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In vitro activity characterization of the tomato SnRK1 complex proteins

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ABSTRACT

Plant Sucrose non-Fermenting 1-Related Protein Kinase1 (SnRK1) complexes are members of the Snf1/AMPK/SnRK protein kinase family and play important roles in many aspects of metabolism. In tomato (*Solanum lycopersicum*, *Sl*), only one α-subunit of the SnRK1 complex, *Sl*SnRK1.1, has been characterized to date. In this study, the phylogenetic placement and *in vitro* kinase activity of a second tomato SnRK1 α-subunit, *Sl*SnRK1.2, were characterized. Interestingly, in the phylogenetic analysis of SnRK1 sequences from monocots and dicots *Sl*SnRK1.2 clusters only with other Solanaceae SnRK1.2 sequences, suggesting possible functional divergence of these kinases from other SnRK1 kinases. For analysis of kinase activity, *Sl*SnRK1.2 was able to autophosphorylate, phosphorylate the complex β-subunits, and phosphorylate the SnRK1 AMARA peptide substrate, all with drastically lower overall kinase activity compared to *Sl*SnRK1.1. Activation by the upstream kinase *Sl*SnAK was able to increase the kinase activity of both *Sl*SnRK1.1 and *Sl*SnRK1.2, although the increase is less dramatic for *Sl*SnRK1.2. The highest kinase activity on the AMARA peptide for *Sl*SnRK1.2 was seen when reconstituting the complex *in vitro* with *Sl*Sip1 as the β-subunit. In comparison, *Sl*SnRK1.1 showed the lowest kinase activity on the AMARA peptide when *Sl*Sip1 was used. These studies suggest the *Sl*SnRK1.2 phylogenetic divergence and lower *Sl*SnRK1.2 kinase activity compared to *Sl*SnRK1.1 may be indicative of different *in vivo* roles for each kinase.

1. Introduction

SnRK1 (Sucrose non-Fermenting Related Kinase1) is the plant homolog of the mammalian AMP-activated protein kinase (AMPK) and the yeast sucrose non-fermenting 1 (Snf1) kinase, and these kinases regulate cellular carbon metabolism such as usage of glucose in yeast and mammals, ATP production in mammals, and sucrose utilization in plants [1]. SnRK1 was originally discovered through its sequence homology to Snf1 and ability to complement the yeast *SNF1* knockout [2]. Like AMPK and Snf1, SnRK1 exists as a heterotrimeric complex consisting of a kinase active α-subunit termed SnRK1, a β-subunit for regulating substrate specificity, and a γ-subunit needed for full kinases activity [3]. Recent studies indicate the plant SnRK1 complex regulates a variety of metabolic processes besides sucrose metabolism, including nitrogen assimilation, sterol synthesis, starch synthesis, and photosynthate partitioning [4].

The SnRK1 α-subunit has been studied from many plant species including *Arabidopsis thaliana*, *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Oryza sativa* (rice), *Sorghum biocolor* (sorghum), *Hordeum vulgare* (barley), and *Nicotiana tabacum* (tobacco) [5–9]. The SnRK1 protein contains an N-terminal kinase domain and a C-terminal β-subunit interaction domain (β-SID), both of which are conserved in AMPK and Snf1 (Supplemental Fig. 1). Plant SnRK1s also contain a kinase associated (KA1) domain embedded within the β-SID domain (Supplemental Fig. 1), which was identified by homology to the KA1 domains in mammalian microtubule-associated proteins [10]. The KA1 domain is a common motif in protein kinases and has been shown to bind to anionic phospholipids and upstream phosphatases, which could play a role in membrane localization and regulation of kinase activity [11,12]. Additionally, SnRK1 contains an ubiquitin-associated domain (UBA), which has been suggested to bind to ubiquitinated proteins [13] and is not found in AMPK or Snf1 (Supplemental Fig. 1). AMPK and Snf1 contain an autoinhibitory domain (AID) for regulation of α-subunit kinase activity [14] that is not found in SnRK1 (Supplemental Fig. 1). Finally, only AMPK has an α-hook domain (Supplemental Fig. 1), which regulates interaction with the γ-subunit in an ADP/AMP dependent manner [15].

The SnRK1 α-subunit from *Arabidopsis* has been studied the most in plants in relation to regulation of kinase activity. Three *Arabidopsis* SnRK1 α-subunits have been identified so far and only two of them, *At*SnRK1.1 (a.k.a. AKIN10) and *At*SnRK1.2 (a.k.a. AKIN11), have been found to be active kinases [16]. The third Arabidopsis α-subunit, *At*SnRK1.3 (a.k.a. AKIN12), has been shown to be expressed at low levels in pollen, developing embryos, and seeds [17,18]. *At*SnRK1.1 and

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*At*SnRK1.2 are activated *in vitro* and *in vivo* by the upstream kinases *At*SnAK1 and *At*SnAK2 (a.k.a. GRIK1 and GRIK2), which phosphorylate the conserved Thr175 or Thr176 residues in the kinase domain T-loop region of *At*SnRK1.1 or *At*SnRK1.2, respectively [19]. Compared to *At*SnAK1, *At*SnAK2 shows higher kinase activity on both *At*SnRK1.1 and *At*SnRK1.2 [20,21]. *At*SnAK1 and *At*SnAK2 can autophosphorylate *in vitro* on Thr153 or Thr154, respectively, while phosphorylation of Ser260 or Ser261 in *At*SnAK1 or *At*SnAK2, respectively, by *At*SnRK1.1 inhibits their activity and functions as a negative feedback control [21].

In tomato, only one α-subunit, *Sl*SnRK1.1, has been functionally studied [7,22]. *Sl*SnRK1.1 was first isolated from a tomato seed cDNA library by hybridization analysis using the tobacco SnRK1 α-subunit NPK5 as a probe, and was shown to be constitutively expressed in seed and leaves [22]. *Sl*SnRK1.1 can bind to and phosphorylate the tomato yellow leaf curl China virus β-satellite βC1 protein at Ser33 and Thr78 to attenuate the viral infection [23]. Later studies found *Sl*SnRK1.1 was also able to interact with the tomato cell death suppressor AGC Ser/Thr protein kinase Adi3, which plays a role in the defense against the bacterial pathogen *Pseudomonas syringae* [7]. While the βC1 protein interaction did not affect *Sl*SnRK1.1 function [23], Adi3 has been proposed to inhibit *Sl*SnRK1 complex kinase activity *in vitro* and *in vivo* [7]. These studies indicate SnRK1 may also play a role in pathogen resistance.

Four tomato SnRK1 complex β-subunits have been identified: *Sl*Gal83, *Sl*Sip1, *Sl*Tau1, and *Sl*Tau2 [7]. Plant SnRK1 β-subunits have been shown to have three conserved protein domains: an N-terminal variable region containing a myristoylation motif (N-Myr), a C-terminal association with Snf1 complex (ASN) domain, and a carbohydrate binding domain (CBD) in the middle of the protein [24]. All four tomato β-subunits have these conserved domains (Supplemental Fig. 2).

Regulation of the SnRK1 β-subunits by phosphorylation has been shown in mammals, yeast, and plants, but has been best studied in mammals and yeast. The mammalian β-subunit AMPK β1 is phosphorylated by the α-subunit AMPKα1 at Ser24/Ser25 and Ser108, as well as Ser182 by an unknown upstream kinase [25,26]. The phosphorylation at Ser108 increases AMPK complex kinase activity [26], while Ser24/Ser25 and Ser182 phosphorylation causes nuclear exclusion of the complex [25]. The yeast β-subunit *Sc*Gal83 can be phosphorylated by the yeast α-subunit Snf1 and casein kinase 2, however, the exact phosphorylation sites and the function of the phosphorylation is still unknown [27]. In plants the tomato β-subunit *Sl*Gal83 was shown to be phosphorylated at Ser26 by Adi3, while the other three tomato β-subunits did not show detectable phosphorylation by Adi3 [7]. This phosphorylation of *Sl*Gal83 by Adi3 has been shown to be the trigger for down regulating the kinase activity of the SnRK1 complex in tomato, possibly during pathogen defense responses [7].

Here we report the identification and characterization of a second SnRK1 α-subunit from tomato, *Sl*SnRK1.2. We show that *Sl*SnRK1.2 has substantially different kinase activity in terms of autophosphorylation and *trans*-phosphorylation of the tomato β-subunits compared to the *Sl*SnRK1.1 α-subunit. Interestingly, this lower activity of *Sl*SnRK1.2 may be conserved among solanaceous plants. Potential implication of these findings for *in vivo* function of *Sl*SnRK1.2 are discussed.

2. Materials and methods

2.1. Cloning, expression, and protein purification of SlSnRK1.1, SlSnRK1.2, and SlSnAK

The *Sl*SnRK1.2 α-subunit was identified by BLAST against the tomato proteome at the Sol Genomics Network (https://solgenomics.net) using the full-length amino acid sequence of *Sl*SnRK1.1 (NP_001304105.1) [7] as the query. The identified *SlSnRK1.2* sequence was used to design primers for cloning of the ORF. Using tomato leaf total RNA and RT-PCR, a 1512 bp full-length ORF for *Sl*SnRK1.2 was cloned that matched the *SlSnRK1.2* sequence (NM_001247396.3) identified in GenBank from a tomato cDNA sequencing project [28]. For the RT-PCR, first strand cDNA was produced using the SuperScript™ IV First-Strand Synthesis System (Invitrogen), and the *SlSnRK1.2* ORF was amplified from first strand cDNA using GoTaq Green (Promega) with forward primer 5′-**ATG**AGTTCCAGAGGTGGTGG-3′ (start codon in bold) and reverse primer 5′-**TCA**TTGTGGCCCCTCTAGCTG-3′ (stop codon in bold). The *SlSnRK1.2* ORF was first cloned into the pGEMT vector by TA cloning, identity confirmed by sequencing, and the ORF sub-cloned into the pMAL-c2x vector using the *Eco*RI and *Sal*I restriction sites for expression of an N-terminal maltose binding protein (MBP) translational fusion protein in *E. coli*. The MBP-*Sl*SnRK1.2 recombinant protein was purified using amylose resin (NEB) in a gravity-fed column according to manufacturer instructions.

The *Sl*SnAK sequence was identified by BLAST against the tomato proteome in the NCBI database using the full-length amino acid sequence of *At*SnAK1 (NP_200863.2) and *At*SnAK2 (NP_566876.3) [20,21]. The *Arabidopsis* sequences identified two possible splicing forms of a single *SlSnAK* (XM_010315107, XM_010315106). Although the two splice variants are different in the 5′-UTR regions, both contained the same full length 1254 bp ORF and 3′-UTR regions. The *SlSnAK* ORF was isolated and cloned into pGEMT as described above for *SlSnRK1.2* using forward primer 5′-GGATGTCTGTG**ATG**ATGC-3′ (5′ UTR sequence underlined, start codon in bold) and reverse primer 5′-**TCA**AGTAGGGGTATCCTCTG-3′ (stop codon in bold). The *SlSnAK* ORF was sub-cloned into the pET28a vector using the *Eco*RI and *Sal*I restriction sites for expression of an N-terminal 6xHis translational fusing protein in *E. coli*. The His-*Sl*SnAK recombinant protein was purified using Ni-NTA agarose resin (QIAGEN) in a gravity-fed column according to manufacturer instructions.

The *SlSnRK1.1* ORF was previously cloned [7] and purification of MBP-*Sl*SnRK1.1 followed published protocols.

2.2. Yeast knockout complementation assay

SlSnRK1.1 and *SlSnRK1.2* were subcloned for a C-terminal FLAG tag into the MBB263 vector (uracil selection) modified for expression control under the *glyceraldehyde-3-phosphate dehydrogenase* promotor. The yeast *SNF1* knockout strain BY4741 (*MATa snf1Δ::KanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was obtained from Dharmacon, and was transformed with the above *SlSnRK1.1* and *SlSnRK1.2* constructs using the lithium acetate/PEG method. The transformants were screened on synthetic complete (SC) media lacking uracil with 2% glucose at 30 °C. Recovered colonies were grown in liquid SC-uracil media with 2% glucose for 40 h, adjusted to the same OD_{600}, spotted on SC-uracil plates containing either 2% glucose or 2% sucrose in 5-fold serial dilutions, and incubated at 30 °C for 48 h or 144 h, respectively, before imaging.

