnewald et al., 1997) and in combination with a GLK (Trethewey et al., 1998) indicated that there was a switch in partitioning in tubers of these lines away from starch biosynthesis and toward glycolysis. Investigation of several other transgenic lines (antisense against ADP-Glc pyrophosphorylase alone and in combination with yeast invertase in the cytosol or apoplast) indicated that this change of partitioning is most closely associated with an increased cytosolic cleavage of Suc (Trethewey et al., 1999). In lines that show enhanced glycolysis, this is mediated in part by an induction in the activity of some key enzymes of glycolysis (e.g. phosphofructokinase, pyruvate kinase, triose-P isomerase, and glyceraldehyde 3-P dehydrogenase).

The question of the nature of the signal that connects the altered metabolism at the level of Suc cleavage to an induction in the activity of the glycolytic pathway is a fascinating one. The signal might conceivably be low Suc, high Glc, or an elevated flux from Suc to Glc-6-P. This last possibility is an interesting one in view of the evidence from yeast systems that HK might be capable of sensing the flux that it is catalyzing. However, the results of this study do not provide any support for the hypothesis that HK1 is a central regulatory element in potato tubers. We now believe that it is more likely that a signal related to the cytosolic Suc levels is the decisive factor controlling carbohydrate partitioning in potato tubers.

In conclusion, the studies presented in this paper provide convincing evidence that HK1 is involved in the pathway of carbon export from chloroplasts at night. However, we have not found any evidence that HK1 is involved in

Glc sensing in potato, nor do we have any indication that this enzyme catalyzes is a decisive step in the metabolism of potato tubers.


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