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# Interaction between SNF1-related kinases and a cytosolic pyruvate kinase of potato

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ABSTRACT

SNF1-related protein kinases (SnRKs) are widely conserved in plants. Previous studies have shown that members of the SnRK1 subfamily phosphorylate and inactivate at least four important plant metabolic enzymes: 3-hydroxy-3-methylglutaryl-CoA reductase, sucrose phosphate synthase, nitrate reductase, and trehalose phosphate synthase 5. In this paper, we demonstrate that two SnRK1 proteins of potato, PKIN1 and StubSNF1, interact with a cytosolic pyruvate kinase ($PK_c$) of potato in a yeast two-hybrid assay. The interacting domain of $PK_c$ is located in its C-terminal region and contains the putative SnRK1 recognition motif ALHRIGS$^{500}$ASVI. Our results indicate that both SnRK1s influence $PK_c$ activity *in vivo*. Antisense repression of SnRK1s alters the intensity and light/dark periodicity of PK activity in leaves. However, the differences between PK activity curves in antisense PKIN1 and antisense StubSNF1 lines indicated that the function of the two kinases is not identical in potato.



## Introduction

The SNF1 (sucrose non-fermenting 1) family of protein kinases (EC 2.7.11.1) is conserved in fungi, animals and plants, and plays diverse roles ranging from energy metabolism to transcriptional control. In the yeast *Saccharomyces cerevisiae*, SNF1 kinase is activated by glucose limitation and is required for the utilization of alternative carbon sources. SNF1 is a heterotrimeric enzyme consisting of a serine/threonine kinase, an activating subunit, SNF4, and one of the three cofactors, SIP1, SIP2, or GAL83, which is required for kinase function and confer substrate specificity (Hedbacker and Carlson, 2008). The animal homologue of yeast SNF1 is the AMP-activated protein kinase (AMPK). In mammals, AMPK coordinates energy homeostasis and regulates lipid and glucose metabolism (Misra, 2008).

In plants, the SNF1-related protein kinases (SnRKs) are central integrators of transcription networks in stress and energy signaling (Baena-González et al., 2007). The family of SnRKs has subfamilies in plants, of which SnRK1 is the most closely related to yeast SNF1. *SnRK1* genes have been cloned from many plant species, and it has been shown that the encoded proteins, which are very likely heterotrimeric enzymes similar to yeast's SNF1, can completely complement yeast *snf1* mutants. SnRK1 is regulated both transcriptionally and post-transcriptionally, activated via phosphorylation and inactivated by dephosphorylation that is inhibited by low concentrations of AMP (Polge and Thomas, 2007).

SnRK1s respond to energy supply and demand by directly phosphorylating rate-limiting enzymes of metabolic pathways, and by controlling gene expression. Gene expression profiling in *Arabidopsis* under various sugar and energy starvation conditions identified 1021 putative SnRK1 target genes (Baena-González et al., 2007). SnRK1s phosphorylate and inactivate at least four important plant metabolic enzymes: 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; EC 1.1.1.34), sucrose phosphate synthase (SPS; EC 2.4.1.14), nitrate reductase (NR; EC 1.7.2.1), and trehalose phosphate synthase 5 (TPS5; EC 2.4.1.36) (Dale et al., 1995; Sugden et al., 1999; Harthill et al., 2006). In addition, SnRK1s phosphorylate a class I heat shock protein (Slocombe et al., 2004).

Pyruvate kinase (EC 2.7.1.40) is a key enzyme of glycolysis and takes both cytoplasmic ($PK_c$) and plastidic ($PK_p$) forms in plants (Plaxton, 1996). Sequence analysis revealed two putative phosphorylation sites in $PK_c$ of soybean, FVRKGS$^{220}$DLVN and VLTRGGS$^{407}$TAKL (Tang et al., 2003). It was shown that post-translational modification of soybean $PK_c$ occurs by two mechanisms. The first is a C-terminal truncation of the 55 kDa full-length $PK_c$ to form an approximately 51 kDa polypeptide whose amount increases during seed development. The second mechanism is phosphorylation, which results in targeting the enzyme to the ubiquitin/26S proteasome pathway for degradation. Because

*Abbreviations:* AMPK, AMP-activated protein kinase; aP, PKIN1-silenced potato; aPS, PKIN1- and StubSNF1-silenced potato; aS, StubSNF1-silenced potato; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; NR, nitrate reductase; $PK_c$, cytosolic pyruvate kinase; PKIN1, potato kinase 1; SNF1, sucrose non-fermenting 1; SnRK1, SNF1-related kinase 1; SPS, sucrose phosphate synthase; StubSNF1, SNF1-related kinase of potato; TPS5, trehalose phosphate synthase 5.