2.3. Autophosphorylation and phosphorylation of the β-subunits

*Sl*SnRK1.1 autophosphorylation and phosphorylation of the β-subunits was carried out in a buffer containing 10 mM DDT, 10 mM Tris-HCl pH 8.0, and 10 mM $MgCl_2$. *Sl*SnRK1.2 autophosphorylation and phosphorylation of the β-subunits was carried out in a buffer containing 10 mM DDT, 10 mM Tris-HCl pH 7.5, and 10 mM $MnCl_2$. 4 μg of *Sl*SnRK1.1 or *Sl*SnRK1.2 and 4 μg of *Sl*Gal83, *Sl*Sip1, *Sl*Tau1, or *Sl*Tau2 were used in each reaction as indicated in the figure legends. Reactions were initiated by addition of 2 μCi of γ-[^{32}P]ATP (6000 Ci/mmol) and non-radioactive ATP to a final concentration of 100 μM. The reactions were carried out at either 30 °C for 15 min for *Sl*SnRK1.1 or 14 h at 20 °C for *Sl*SnRK1.2, after which the reaction was terminated with the addition of 10ul 4x SDS-PAGE sample buffer. Proteins were resolved by 8% SDS-PAGE, the gel dried, and the gel exposed to a phosphorimager screen for different lengths of time as specified in the figure legends. Incorporated radioactivity was visualized and quantified using a phosphorimager (Typhoon FLA7000, GE Healthcare Life Sciences) and quantification software (ImageQuant TL, GE Healthcare Life Sciences).

Band volume of each phosphorylated protein was calculated by integrating the area below the peak of each band and above the background as determined using the rolling ball method (see Supplemental Fig. 3 for details on this approach). The band volume was then normalized to the corresponding protein levels as determined by densitometry analysis of the Coomassie stained gel. In order to determine suitable exposure times for different kinase assays, an initial 24 h exposure was analyzed and subsequent exposure times were adjusted so that the signal from different reactions were all in the same range.

2.4. SlSnAK activation of SlSnRK1.1 and SlSnRK1.2 for β-subunit phosphorylation

For *Sl*SnAK activation reactions, 4 μg of *Sl*SnRK1.1 or *Sl*SnRK1.2 were pre-incubated with 1 μg of *Sl*SnAK in the presence of 50 μM non-radioactive ATP at 30 °C for 15 min. Each β-subunit was then added and the phosphorylation reactions initiated by the addition of 2 μCi of γ-[^{32}P]ATP (6000 Ci/mmol) and non-radioactive ATP to a final concentration of 100 μM, including the initial 50 μM non-radioactive ATP used for *Sl*SnAK activation. Due to the relatively high level of *Sl*SnAK autophosphorylation, *Sl*SnRK1.2 reactions that contain *Sl*SnAK were only carried out for 30 min at 30 °C instead of the usual 14 h at 20 °C to avoid the overly strong signal from *Sl*SnAK autophosphorylation. Reactions were terminated with the addition of 10 μl 4x SDS-PAGE sample buffer and protein phosphorylation levels were analyzed as described above.

2.5. SlSnAK activation of SlSnRK1.1 and SlSnRK1.2 complexes for peptide substrate-based kinase assays

4 μg of *Sl*SnRK1.1 or *Sl*SnRK1.2, 4 μg of *Sl*Gal83, *Sl*Sip1, *Sl*Tau1, or *Sl*Tau2, and 4 μg of *Sl*Snf4 were used to reconstitute the *Sl*SnRK1 complex. The complexes were activated by the addition of 1 μg of *Sl*SnAK and 50 μM non-radioactive ATP at 30 °C for 15 min. After activation, 2 μCi of γ-[^{32}P]ATP (6000 Ci/mmol) with non-radioactive ATP to a final concentration of 100 μM, including the initial 50 μM non-radioactive ATP used for *Sl*SnAK activation, and 100 μM AMARA peptide [29] (GenScript) were added to each reaction. The *Sl*SnRK1.1 reaction was incubated at 30 °C for 15 min, while *Sl*SnRK1.2 was incubated at 30 °C for 30 min. The reactions were terminated by heating at 95 °C for 10 min. Each reaction was spotted on a 2 cm × 2 cm piece of P81 paper (Reaction Biology Corp), washed three times with 74 mM phosphoric acid, and washed one time with acetone to bind the phosphorylated peptide to the filter paper while removing all other proteins. The filter paper was then submerged in scintillation fluid (Bio Safe II, RPI) in a scintillation vial and the incorporated radioactivity detected using a liquid scintillation counter (LSC) (Tri-Carb 2910R, Perkin Elmer). The count per minute (CPM) reading was converted to nmol of ATP incorporated per minute per μg of *Sl*SnRK1.1 or *Sl*SnRK1.2 as previously described [30]. Due to the possibility of residual phosphorylated proteins adhering to the filter paper, CPMs from reactions containing each protein combination but no AMARA peptide were used as a background level, and this background from each protein combination was subtracted from each corresponding full reaction CPM to obtain net AMARA phosphorylation levels. Reactions containing all components except *Sl*SnRK1.1 or *Sl*SnRK1.2 were carried out as negative controls.

3. Results

3.1. Identification of a second tomato SnRK1 complex α-subunit

Most plants have at least two α-subunits for the SnRK1 complex including *Arabidopsis*, potato, rice, sorghum, and barley [5,6]. However, for tomato only one α-subunit has been characterized at the protein level [7,22]. Thus, we undertook a search for additional tomato *Sl*SnRK1 complex α-subunits. A BLAST search of the tomato proteome using the Sol Genome Database with *Sl*SnRK1.1 as the query returned one additional α-subunit sequence we termed *Sl*SnRK1.2. The *Sl*SnRK1.2 protein showed conservation of all SnRK1 domains and showed 69% identity to *Sl*SnRK1.1, with high conservation within the catalytic domain, the UBA domain, and the KA1 domain (Supplemental Fig. 4). The *Sl*SnRK1.2 sequence also showed very high identity (98%) to the potato homolog, PKIN1 [31], and to (99%) the predicted SnRK1 from wild tomato (*Solanum pennellii*), *Sp*Kin10like (Supplemental Fig. 4).

A phylogenetic analysis of SnRK1.1 and SnRK1.2 sequences from 28 plant species showed the monocot and dicot sequences could be distinguished from each other, and the Solanaceae sequences formed two distinct Group A and Group B clades (Supplemental Fig. 5). Interestingly, while the monocot SnRK1.1 and SnRK1.2 sequences clustered together, as well as the *Arabidopsis* homologs *At*SnRK1.1 and *At*SnRK1.2 [32], the Solanaceae SnRK1.1 and SnRK1.2 sequences distinctly clustered into the distantly related Group B and Group A clades, respectively (Supplemental Fig. 5). Also, the three *Solanum* SnRK1.2 sequences *Sl*SnRK1.2, potato PKIN1, and *S. pennellii Sp*Kin10like all clustered together in a subclade (Supplemental Fig. 5). This may suggest the Solanaceae SnRK1.2 proteins have functions divergent from those proposed for SnRK1.1.

Most plant SnRK1 α-subunit proteins have been confirmed as functional Snf1-like proteins by successfully complementing the yeast *SNF1* knockout line (*snf1Δ*) [6,9,33]. Thus, the ability of *Sl*SnRK1.2 to complement *snf1Δ* was tested to confirm it as a functional Snf1-like protein. Indeed, *Sl*SnRK1.2 was able to confer at least partial complementation for growth on sucrose when expressed in the yeast *snf1Δ* line (Supplemental Fig. 6). However, *Sl*SnRK1.2 was not able to complement to the extent of *Sl*SnRK1.1 (Supplemental Fig. 6). This suggests that while *Sl*SnRK1.2 may function as an SnRK1 α-subunit, it may not have full Snf1-like activity.

3.2. SlSnRK1.2 has weak kinase activity compared to SlSnRK1.1

Autophosphorylation is prevalent in eukaryotic protein kinases [34] and both *Arabidopsis* and tomato SnRK1 α-subunit proteins have been shown to autophosphorylate [7,33]. In order to confirm *Sl*SnRK1.2 as a functional protein kinase, we first determined its autophosphorylation ability under several different reaction conditions by varying the pH, temperature, reaction time, and preference for divalent cations of Mg^{+2} or Mn^{+2}. While the differences between the conditions were minimal, the experiments (not shown) showed the optimal conditions to be pH 7.5, 20 °C, 14 h reaction time, and preference for Mn^{+2}. This differs from the *Sl*SnRK1.1 reaction conditions of pH 8.0, 30 °C, 30 min reaction time, and preference for Mg^{+2} [7]. The main difference between the reaction conditions for the two kinases is the reaction time, which suggests *Sl*SnRK1.2 is a much weaker kinase than *Sl*SnRK1.1.

Using the optimal reaction conditions, *Sl*SnRK1.2 autophosphorylation activity was analyzed for comparison to *Sl*SnRK1.1. Wild-type (WT) *Sl*SnRK1.2 autophosphorylation was shown to be much weaker than that of WT *Sl*SnRK1.1 (Fig. 1A and B, lane 1). In fact, the *Sl*SnRK1.2 autophosphorylation signal was only slightly detectable after 168 h of exposure to the phosphorimager screen (Fig. 1B, lane 1). We previously identified *Sl*SnRK1.1 Lys48 as the key amino acid involved in ATP binding and mutation of this residue to Gln eliminates autophosphorylation [7] (Fig. 1A, lane 2). The corresponding residue in *Sl*SnRK1.2 is Lys46 (Supplemental Fig. 4) and mutation to Gln also eliminated autophosphorylation activity (Fig. 1B, lane 2).

In *Arabidopsis*, upstream kinases are known to phosphorylate *At*SnRK1.1 and *At*SnRK1.2 at the conserved Thr175 or Thr176, respectively, for activation [21]. In the case of *Sl*SnRK1.1 and *Sl*SnRK1.2 this residue is Thr175 and Thr173, respectively (Supplemental Fig. 4). Mutation of these residues in *Sl*SnRK1.1 and *Sl*SnRK1.2 to the phosphomimetic Asp or Glu significantly increased autophosphorylation of

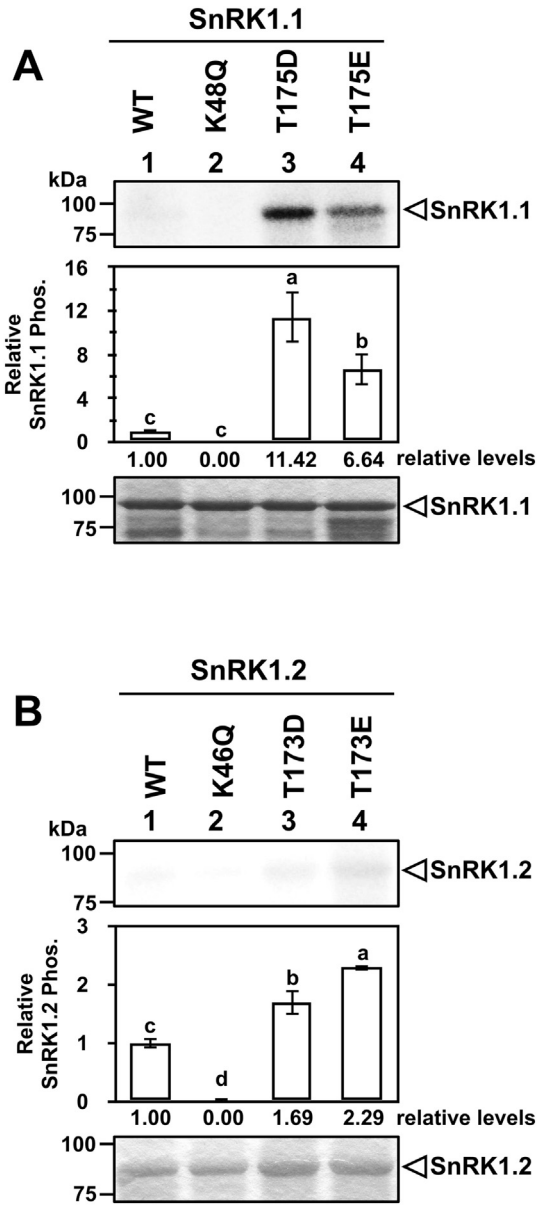

Fig. 1. Autophosphorylation of *Sl*SnRK1.1 and *Sl*SnRK1.2. In (A) and (B), 4 μg of the *Sl*SnRK1.1 and *Sl*SnRK1.2 wild-type (WT), kinase inactive K48Q or K45Q mutants, or the phosphomimetic T175D, T175E or T173D, T173E mutants, respectively, were incubated with γ-[^{32}P]ATP in an *in vitro* kinase assay. Top panel, phosphorimage; middle panel, quantification of autophosphorylation from four repeats, error bars indicate standard error, average value is shown under each column; bottom panel, Coomassie blue-stained gel. Statistical analysis was carried out using the Fisher LSD test and samples with the same letter above the bars are not significantly different ($p < 0.05$). (A) *Sl*SnRK1.1 autophosphorylation. The reaction was carried out at 30 °C for 30 min and the gel was exposed to the phosphorimager screen for 24 h. (B) *Sl*SnRK1.2 autophosphorylation. The reaction was carried out at 20 °C for 14 h and the gel was exposed to the phosphorimager screen for 168 h.

both kinases (Fig. 1A and B, lanes 3, 4). The T175D mutation in *Sl*SnRK1.1 conferred higher autophosphorylation activity compared to the T175E mutation (Fig. 1A, lanes 3, 4), while the *Sl*SnRK1.2 T173E mutation resulted in higher autophosphorylation activity compared to the T173D mutation (Fig. 1B, lanes 3, 4). It should be noted that in all the kinase assays in Fig. 1 the *Sl*SnRK1.1 activity was much higher than that of *Sl*SnRK1.2.

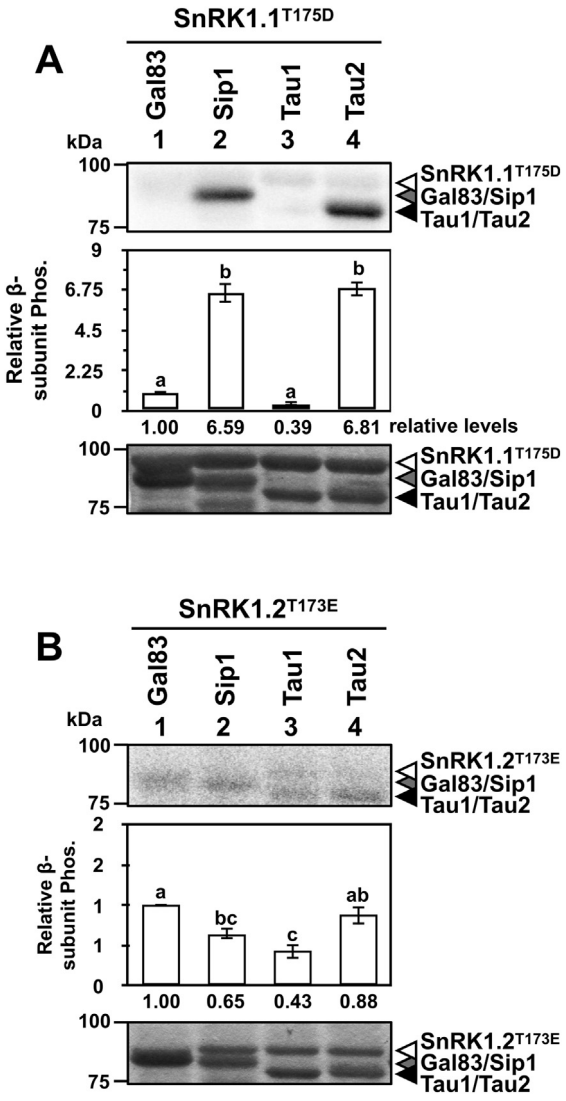

Fig. 2. *Sl*SnRK1.1^{T175D} and *Sl*SnRK1.2^{T173E} have different preferences for β-subunit phosphorylation. In (A) and (B), the indicated proteins were incubated with γ-[^{32}P]ATP in an *in vitro* kinase assay. Top panel, phosphorimage; middle panel, quantification of β-subunit phosphorylation from four repeats, error bars indicate standard error, average value is shown under each column; bottom panel, Coomassie blue-stained gel. Statistical analysis was carried out using the Fisher LSD test and samples with the same letter above the bars are not significantly different ($p < 0.05$). (A) *Sl*SnRK1.1^{T175D} phosphorylation of different β-subunits. The reaction was carried out at 30 °C for 15 min and the gel was exposed to the phosphorimager screen for 24 h. (B) *Sl*SnRK1.2^{T173E} phosphorylation of different β-subunits. The reaction was carried out at 20 °C for 14 h and the gel was exposed to the phosphorimager screen for 168 h.

3.3. *SlSnRK1.1 and SlSnRK1.2 differentially phosphorylate the β-subunits in vitro*

Previous studies have shown that the α-subunits of the Snf1 and AMPK complexes are capable of phosphorylating their respective β-subunits [26,27]. Thus, given the different kinase activity levels between *Sl*SnRK1.1 and *Sl*SnRK1.2 the ability of each of these kinases to phosphorylate the tomato β-subunits was analyzed using the phosphomimetic kinases with the highest activity, *Sl*SnRK1.1^{T175D} and *Sl*SnRK1.1^{T173E}. While *Sl*SnRK1.1^{T175D} was able to phosphorylate each β-subunit, Sip1 and Tau2 (Fig. 2A, lanes 2, 4) were phosphorylated over 6 times stronger than Gal83 and Tau1 (Fig. 2A, lanes 1, 3). For *Sl*SnRK1.2^{T173E} all β-subunits were phosphorylated within 50% of each other (Fig. 3B) with Gal83 and Tau2 having the highest

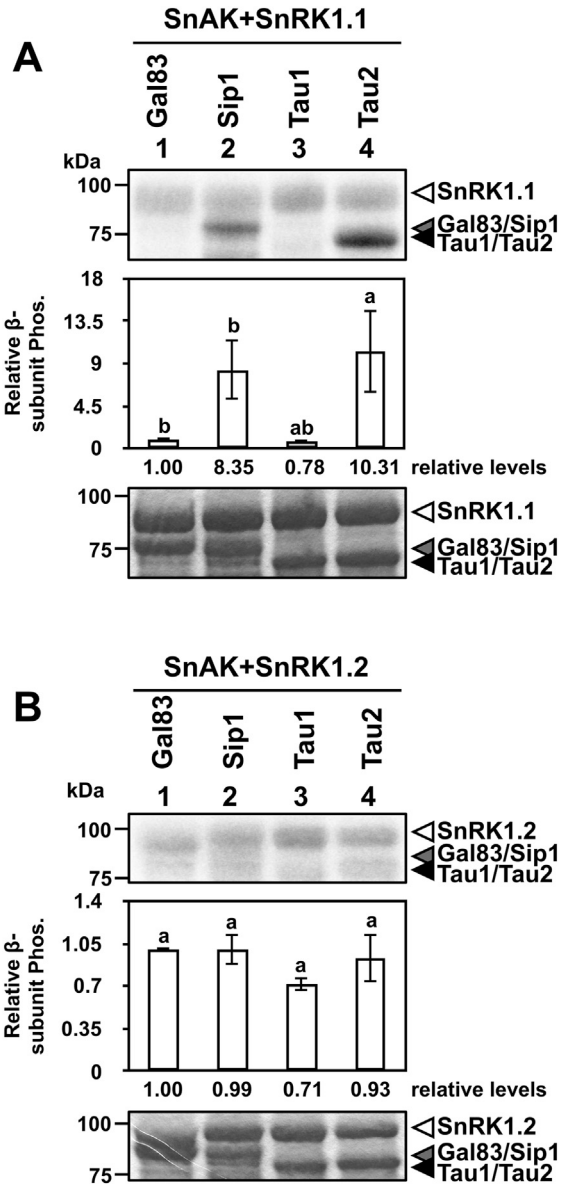

Fig. 3. *Sl*SnAK activated *Sl*SnRK1.1 and *Sl*SnRK1.2 phosphorylation of β-subunits. In (A) and (B), *Sl*SnRK1.1 or *Sl*SnRK1.2 were pre-incubated with *Sl*SnAK in the presence of non-radioactive ATP at 30 °C for 15 min, followed by addition of the indicated β-subunits and γ-[^{32}P]ATP with incubation at 30 °C for 15 min for *Sl*SnRK1.1 or 30 min for *Sl*SnRK1.2. Top panel, phosphorimage; middle panel, quantification of β-subunit phosphorylation from four repeats, error bars indicate standard error, average value is shown under each column; bottom panel, Coomassie blue-stained gel. Statistical analysis was carried out using the Fisher LSD test and samples with the same letter above the bars are not significantly different ($p < 0.05$). (A) *Sl*SnAK activated *Sl*SnRK1.1 phosphorylation of different β-subunits. After the assay the gel was exposed to the phosphorimager screen for 2 h. (B) *Sl*SnAK activated *Sl*SnRK1.2 phosphorylation of different β-subunits. After the assay the gel was exposed to the phosphorimager screen for 48 h. The phosphorylation levels of different β-subunits are not significantly different according to the Fisher LSD test.

phosphorylation levels (Fig. 2B, lanes 1, 4). As was seen in the autophosphorylation assays, *Sl*SnRK1.2 β-subunit phosphorylation was much weaker than that of *Sl*SnRK1.1 (Fig. 2A, B).

3.4. Identification of the tomato upstream kinase for the SlSnRK1 complex α-subunits

It has been shown in *Arabidopsis* that replacing Thr175 or 176 in *At*SnRK1.1 or *At*SnRK1.2, respectively, with a phosphomimetic mutation is not enough to fully activate these kinases, and the upstream kinase *At*SnAK2 is required for full activation [35]. Since the *Sl*SnRK1.2 phosphomimetic mutations contained quite low auto- and *trans*-phosphorylation activity (Figs. 1 and 2), a tomato *SnAK* sequence was identified to test the ability of the encoded kinase to confer higher *Sl*SnRK1.2 kinase activity. The *Arabidopsis* SnAK1 and SnAK2 sequences (*At*SnAK1, *At*SnAK2; [20,21]) were used in a BLAST search of the tomato proteome to identify any homologous tomato sequences. The results identified only a single *Sl*SnAK sequence that showed 62% identity to *At*SnAK1 and 65% identity to *At*SnAK2 (Supplemental Fig. 7). *Sl*SnAK was able to phosphorylate both *Sl*SnRK1.1 and *Sl*SnRK1.2 as determined by using the kinase-inactive *Sl*SnRK1.1^{K48Q} and *Sl*SnRK1.2^{K45Q} in kinase assays with *Sl*SnAK (Supplemental Fig. 8).

3.5. SlSnAK activation of SlSnRK1.1 and SlSnRK1.2 kinase activity

The ability of *Sl*SnAK phosphorylation to activate *Sl*SnRK1.1 and *Sl*SnRK1.2 *trans*-phosphorylation was tested using the β-subunits as substrate. The *Sl*SnAK activated *Sl*SnRK1.1 showed a dramatic increase in its ability to phosphorylate all β-subunits, reducing the phosphorimager screen exposure time from 24 h for the phosphomimetic *Sl*SnRK1.1^{T175D} (Fig. 2A) to 2 h (Fig. 3A). The preference of *Sl*SnAK activated *Sl*SnRK1.1 for phosphorylating the different β-subunits did not change when compared to the phosphomimetic *Sl*SnRK1.1^{T175D} (Figs. 2A and 3A). For *Sl*SnRK1.2, activation by *Sl*SnAK also conferred a significant increase in the ability of *Sl*SnRK1.2 to phosphorylate the β-subunits, decreasing the optimal phosphorimager screen exposure time to 48 h (Fig. 3B) from 168 h for the phosphomimetic *Sl*SnRK1.2^{T173E} (Fig. 2B). The *Sl*SnAK activated *Sl*SnRK1.2 showed decreased phosphorylation specificity for the β-subunits with roughly equal phosphorylation of all β-subunits expect Tau1 (Fig. 3B) compared to favoring Gal83 and Tau2 for the phosphomimetic *Sl*SnRK1.2^{T173E} (Fig. 2B). While *Sl*SnAK alone was able to weakly phosphorylate the β-subunits, this level of phosphorylation was below the detection limit of the activated *Sl*SnRK1.2 phosphorylated β-subunits (Supplemental Fig. 9).