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the sequence of phosphorylation sites matches the minimal consensus motif that is recognized by calcium-dependent protein kinases and SnRK1, it has been proposed that these kinases are involved in the modification and proteasome-mediated degradation of $PK_c$ (Tang et al., 2003).

The $PK_c$ of potato is 89% identical to the soybean $PK_c$ at the amino acid level. It contains the same putative phosphorylation sites and their surrounding sequences as the soybean enzyme. In addition, we have found that the region around $S^{500}$ in the potato $PK_c$ matches the consensus SnRK1 recognition motif X-[MLIF]-X-[RKY]-X-X-S-X-X-X-[LMVI] (Sugden et al., 1999). Here, we demonstrate that the $PK_c$ of potato interacts with both PKIN1 and StubSNF1, the two SnRK1 isoforms detected in potato (Man et al., 1997; Lakatos et al., 1999). The interacting domain is located in the C-terminal region of $PK_c$ containing $S^{500}$. In addition, we show evidence that both PKIN1 and StubSNF1 contribute to the diurnal changes of PK activity in leaves of potato.

## Materials and methods

### *Plant material and growth conditions*

The antisense PKIN1 (aP1, aP2), antisense StubSNF1 (aS5, aS6), and double antisense (aPS4, aPS7) lines were generated previously from *Solanum tuberosum* cv. Désirée (Sós-Hegedűs et al., 2005). Plants were vegetatively propagated from single-node stem segments on RM medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose at 24 °C under a light regime of 16 h light at 5000 lx intensity and 8 h of dark. Six-week-old plants obtained by tissue culture were transferred into pots and grown further under greenhouse conditions at 18–28 °C.

### *RNA gel blot analysis*

Low molecular weight RNAs were detected according to Szittya et al. (2002). Briefly, RNAs were isolated, separated on polyacrylamide gels, blotted to Hybond N filter (GE Healthcare), and hybridized with $^{32}$P-labelled *in vitro* transcripts of *StubSNF1* (accession number: U83797) and *PKIN1* cDNA (accession numbers: X95997–X96000 and X96372) cloned in pBluescript SK.

Messenger RNAs were separated on agarose gels and hybridized with PCR-amplified $^{32}$P-labelled double-stranded cDNA probes under the conditions specified by Lovas et al. (2003a).

### *Plasmid constructions and yeast two-hybrid analysis*

For the yeast two-hybrid experiments, *StubSNF1* and *PKIN1* cloned into vectors pAD-GAL4 and pBD-GAL4 (Clontech) (Lakatos et al., 1999; Lovas et al., 2003b) were used. To create clones of potato $PK_c$ (accession number X53688) in the same vectors, $PK_c$ DNA cloned previously into pUC19 was PCR-amplified using the primers PKs1 (5′-gggaattcatggccaacatagacatagct-3′; EcoRI site is underlined) and PKas1 (5′-gacacgacgattacttcacg-3′). The obtained fragment, $PK_{c1-510}$, was inserted into the EcoRI–SmaI site of pBD-GAL4 and then it was subcloned to pAD-GAL4 as an EcoRI–SalI fragment. The $PK_{c1-475}$ and $PK_{c476-510}$ fragments were obtained from the same template DNA using the primers PKs1 and PKas2 (5′-ggggtcgacgcttcaaggattacctc-3′; SalI site is underlined), and PKs2 (5′-gggaattcgccctgaagtctgccgtaac-3′; EcoRI site is underlined) and PKas1, respectively. Clones were verified by DNA sequence analysis.