3.6. SlSnRK1.1 and SlSnRK1.2 show differential substrate phosphorylation dependent on the β-subunit used

Finally, the kinase activity of the *Sl*SnAK activated *Sl*SnRK1 complex was tested with either the *Sl*SnRK1.1 or *Sl*SnRK1.2 α-subunit in combinations with the four different β-subunits. The γ-subunit is required for full kinase activity of the SnRK1 complex *in vitro* [7], so the only γ-subunit so far cloned and characterized in tomato, *Sl*Snf4, was included in the *in vitro* assays to reconstitute the *Sl*SnRK1 complex. We previously demonstrated the ability to reconstitute the *Sl*SnRK1 complex *in vitro* for testing complex kinase activity [7]. The complex kinase activity was assessed using the AMARA peptide (AMARAASAAALARRR; phosphorylation site underlined), which is an artificial substrate developed according to the consensus phosphorylation sequence identified for AMPK/Snf1 substrates [29]. Similar to *At*SnAKs [21,36], phosphorylation of the AMARA peptide by *Sl*SnAK was not observed in reactions containing only *Sl*SnAK and the β- and γ-subunits (Fig. 4A). When using *Sl*SnAK activated *Sl*SnRK1.1 as the α-subunit, the reconstituted complex with Sip1 had the lowest kinase activity (Fig. 4B, lane 2), while the complexes reconstituted with the other three β-subunits had an equal level of kinase activity (Fig. 4B, lanes 1, 3, 4). In contrast, when *Sl*SnAK activated *Sl*SnRK1.2 was used as the α-subunit the complex reconstituted with Sip1 showed the highest kinase activity (Fig. 4C, lane 2), while the complex reconstituted with Tau2 showed the lowest kinase activity (Fig. 4C, lane 4). Kinase activity with Gal83 and Tau1 showed an intermediate level of activity (Fig. 4C, lanes 1, 3). Additionally, when comparing kinase activity between complexes with *Sl*SnRK1.1 or *Sl*SnRK1.2 the *Sl*SnRK1.1 containing complexes showed activity 86 to

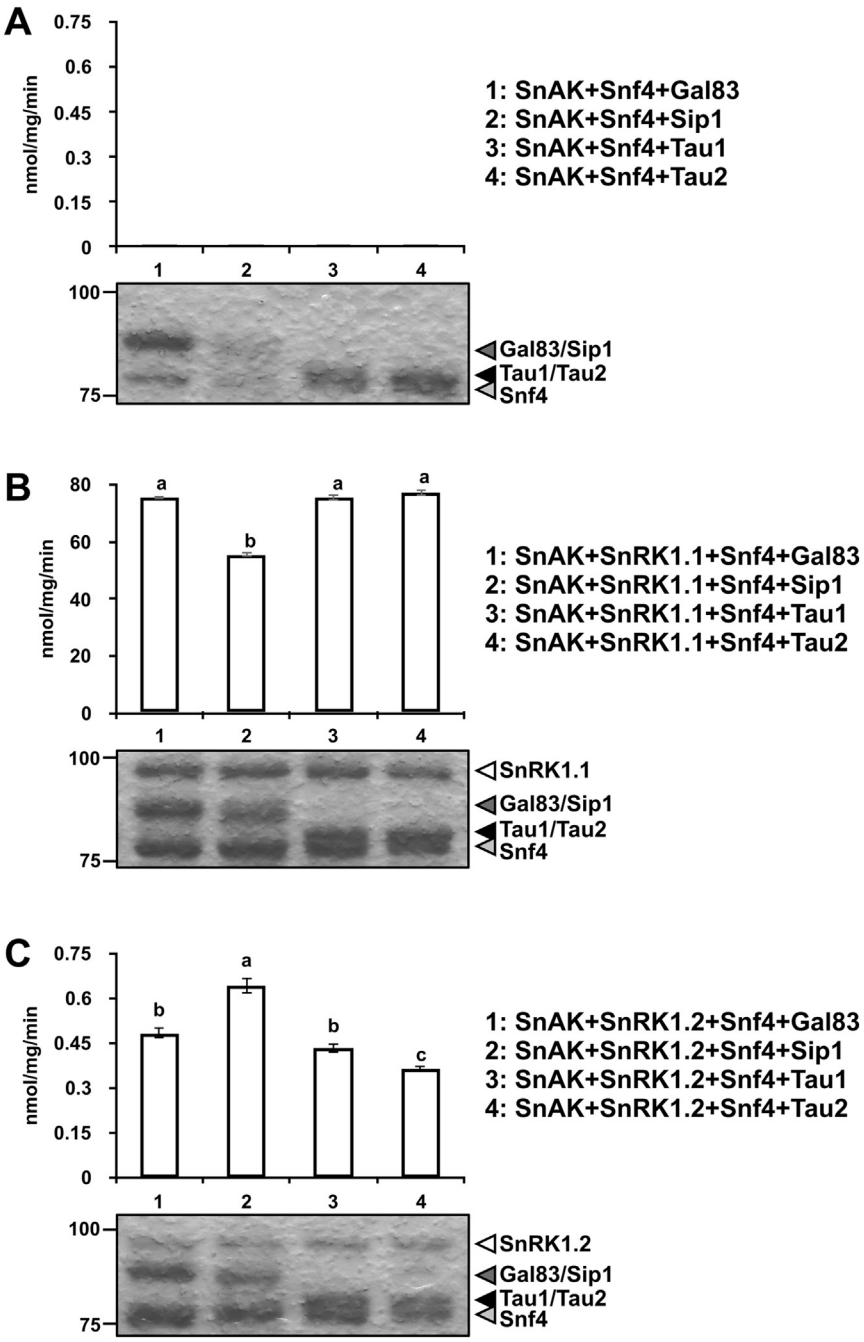

Fig. 4. *Sl*SnAK activated *Sl*SnRK1.1 and *Sl*SnRK1.2 phosphorylation of the AMARA peptide. In (A) and (B), *Sl*SnRK1.1 or *Sl*SnRK1.2 were pre-incubated with *Sl*SnAK in the presence of non-radioactive ATP at 30 °C for 15 min, followed by addition of the indicated β- and γ-subunits, γ-[^{32}P]ATP, and AMARA peptide with incubation at 30 °C for 15 min for *Sl*SnRK1.1 or 30 min for *Sl*SnRK1.2. Top panel, quantification of scintillation counts for AMARA peptide phosphorylation from four repeats, values are shown as nmol phosphate incorporated mg^{-1} SnRK1 protein min^{-1}, error bars indicate standard error; bottom panel, Coomassie blue-stained gel of proteins put in the assay. *Sl*SnAK was run off the end of each gel in order to fully separate the α- and β-subunits. Statistical analysis was carried out using the Fisher LSD test and samples with the same letter above the bars are not significantly different ($p < 0.05$). (A) *Sl*SnAK activated *Sl*SnRK1.1 phosphorylation of the AMARA peptide in the presence of different β-subunits. After the assay the gel was exposed to the phosphorimager screen for 2 h. (B) *Sl*SnAK activated *Sl*SnRK1.2 phosphorylation of the AMARA peptide in the presence of different β-subunits. After the assay the gel was exposed to the phosphorimager screen for 48 h.

210 times higher than *Sl*SnRK1.2 containing complexes (Fig. 4B, C). These data indicate the α-subunit in combination with the β-subunits dictate substrate phosphorylation levels, and the *Sl*SnRK1.1 α-subunit confers overall higher activity for the complex.

4. Discussion

Although many plants have more than one SnRK1 α-subunit, the importance of having multiple SnRK1 α-subunits has not been well studied. In tomato, *Sl*SnRK1.1 is the only α-subunit that had been studied at the protein level. Here, we characterized the *in vitro* activity of an additional tomato α-subunit, *Sl*SnRK1.2. Overall, compared to *Sl*SnRK1.1 *Sl*SnRK1.2 is a much weaker kinase in relation to its ability to autophosphorylate and phosphorylate the β-subunits and AMARA peptide. For *Sl*SnRK1.1, the T175D phosphomimetic mutation increased its kinase activity 11.4 times, while for *Sl*SnRK1.2 the T173E phosphomimetic mutation only increased kinase activity 2.3 times (Fig. 1A, B). Similarly, *Sl*SnRK1.2 kinase activity on the β-subunits or the AMARA peptide is much weaker than that of *Sl*SnRK1.1 even after *Sl*SnAK activation (Fig. 3A, B). One possible reason for the lower kinase activity of *Sl*SnRK1.2 could be the presence of autoinhibitory domains in the protein. While the autoinhibitory domains found in AMPK and Snf1 are not found in plant SnRK1 α-subunits, a recent study raised the possibility that kinase activity of the *Arabidopsis* α-subunit *At*SnRK1.1 could be negatively regulated by its C-terminus, but *At*SnRK1.2 did not show such regulation [35]. It is possible the *Sl*SnRK1.2 C-terminal region negatively regulates its kinase activity similar to *At*SnRK1.1 through a yet to be identified domain, while the *Sl*SnRK1.1 C-terminal region does not have a negative regulatory function. It will be interesting to see whether the C-termini of *Sl*SnRK1.1 and *Sl*SnRK1.2 have different regulatory functions as seen in *Arabidopsis*.

*Sl*SnRK1.2 did show different preferences for β-subunit

phosphorylation compared to *Sl*SnRK1.1. Sip1 and Tau2 are highly phosphorylated by *Sl*SnRK1.1 (Figs. 2, 3), while *Sl*SnRK1.2 could phosphorylate *Sl*Gal83 and *Sl*Tau2 to a higher extent than the other two β-subunits (Figs. 2, 3). On the other hand, when phosphorylating the AMARA peptide, *Sl*SnRK1.2 kinase activity is the highest when the heterotrimer was reconstituted using Sip1 as the β-subunit (Fig. 4B), while *Sl*SnRK1.1 kinase activity is the lowest when Sip1 was used as the β-subunit (Fig. 4A).

There are a few possible ways these differences in *Sl*SnRK1.1 and *Sl*SnRK1.2 activity could have physiological importance. It is possible the activity differences relate to different *in vivo* functions for these two kinases, specifically for *Sl*SnRK1.2, which along with other solanaceous SnRK1.2 sequences appear to be quite different phylogenetically from the other plant SnRK1.2 sequences (Supplemental Fig. 5). In potato, the *Sl*SnRK1.1 α-subunit homolog, StubSNF1, can interact with the potato Gal83 β-subunit and complement the yeast *snf1*Δ knockout [6], similar to what we have seen for *Sl*SnRK1.1 [7]. On the other hand, the potato *Sl*SnRK1.2 α-subunit homolog, PKIN1, does not interact with Gal83 or compliment the yeast *snf1*Δ knockout [6]. These data may suggest unique functions for solanaceous SnRK1.2 α-subunit kinases.

*Sl*SnRK1.1 and *Sl*SnRK1.2 could also be differentially regulated at distinct plant developmental stages. Transcriptome analysis of various tomato tissues showed that while *Sl*SnRK1.1 and *Sl*SnRK1.2 expression levels are similar in the leaf, *Sl*SnRK1.2 expression levels increased more than three fold in developing fruit before reaching the green mature stage [37]. The expression levels for *Sl*SnRK1.1 only increase about 20% at the same fruit developmental stage [37]. This could indicate differences in regulation, and possibly function, for *Sl*SnRK1.1 and *Sl*SnRK1.2 during the carbohydrate storage process in fruit.

Additionally, *Sl*SnRK1.1 and *Sl*SnRK1.2 could be differentially regulated under stress conditions. In *Arabidopsis* under phosphate starvation conditions, *At*SnRK1.2 kinase activity decreased 35%–40% and is selectively degraded, while *At*SnRK1.1 activity increased one fold at the same time [18]. We have found that *Sl*SnRK1.1 interacts with the tomato cell death regulatory kinase Adi3 [7], which helps to regulate the cell death associated with resistance to the causative agent of bacterial spec disease, *P. syringae* [38–40]. This interaction is speculated to regulate *Sl*SnRK1.1 for the reallocation of nutrients during the resistance cell death. Thus, it will be interesting to determine how biotic stresses such as *P. syringae*, or even abiotic stresses, will cause changes in *Sl*SnRK1.1 and *Sl*SnRK1.2 transcript and protein levels as well as differential regulation of kinase activity.

Transparency document

The http://dx.doi.org/10.1016/j.bbapap.2018.05.010 associated this article can be found, in online version.

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Appendix A. Supplemental data

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References

[1] C. Polge, M. Thomas, SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? Trends Plant Sci. 12 (2006) 20–28.
[2] A. Alderson, P.A. Sabelli, J.R. Dickinson, D. Cole, M. Richardson, M. Kreis, P.R. Shewry, N.G. Halford, Complementation of *snf1*, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein kinase cDNA, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8602–8605.
[3] S. Emanuelle, M.S. Doblin, D.I. Stapleton, A. Bacic, P.R. Gooley, Molecular insights into the enigmatic metabolic regulator, SnRK1, Trends Plant Sci. 21 (2016) 341–353.
[4] N.G. Halford, S.J. Hey, Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants, Biochem. J. 419 (2009) 247–259.
[5] M. Takano, H. Kajiya-Kanegae, H. Funatsuki, S. Kikuchi, Rice has two distinct classes of protein kinase genes related to *SNF1* of *Saccharomyces cerevisiae*, which are differently regulated in early seed development, Mol Gen Genet 260 (1998) 388–394.
[6] Á. Lovas, A. Sós-Hegedus, A. Bimbó, Z. Bánfalvi, Functional diversity of potato SNF1-related kinases tested in *Saccharomyces cerevisiae*, Gene 321 (2003) 123–129.
[7] J. Avila, O.G. Gregory, D. Su, T.A. Deeter, S. Chen, C. Silva-Sanchez, S. Xu, G.B. Martin, T.P. Devarenne, The β-subunit of the SnRK1 complex is phosphorylated by the plant cell death suppressor Adi3, Plant Physiol. 159 (2012) 1277–1290.
[8] S.W. Bledsoe, C. Henry, C.A. Griffiths, M.J. Paul, R. Feil, J.E. Lunn, M. Stitt, L.M. Lagrimini, The role of Tre6P and SnRK1 in maize early kernel development and events leading to stress-induced kernel abortion, BMC Plant Biol. 17 (2017) 1–17.
[9] T. Muranaka, H. Banno, Y. Machida, Characterization of tobacco protein kinase NPK5, a homolog of *Saccharomyces cerevisiae* SNF1 that constitutively activates expression of the glucose-repressible *SUC2* gene for a secreted invertase of *S. cerevisiae*, Mol. Cell. Biol. 14 (1994) 2958–2965.
[10] N. Tochio, S. Koshiba, N. Kobayashi, M. Inoue, T. Yabuki, M. Aoki, E. Seki, T. Matsuda, Y. Tomo, Y. Motoda, A. Kobayashi, A. Tanaka, Y. Hayashizaki, T. Terada, M. Shirouzu, T. Kigawa, S. Yokoyama, Solution structure of the kinase-associated domain 1 of mouse microtubule-associated protein/microtubule affinity-regulating kinase 3, Protein Sci. 15 (2006) 2534–2543.
[11] A. Rodrigues, M. Adamo, P. Crozet, L. Margaha, A. Confraria, C. Martinho, A. Elias, A. Rabissi, V. Lumbreras, M. González-Guzmán, R. Antoni, P.L. Rodriguez, E. Baena-González, ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in *Arabidopsis*, Plant Cell 25 (2013) 3871–3884.
[12] K. Moravcevic, J.M. Mendrola, K.R. Schmitz, Y. Wang, D. Slochower, P.A. Janmey, M.A. Lemmon, Kinase associated-1 domains drive MARK/PAR1 kinases to membrane targets by binding acidic phospholipids, Cell 143 (2010) 966–977.
[13] R. Farras, A. Ferrando, J. Jásik, T. Kleinow, L. Ökrész, A. Tiburcio, K. Salchert, C. del Pozo, J. Schell, C. Koncz, SKP1-SnRK protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase, EMBO J. 20 (2001) 2742–2756.
[14] L. Chen, F. Xin, J. Wang, J. Hu, Y. Zhang, S. Wan, L. Cao, C. Lu, P. Li, S.F. Yan, D. Neumann, U. Schlattner, B. Xia, Z. Wang, J. Wu, Conserved regulatory elements in AMPK, Nature 498 (2013) E8–E10.
[15] B. Xiao, M.J. Sanders, E. Underwood, R. Heath, F.V. Mayer, D. Carmena, C. Jing, P.A. Walker, J.F. Eccleston, L.F. Haire, P. Saiu, S.A. Howell, R. Aasland, S.R. Martin, D. Carling, S.J. Gamblin, Structure of mammalian AMPK and its regulation by ADP, Nature 472 (2011) 230–233.
[16] E. Baena-Gonzalez, F. Rolland, J.M. Thevelein, J. Sheen, A central integrator of transcription networks in plant stress and energy signalling, Nature 448 (2007) 938–943.
[17] M. Schmid, T.S. Davison, S.R. Henz, U.J. Pape, M. Demar, M. Vingron, B. Schölkopf, D. Weigel, J.U. Lohmann, A gene expression map of *Arabidopsis thaliana* development, Nat. Genet. 37 (2005) 501–506.
[18] S. Fragoso, L. Espindola, J. Paez-Valencia, A. Gamboa, Y. Camacho, E. Martinez-Barajas, P. Coello, L. Espíndola, J. Páez-Valencia, A. Gamboa, Y. Camacho, E. Martínez-Barajas, P. Coello, SnRK1 isoforms AKIN10 and AKIN11 are differentially regulated in *Arabidopsis* plants under phosphate starvation, Plant Physiol. 149 (2009) 1906–1916.
[19] N. Glab, C. Oury, T. Guerinier, S. Domenichini, P. Crozet, M. Thomas, J. Vidal, M. Hodges, The impact of *Arabidopsis thaliana* SNF1-related-kinase 1 (SnKR1)-activating kinase 1 (SnAK1) and SnRK2 on SnRK1 phosphorylation status: characterization of a SnAK double mutant, Plant J. 89 (2017) 1031–1041.
[20] S. Hey, H. Mayerhofer, N.G. Halford, J.R. Dickinson, DNA sequences from *Arabidopsis*, which encode protein kinases and function as upstream regulators of Snf1 in yeast, J. Biol. Chem. 282 (2007) 10472–10479.
[21] P. Crozet, F. Jammes, B. Valot, F. Ambard-Bretteville, S. Nessler, M. Hodges, J. Vidal, M. Thomas, Cross-phosphorylation between *Arabidopsis thaliana* sucrose nonfermenting 1-related protein kinase 1 (AtSnRK1) and its activating kinase (AtSnAK) determines their catalytic activities, J. Biol. Chem. 285 (2010) 12071–12077.
[22] K.J. Bradford, A.B. Downie, O.H. Gee, V. Alvarado, H. Yang, P. Dahal, Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds, Plant Physiol. 132 (2003) 1560–1576.
[23] Q. Shen, Z. Liu, F. Song, Q. Xie, L. Hanley-Bowdoin, X. Zhou, Tomato SlSnRK1 protein interacts with and phosphorylates βC1, a pathogenesis protein encoded by a geminivirus β-satellite, Plant Physiol. 157 (2011) 1394–1406.
[24] P. Crozet, L. Margalha, A. Confraria, A. Rodrigues, C. Martinho, M. Adamo, C.A. Elias, E. Baena-González, Mechanisms of regulation of SNF1/AMPK/SnRK1 protein kinases, Front. Plant Sci. 5 (2014) 190.
[25] S.M. Warden, C. Richardson, J. O'Donnell Jr., D. Stapleton, B.E. Kemp, L.A. Witters, Post-translational modifications of the β-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization, Biochem. J. 354 (2001) 275–283.
[26] K.I. Mitchelhill, B.J. Michell, C.M. House, D. Stapleton, J. Dyck, J. Gamble, C. Ullrich, L.A. Witters, B.E. Kemp, Posttranslational modifications of the 5′-AMP-activated protein kinase β1 subunit, J. Biol. Chem. 272 (1997) 24475–24479.
[27] S. Mangat, C. Dakshayini, R.R. McCartney, K. Elbing, M.C. Schmidt, Differential roles of the glycogen-binding domains of β-subunits in regulation of the Snf1 kinase complex, Eukaryot. Cell 9 (2010) 173–183.

[28] K. Aoki, K. Yano, A. Suzuki, S. Kawamura, N. Sakurai, K. Suda, A. Kurabayashi, T. Suzuki, T. Tsugane, M. Watanabe, K. Ooga, M. Torii, T. Narita, T. Shin-i, Y. Kohara, N. Yamamoto, H. Takahashi, Y. Watanabe, M. Egusa, M. Kodama, Y. Ichinose, M. Kikuchi, S. Fukushima, A. Okabe, T. Arie, Y. Sato, K. Yazawa, S. Satoh, T. Omura, H. Ezura, D. Shibata, Large-scale analysis of full-length cDNAs from the tomato (*Solanum lycopersicum*) cultivar Micro-Tom, a reference system for the Solanaceae genomics, BMC Genomics 11 (2010) 2–6.

[29] S.A. Hawley, M. Davison, A. Woods, S.P. Davies, R.K. Beri, D. Carling, D.G. Hardie, Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase, J. Biol. Chem. 271 (1996) 27879–27887.

[30] C.J. Hastie, H.J. McLauchlan, P. Cohen, Assay of protein kinases using radiolabeled ATP: a protocol, Nat. Protoc. 1 (2006) 968–971.

[31] A.L. Man, P.C. Purcell, U. Hannappel, N.G. Halford, Potato SNF1-related protein kinase: molecular cloning, expression analysis and peptide kinase activity measurements, Plant Mol. Biol. 34 (1997) 31–43.

[32] E.M. Hrabak, C.W.M. Chan, M. Gribskov, J.F. Harper, J.H. Choi, N. Halford, J. Kudla, S. Luan, H.G. Nimmo, M.R. Sussman, M. Thomas, K. Walker-Simmons, J.-K. Zhu, A.C. Harmon, The *Arabidopsis* CDPK-SnRK superfamily of protein kinases, Plant Physiol. 132 (2003) 666–680.

[33] R.P. Bhalerao, K. Salchert, L. Bakó, L. Okrész, L. Szabados, T. Muranaka, Y. Machida, J. Schell, C. Koncz, Regulatory interaction of PRL1 WD protein with *Arabidopsis* SNF1-like protein kinases, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 5322–5327.

[34] J. Beenstock, N. Mooshayef, D. Engelberg, How do protein kinases take a selfie (autophosphorylate)? Trends Biochem. Sci. 41 (2016) 938–953.

[35] J.L. Maya-Bernal, A. Ávila, A. Ruiz-Gayosso, R. Trejo-Fregoso, N. Pulido, A. Sosa-Peinado, E. Zúñiga-Sánchez, E. Martínez-Barajas, R. Rodríguez-Sotresa, P. Coello, Expression of recombinant SnRK1 in *E. coli*. Characterization of adenine nucleotide binding to the SnRK11/AKIN βγ-β3 complex, Plant Sci. 263 (2017) 116–125.

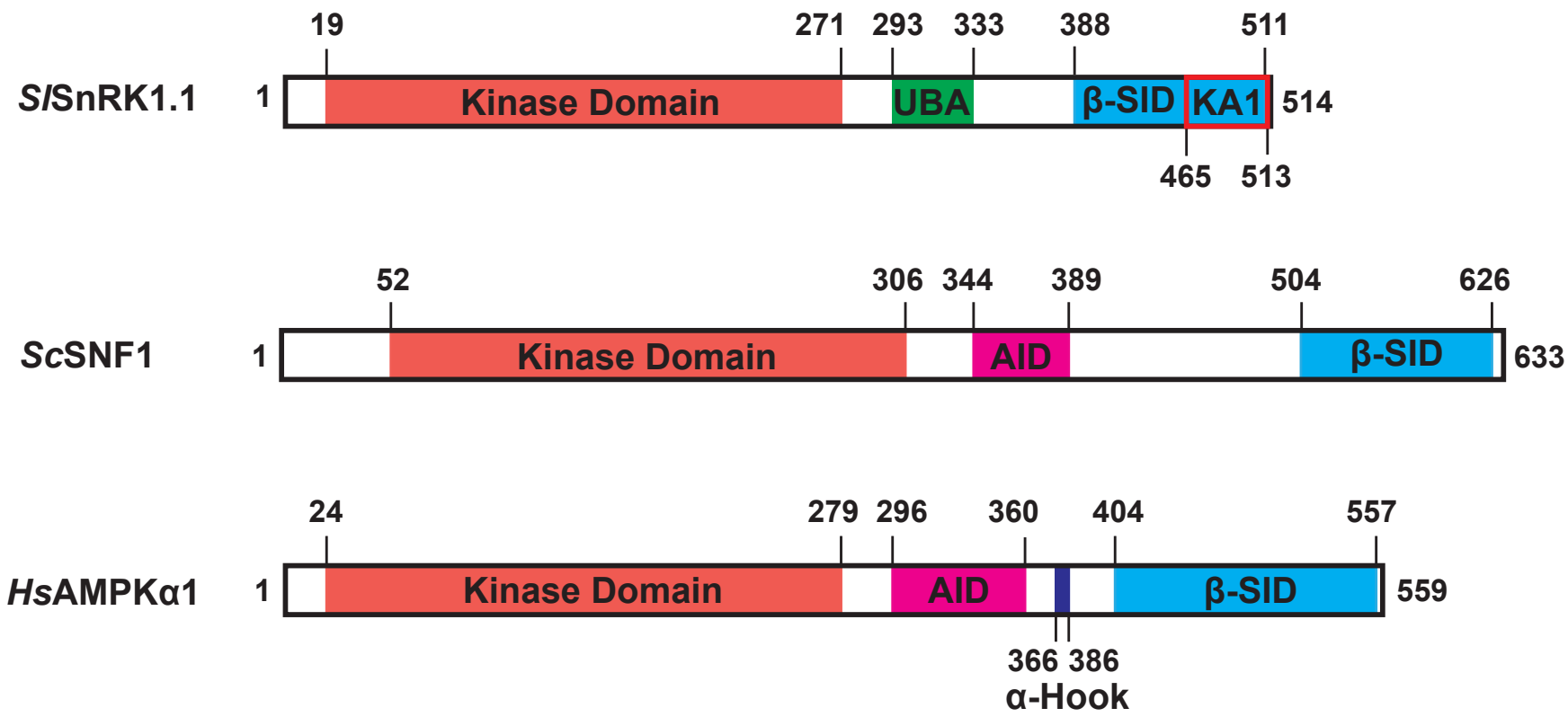
[36] W. Shen, M.I. Reyes, L. Hanley-Bowdoin, *Arabidopsis* protein kinases GRIK1 and GRIK2 specifically activate SnRK1 by phosphorylating its activation loop, Plant Physiol. 150 (2009) 996–1005.