*Saccharomyces cerevisiae* strain Y190, harboring the appropriate combinations of *StubSNF1*, *PKIN1* and $PK_c$ clones, was grown in standard media at 30 °C (Rose et al., 1990). Protein–protein interactions were detected as β-galactosidase activities using a filter lift assay (Jiang and Carlson, 1996) and quantified as described by Miller (1972). One unit of β-galactosidase was defined as the amount of enzyme which hydrolyzes 1 μmol of *o*-nitrophenyl β-*o*-galactopyranoside (ONPG) to *o*-nitrophenol and D-galactose per min per cell.

### *SAMS and SAS peptide kinase activity assays*

Partial purification of SnRK1s was performed as described previously (Man et al., 1997) using 1 g leaf tissue. Protein concentration of the extracts was determined by the Bradford (1976) method applying BSA as standard. SAMS (HMRSAMSGLHLVKRR) and the $PK_c$-specific SAS (VALHRIGSASVIKRR) peptides were used as substrates to detect kinase activity. Peptide phosphorylation was performed using the 15–40% saturated ammonium sulphate fraction of leaf extracts by established methods employing $\gamma^{32}$P ATP (Davies et al., 1989; Man et al., 1997), except that quantification was performed by PhosphorImage analysis (Storm 860 Gel and Blot Imaging System, GE Healthcare) of the filters on which the reaction mixtures were spotted. A dilution series was made from leaf extracts and kinase activity of each dilution step was measured using both SAMS and SAS peptides. The sum intensity of all pixels of each radioactive spot was measured, corrected by the parallel negative control's (no substrate) intensity, and normalized to the protein concentration. From the corrected data, an activity curve was created and kinase activity of the plants was calculated in that dilution range where dilution and activity displayed a linear correlation.

### *Pyruvate kinase activity assay*

The PK assays were performed using previously described methods (Burrell et al., 1994; Turner and Plaxton, 2000) with some modifications. Briefly, 0.5 g leaf tissue frozen in liquid nitrogen was homogenized in 2.5 mL of extraction buffer containing 100 mM Tris (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 28 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 40 mg mL$^{-1}$ polyvinyl polypyrollidone, and 1 mg mL$^{-1}$ bovine serum albumin, and clarified by centrifugation at 13,000 × *g* for 5 min at 4 °C. After centrifugation, 0.5 mL of the supernatant was applied to a NAP5 anion exchange column (GE Healthcare) and PK was eluted with 1 mL extraction buffer. PK activity of 30 μL fractions was measured in 250 μL total volume containing 100 mM HEPES (pH 7.1), 40 mM KCl, 10 mM $MgCl_2$, 1 mM ADP, 0.15 mM NADH, 1 U lactate dehydrogenase, in a 96-well microplate. Assays were initiated by the addition of 5 mM phosphoenolpyruvate. The decrease in absorbance at 340 nm as a result of the oxidation of NADH was monitored for 30 min in a Multiskan EX (Labsystems) microplate reader. PK activity was calculated from the linear part of the absorbance curve and expressed as μmol $NAD^+$ produced by 1 mg of protein per hour.

## Results

### *Detection of interaction of $PK_c$ with potato SnRK1s in yeast two-hybrid system*

Comparison of SnRK1 recognition sites of HMGR, NR and SPS revealed a consensus motif with a sequence of X-[MLIF]-X-[RKY]-X-X-S-X-X-X-[LMVI] (Sugden et al., 1999). To identify the putative SnRK1 recognition sites in potato $PK_c$, we surveyed the amino acid sequence of the enzyme with the consensus motif using the ScanProsite software. The survey resulted in identification of two putative SnRK1 recognition sites: VLTRGGS$^{406}$TAKL and ALHRIGS$^{500}$ASVI. The region surrounding $S^{406}$ was identical to that of the surrounding $S^{407}$ phosphorylation site in soybean $PK_c$ (Tang et al., 2003). The difference in the position of the motif is due to an extra $Q^{14}$ in soybean $PK_c$. The sequence surrounding $S^{219}$ in potato is identical to the FVRKGS$^{220}$DLVN motif in soybean, which was