[37] Tomato Functional Genomics Database, http://ted.bti.cornell.edu.

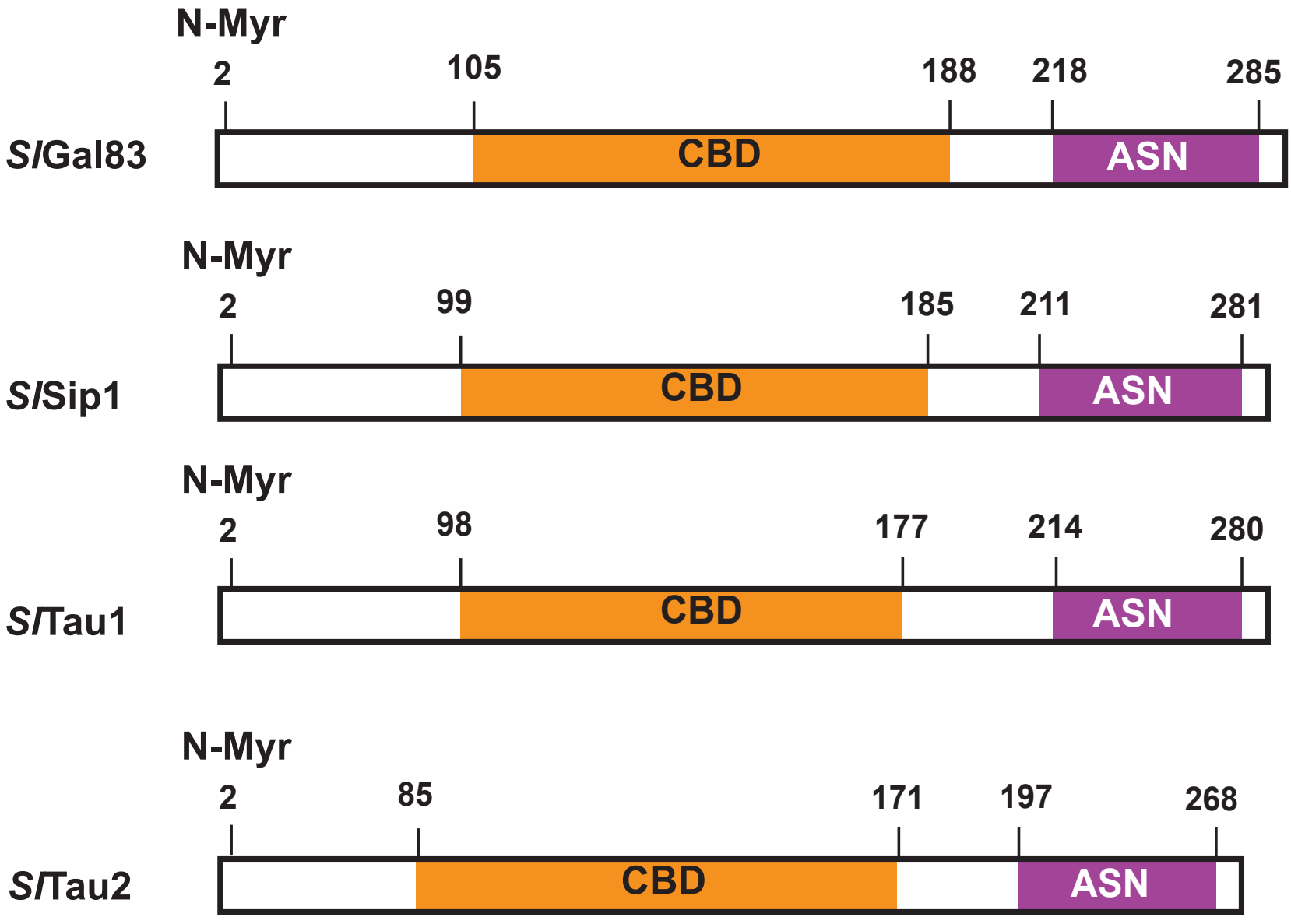
[38] T.P. Devarenne, S.K. Ekengren, K.F. Pedley, G.B. Martin, Adi3 is a Pdk1-interacting AGC kinase that negatively regulates plant cell death, EMBO J. 25 (2006) 255–265.

[39] M.J. Ek-Ramos, J. Avila, A.C. Nelson, D. Su, J.W. Gray, T.P. Devarenne, The tomato cell death suppressor Adi3 is restricted to the endosomal system in response to the *Pseudomonas syringae* effector protein AvrPto, PLoS One 9 (2014) e110807.

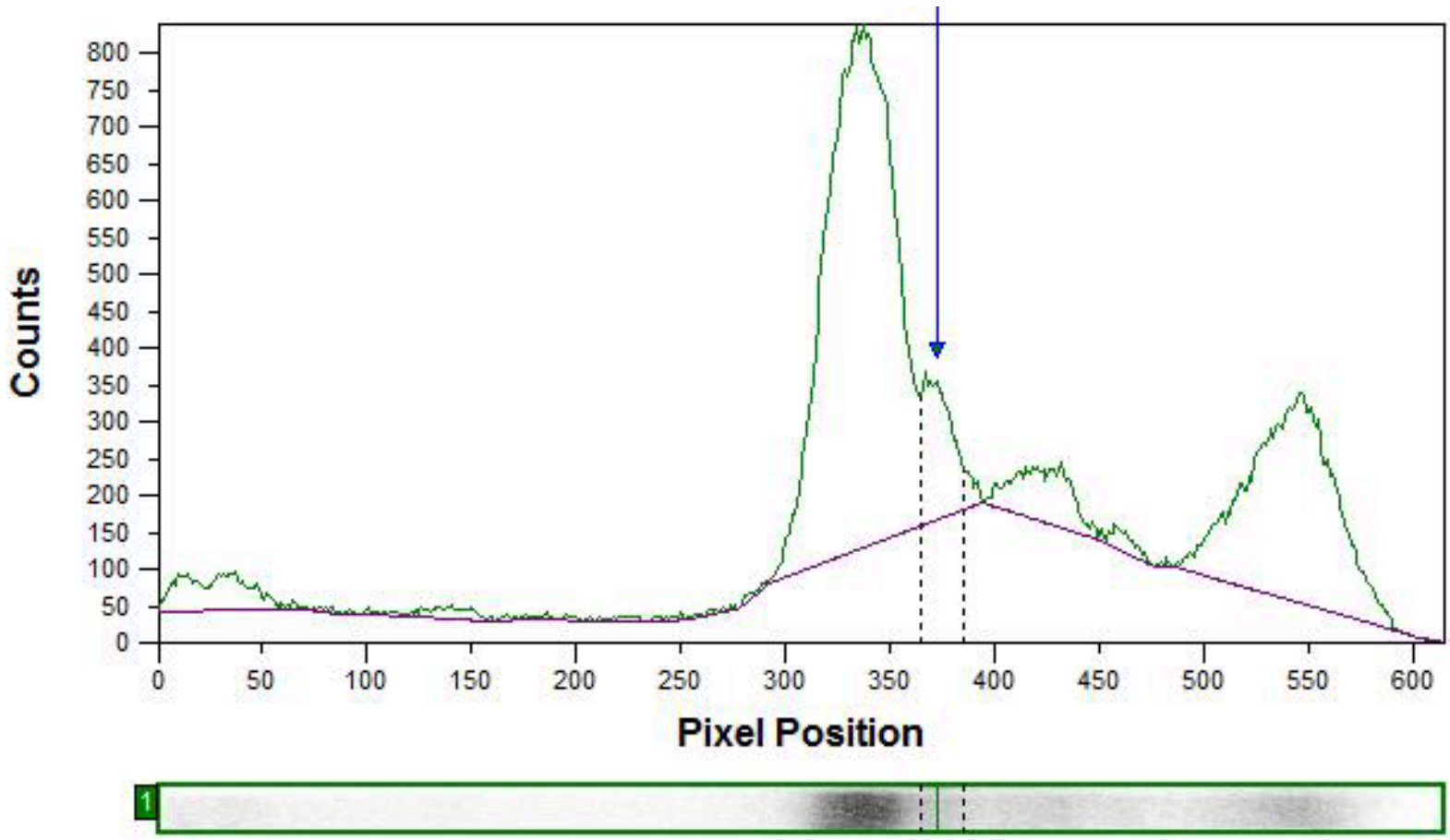
[40] M.J. Ek-Ramos, J. Avila, C. Cheng, G.B. Martin, T.P. Devarenne, The T-loop extension of the tomato protein kinase AvrPto-dependent Pto-interacting protein 3 (Adi3) directs nuclear localization for suppression of plant cell death, J. Biol. Chem. 285 (2010) 17584–17594.



Supplemental Fig. 1. Conserved protein domains of plant SnRK1, yeast SNF1, and mammalian AMPK. Salmon colored box, kinase domain; green box, ubiquitin associated domain (UBA); magenta box, auto-inhibitory domain (AID); light blue box, β-subunit interaction domain (β-SID); dark blue box, α-hook domain, red box, KA1 motif. The kinase domain and β-SID are conserved across yeast, plant and mammals. In plants, there is a conserved KA1 domain within the β-SID domain. Only yeast and mammals have the AID domain that can bind to the unphosphorylated kinase domain for inhibition of kinase activity in the absence of ADP and AMP. The mammalian AMPK also contains an α-hook domain that interacts with the γ-subunit in the presence of ADP or AMP and regulates kinases activity.



Supplemental Fig. 2. Conserved protein domains of tomato SnRK1 β-subunits *Sl*Gal83, *Sl*Sip1, *Sl*Tau1, and *Sl*Tau2. There is a proposed myristoylation (N-Myr) site at N-terminus. Orange box, carbohydrate binding domain (CBD); purple box, association with Snf1 complex (ASN) domain.



Supplemental Fig. 3. Analysis of phosphorimage band volume using ImageQuant TL. Top panel, signal counts for Fig. 3B, lane 1. Green peaks, signal counts at each pixel position of a single gel lane; purple line, background subtraction using rolling ball method; blue arrow, detection peak for Gal83 phosphorylation signal; dotted line, cut-off for Gal83 peak by slope detection. Band volume of Gal83 phosphorylation is calculated by integrating the area below the green peak and above the purple line. Bottom panel, a single phosphor image scan lane in horizontal orientation for Fig. 3B, lane 1.

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PKIN1           ---MSSRGGGIAESPYLRNYRVGKTLGHGSFGKVKIAEHLLTGHKVAIKILNRRKMKTPD 57
SlSnRK1.2       ---MSSRGGGIAESPYLRNYRVGKTLGHGSFGKVKIAEHLLTGHKVAIKILNRRKMKTPD 57
SpKIN10like     ---MSSRGGGIAESPYLRNYRVGKTLGHGSFGKVKIAEHLLTGHKVAIKILNRRKMKTPD 57
AtSnRK1.2       MDHSSNRFGNNGVESILPNYKLGKTLGIGSFGKVKIAEHVVTGHKVAIKILNRRKIKNME 60
AtSnRK1.1       -MDGSGTGSRSGVESILPNYKLGRTLGIGSFGRVKIAEHALTGHKVAIKILNRRKIKNME 59
SlSnRK1.1       -MDGTAVQGTSSVDSFLRNYKLGKTLGIGSFGKVKIAEHTLTGHKVAVKILNRRKIRNMD 59
StubSNF1        -MDGTAVQGTSSVDSFLRNYKLGKTLGIGSFGKVKIAEHTLIGHKVAVKILNRRKIRNMD 59
                    :   .  . .  * **::*:*** ****:****** : *****:********::. :

PKIN1           MEEKLRREIKICRLFVHPHVIRLYEVIETPTDIYVVMEYVKSGELFDYIVEKGRLQEDEA 117
SlSnRK1.2       MEEKLRREIKICRLFVHPHVIRLYEVIETPTDIYVVMEYVKSGELFDYIVEKGRLQEDEA 117
SpKIN10like     MEEKLRREIKICRLFVHPHVIRLYEVIETPTDIYVVMEYVKSGELFDYIVEKGRLQEDEA 117
AtSnRK1.2       MEEKVRREIKILRLFMHPHIIRQYEVIETTSDIYVVMEYVKSGELFDYIVEKGRLQEDEA 120
AtSnRK1.1       MEEKVRREIKILRLFMHPHIIRLYEVIETPTDIYLVMEYVNSGELFDYIVEKGRLQEDEA 119
SlSnRK1.1       MEEKVRREIKILRLFMHPHIIRLYEVIETPSDIYVVMEYVKSGELFDYIVEKGRLQEDEA 119
StubSNF1        MEEKVSREIKILRLFMHGHISRLYEVIETPSDIYVVMEYVKSGELFDYIVEKGRLQEDEA 119
                ****: ***** ***:* *: * ****** :***:*****:********************

PKIN1           RKIFQQIIAGVEYCHKNMVVHRDLKPENLLLDARRNVKIADFGLGNIMRDGHFLKTSCGS 177
SlSnRK1.2       RKFFQQIIAGVEYCHRNMVVHRDLKPENLLLDARRNVKIADFGLGNIMRDGHFLKTSCGS 177
SpKIN10like     RKFFQQIIAGVEYCHRNMVVHRDLKPENLLLDARRNVKIADFGLGNIMRDGHFLKTSCGS 177
AtSnRK1.2       RNFFQQIISGVEYCHRNMVVHRDLKPENLLLDSRCNIKIADFGLSNVMRDGHFLKTSCGS 180
AtSnRK1.1       RNFFQQIISGVEYCHRNMVVHRDLKPENLLLDSKCNVKIADFGLSNIMRDGHFLKTSCGS 179
SlSnRK1.1       RNFFQQIISGVEYCHRNMVVHRDLKPENLLLDSKWNVKIADFGLSNIMRDGHFLKTSCGS 179
StubSNF1        RNFFQQIISGVEYCHINMVVHRDLKPENLLLDSKWNVKIADFGLSNIMRDGHFLKTSCGS 179
                *::*****:****** ****************:: *:*******.*:************
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PKIN1           PNYAAPEVVSGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKSGVYTLPSH 237
SlSnRK1.2       PNYAAPEVVSGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKSGVYTLPSH 237
SpKIN10like     PNYAAPEVVSGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKSGVYTLPSH 237
AtSnRK1.2       PNYAAPEVISGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKGGIYTLPSH 240
AtSnRK1.1       PNYAAPEVISGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKGGIYTLPSH 239
SlSnRK1.1       PNYAAPEVISGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKGGIYTLPSH 239
StubSNF1        PNYAAPEVISGKLYAGPEVDVWSCGVILYALLCGTLPFDDENIPNLFKKIKGGYITLPSH 239
                ********:*******************************************.*  *****

PKIN1           LSPLARDLIPRMLIVDPMKRISVPDIRQHQWFKIHLPRYLAVPPPDARQHLKKLDEEILQ 297
SlSnRK1.2       LSPLARDLIPRMLIVDPMKRISVADIRQHQWFKIHLPRYLAVPPPDARQHLKKLDEEILQ 297
SpKIN10like     LSPLARDLIPRMLIVDPMKRISVADIRQHQWFKIHLPRYLAVPPPDARQHLKKLDEEILQ 297
AtSnRK1.2       LSSEARDLIPRMLIVDPVKRITIPEIRQHRWFQTHLPRYLAVSPPDTVEQAKKINEEIVQ 300
AtSnRK1.1       LSPGARDLIPRMLVVDPMKRVTIPEIRQHPWFQAHLPRYLAVPPPDTVQQAKKIDEEILQ 299
SlSnRK1.1       LSAGARDLIPRMLIVDPMKRMTIPEIRLHPWFQAHLPRYLAVPPPDTTQQAKKIDEEILQ 299
StubSNF1        LSAGARDLIPRMLIVDPMKRMTIPEIRLHPWFQAHLPRYLAVPPPDTMQQAKKIDEEILQ 299
                **  *********:***:**::: :** * **: ******** ***: :: **::***:*

PKIN1           QVSRMGLDRDQLLDSLQKRIQDDATVAYYLLYDNRSMASSGYLGAEFQESVDCYSPGLFP 357
SlSnRK1.2       QVTRMGLDRDQLLDSLQKRIQDDATVAYYLLYDNRSMASSGYLGAEFQESVDGYSSGLFP 357
SpKIN10like     QVTRMGLDRDQLLDSLQKRIQDDATVAYYLLYDNRSMASSGYLGAEFQESVDCYSPGLFP 357
AtSnRK1.2       EVVNMGFDRNQVLESLRNRTQNDATVTYYLLLDNRFRVPSGYLESEFQETTDSGSNPMRT 360
AtSnRK1.1       EVINMGFDRNHLIESLRNRTQNDGTVTYYLILDNRFRASSGYLGAEFQETME-GTPRMHP 358
SlSnRK1.1       EVVKMGFDRNNLTESLRNRVQNEGTVAYYLLLDNRHRVSTGYLGAEFQESMEYGYNRINS 359
StubSNF1        EVVKMGFDRNNLTESLRNRVQNEGTVPYYLLLDNRHRVSTGYLGAEFQESMEYGYNRINS 359
                :* .**:**::: :*::** *::.** ***: ***  . :*** :****: :     :
```

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PKIN1            NLDLQLSTGNG------VSEESLRRPFRKEKMWLVGLQSPANPKEIMNQVLGTLLELNVR411
SlSnRK1.2        NLDLQLSSGNG------VSEESLRRPFRKEKTWLVGLQSPANPKEIMNQVLGTLLELNVR411
SpKIN10like      NLDLQLSTGNG------VSEESLRRPFRKEKTWLVGLQSPANPKEIMNQVLGTLLELNVR411
AtSnRK1.2        PEAGASPVGHWIPAHVDHYGLGARSQVPVDRKWALGLQSHAHPREIMNEVLKALQELNVC420
AtSnRK1.1        AESVASPVSHRLPGLMEYQGVGLRSQYPVERKWALGLQSRAHPREIMTEVLKALQDLNVC418
SlSnRK1.1        NETAASPVGQRFPGIMDYQQAGAR-QFPIERKWALGLQSRAHPREIMTEVLKALQELNVC418
StubSNF1         NETAASPVGQRFPGIMDYQQAGAR-QFPIERKWALGLQSRAHPREIMTEVLKALQELNVC418
                        .:           . *     :: * :**** *:*:***.:** :* :***

PKIN1            WKKIGHYNMKCLWCHDLH--LHSMANNHMNDDDHFISNATAIST--HLQPQPTVKFEMQL467
SlSnRK1.2        WKKIGHYNMKCLWCHDLH--LHSMASNHMNDDDHFISNATAIST--HLQPLPTVKFEMQL467
SpKIN10like      WKKIGHYNMKCLWCHDLH--LHSMASNHMNDDDHFISNATAIST--LLQPLPTVKFEMQL467
AtSnRK1.2        WKKIGHYNMKCRWVPGLADGQNTMVNNQ----LHFRDESSIIEDDCAMTSPTVIKFELQL476
AtSnRK1.1        WKKIGHYNMKCRWVPNSS--ADGMLSNSMHDNNYFGDESSIIENEAAVKSPNVVKFEIQL476
SlSnRK1.1        WKKIGQYNMKCRWVPSLPGHHEGMGVNSMHGNQFFGDDSSIIENDGATKLTNVVKFEVQL478
StubSNF1         WKKIGQYNMKCRWVPSVPGHHEGMGVNSMHGNQFFGDDSSIIENDGDTKLTNVVKFEVQL478
                 *****:***** *  .     . *  *      .* .::: *.          .:***:**

PKIN1            YKTEDEKYLLDLQRISGPQFLFLDFCAGFIRQLEGPQ  504
SlSnRK1.2        YKTEDEKYLLDLQRISGPQFLFLDFCAGFIRQLEGPQ  504
SpKIN10like      YKTEDEKYLLDLQRISGPQFLFLDFCAGFIRQLEGPQ  504
AtSnRK1.2        YKAREEKYLLDIQRVNGPQFLFLDLCAAFLTELRVI-  512
AtSnRK1.1        YKTRDDKYLLDLQRVQGPQFLFLDLCAAFLAQLRVL-  512
SlSnRK1.1        YKTREEKYLLDLQRLQGPQFLFLDLCAAFLAQLRVL-  514
StubSNF1         YKTREEKYLLDLQRIQGPQFLFLDLCAAFLAQLRVL-  514
                 **:.::******:**:.********:**.*: :*.
```
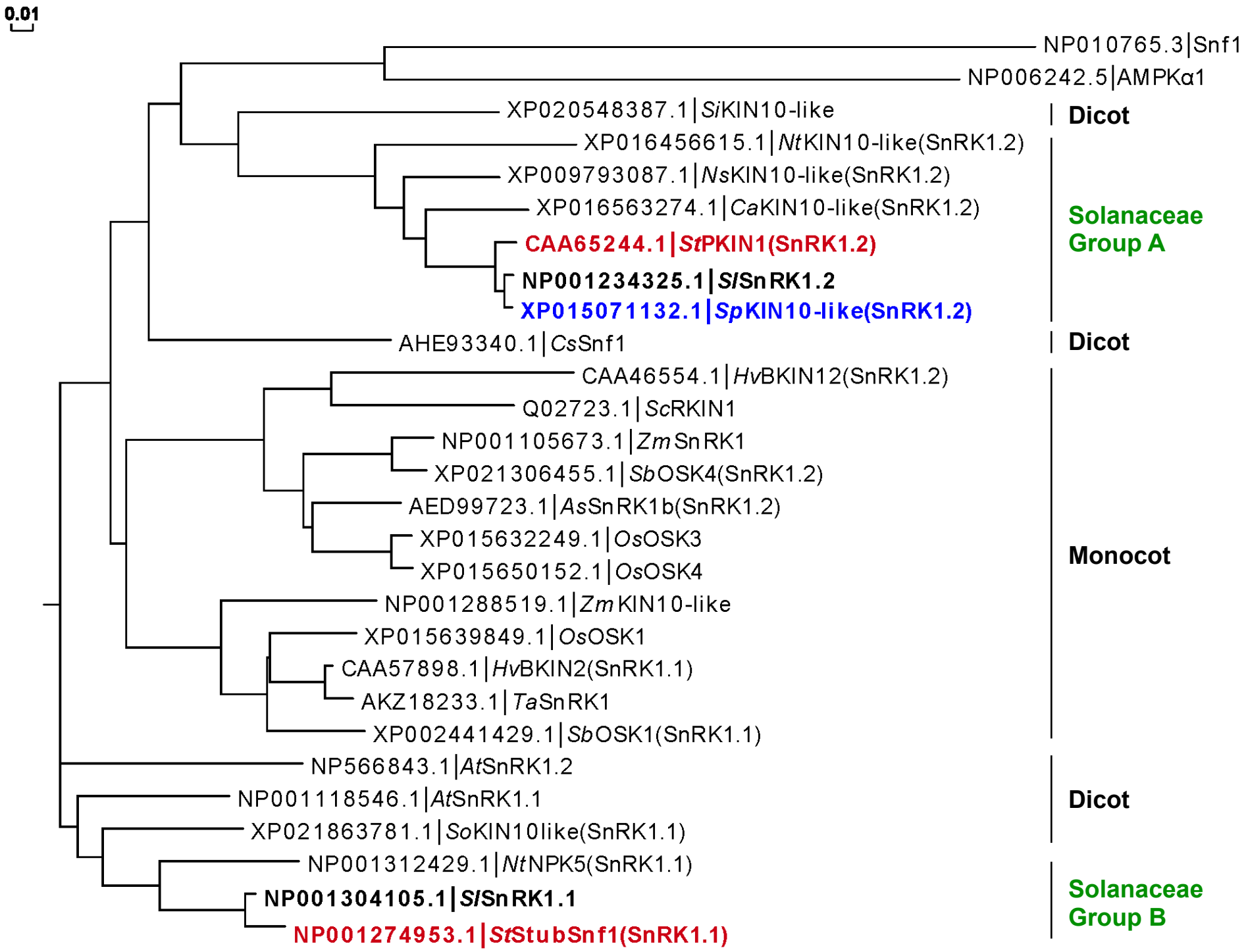
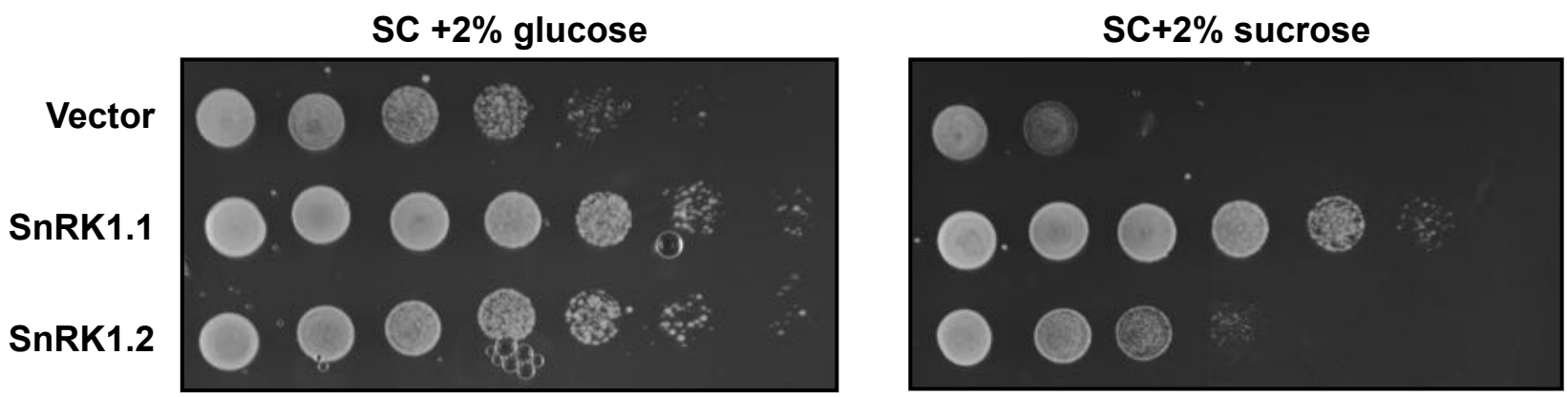

Percent Identity Matrix

	PKIN1	*Sl*SnRK1.2	*Sp*KIN10like	*At*SnRK1.2	*At*SnRK1.1	*Sl*SnRK1.1	*St*ubSNF1
PKIN1	100.00	98.02	98.41	67.74	68.73	68.92	67.73
*Sl*SnRK1.2	98.02	100.00	99.21	67.74	68.92	69.12	67.73
*Sp*KIN10like	98.41	99.21	100.00	67.74	69.12	69.12	67.73
*At*SnRK1.2	67.74	67.74	67.74	100.00	81.10	79.02	77.25
*At*SnRK1.1	68.73	68.92	69.12	81.10	100.00	84.93	83.37
*Sl*SnRK1.1	68.92	69.12	69.12	79.02	84.93	100.00	97.67
*St*ubSNF1	67.73	67.73	67.73	77.25	83.37	97.67	100.00

Supplemental Fig. 4. Alignment of *Arabidopsis*, tomato, and potato SnRK1 α-subunits. Protein sequences of SnRK1 α-subunits from *Arabidopsis* (*At*SnRK1.1, *At*SnRK1.2), tomato (*Sl*SnRK1.1, *Sl*SnRK1.2), wild tomato (*Solanum pennellii*, *Sp*Kin10like), and potato (PKIN1, StubSnf1) were aligned using Clustal Omega. The lysine in Green indicates the conserved key residue for ATP binding. The threonine in dark blue indicates the conserved phosphorylation site for activation. The kinase domain is highlighted in salmon, the UBA domain is highlighted in green, the β-SID domain is highlighted in light blue, and the KA1 domain is highlighted by the red box.



Supplemental Fig. 5. Phylogenetic tree of SnRK1 family proteins. Shown is a neighbor-joining tree without distance corrections generated using Clustal Omega and visualized by EvolView. Scale bar indicates branch length. If SnRK1.1 or SnRK1.2 is not given in the protein name this designation is indicated in parentheses after the protein name. This designation is not known for some proteins. *At*, *Arabidopsis thaliana*; *As*, *Avena sativa*; *Ca*, *Capsicum annuum*; *Cs*, *Camellia sinensis*; *Hv, Hordeum vulgare*; *Nt*, *Nicotiana tabacum*; *Ns*, *Nicotiana sylvestris*; *Os, Oryza sativa*; *Sb*, *Sorghum bicolor*; *Sc, Secale cereale*; *Si*, *Sesamum indicum*; *Sl*, *Solanum lycopersicum*; *So*, *Spinacia oleracea*; *Sp*, *Solanum pennellii*; *St, Solanum tuberosum*; *Ta*, *Triticum aestivum*; *Zm*, *Zea mays*.



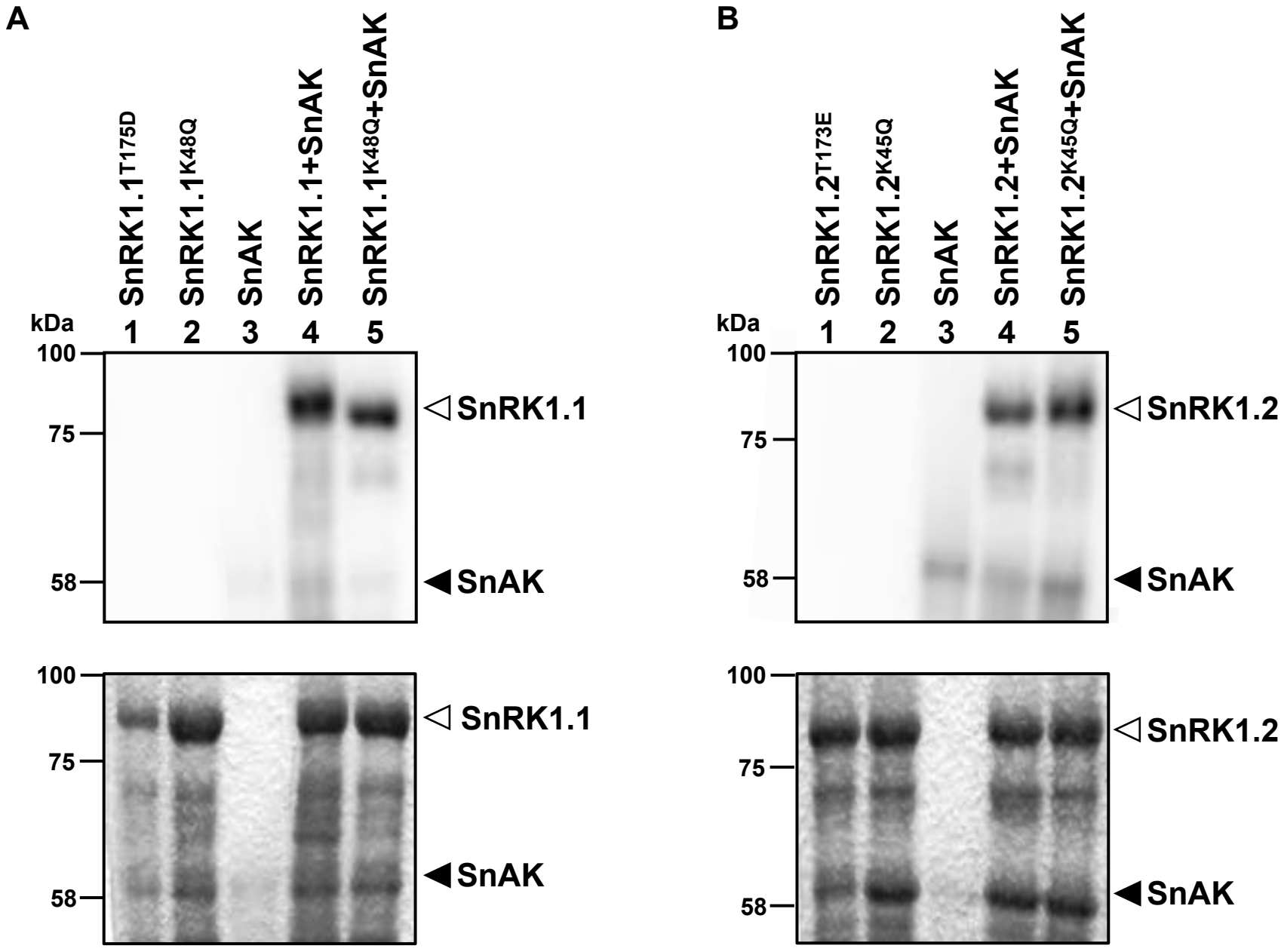
Supplemental Fig. 6. *Sl*SnRK1.2 is able to at least partially compliment a yeast *SNF1* knock-out. *snf1Δ* yeast cells were transformed with empty vector, FLAG-tagged *Sl*SnRK1.1, or FLAG-tagged *Sl*SnRK1.2 and spotted in 5-fold dilutions on SC-Ura plates containing either 2% glucose (left panel) or 2% sucrose (right panel).

```
SlSnAK          MSVMMHSVDEVTEMGCCGCFGFSFARKPKKEIRPNRGYGNSWSHEPLLQQEAEEVEDDGF    60
AtSnAK1         --MFRDSFLFARTIGCFGCFGSSGSRNQQS---PKPYDDDTHSCDSDVTSTAR-------    48
AtSnAK2         --MFCDSFAFAQVMSCFGCFGGSE-RSRHS---PNPYDDDTYSHDSGETSNPG-------    47
                  :: .*.  .  :.* **** *  *. :.   *:   .:: * :    .

SlSnAK          DSGDIIDTGSE-DDEVCHSPVKRYQEILMERAQNGLICREIPVKETHKVVRTEDEDGNKM    119
AtSnAK1         --GEE-E--EDEEEVEQKSRSKRSEEILKYRLDNGLICRHIPVRETNELIRGEDENGDKT    103
AtSnAK2         --GDD-EEGEEEEEVEELSRSKRSEEILKCKLQNGLVCRQFPVKETNKLTRGEDEDGNKT    104
                  *:  :  .: ::    *  ** :***  : :***:**.:**:**::: * ***:*:*

SlSnAK          VNEYVREHKIGAGSYGKVVLYRSCTDGKHYAIKAFHKSHLSKMRVAPSETAMGDVLREVS    179
AtSnAK1         INEYVRVCKIGSGSYGKVVLYRSTLDGQYYAIKAFHKSHLLRLRVAPSETAMSDVLREVM    163
AtSnAK2         INEFVRERKIGSGSYGKVVLYRSTVDDKHYAIKAFHKSHLSRLRVAPSETAMGDVLREVM    164
                :**:**  ***:***********  *.::*********** ::*********.******

SlSnAK          IMKMLCHPNIVNLVEVIDDPETDNFYMVLEYVEGKWVCEDSGPPCVLEENKARLYLRDIV    239
AtSnAK1         IMKILEHPNIVNLIEVIDDPETDHFYMVLEYVDGKWVYDGSGPPGALGEKTARKYLRDIV    223
AtSnAK2         IMKTLEHPNIVNLIEVIDDPEFDDFYMVLEYVDGKWAYDDSGPPGALGEITARKYLRDVV    224
                *** * *******:******* *.********:***. :.**** .* * .** ****:*

SlSnAK          SGLMYLHSHNIIHGDIKPDNLLVSAAGKVKIGDFSVSQVFEDDNDKLRRSPGTPVFTAPE    299
AtSnAK1         TGLMYLHAHDVIHGDIKPDNLLVTSSGTVKIGDFSVSQVFKDDDDQLRRSPGTPVFTAPE    283
AtSnAK2         AGLMYLHAHNVIHGDIKPDNLLVTSTGRVKIGDFSVSQVFKDDDDQLRRSPGTPVFTAPE    284
                :*******:*::**************:* ************:**:*:**********:** *

SlSnAK          CCV--GDRYHGKCADTWAVGVTLYCMILGKYPFLGETLQDTYDKIVNNPIILPDDMNPLL    357
AtSnAK1         CCLVSGITYSGRAADTWAVGVTLYCMILGQYPFLADTLQDTYDKIVNNPLIIPDGLNPLL    343
AtSnAK2         CCL--GITYSGRSADTWAVGVTLYCMILGQYPFLGDTLQDTYDKIVHNPLIIPEGLNPRL    342
                **:  *  * *:.*****************:****.:**********:**:*:*:.:** *

SlSnAK          KNLLEGLLCKDPTQRLTLESVCQHEWFLGDEGPIPQFSCWCQRQKLQKDVQDGSAEDTPT    417
AtSnAK1         RDLIEGLLCKDPSQRMTLKNVSEHPWVIGEDGHVPEYFCWCKRNAASKIEEGEANGISET    403
AtSnAK2         RDLIEGLLCKDPNQRMTLKAVAEHPWITGEDGAISEYCCWCKRKAEEEEDQNHS------    396
                ::*:********.**:**: *.:* *. *::* : :: ***:*:  .:  :. :

SlSnAK          ----417
AtSnAK1         SDPN407
AtSnAK2         ----396
```

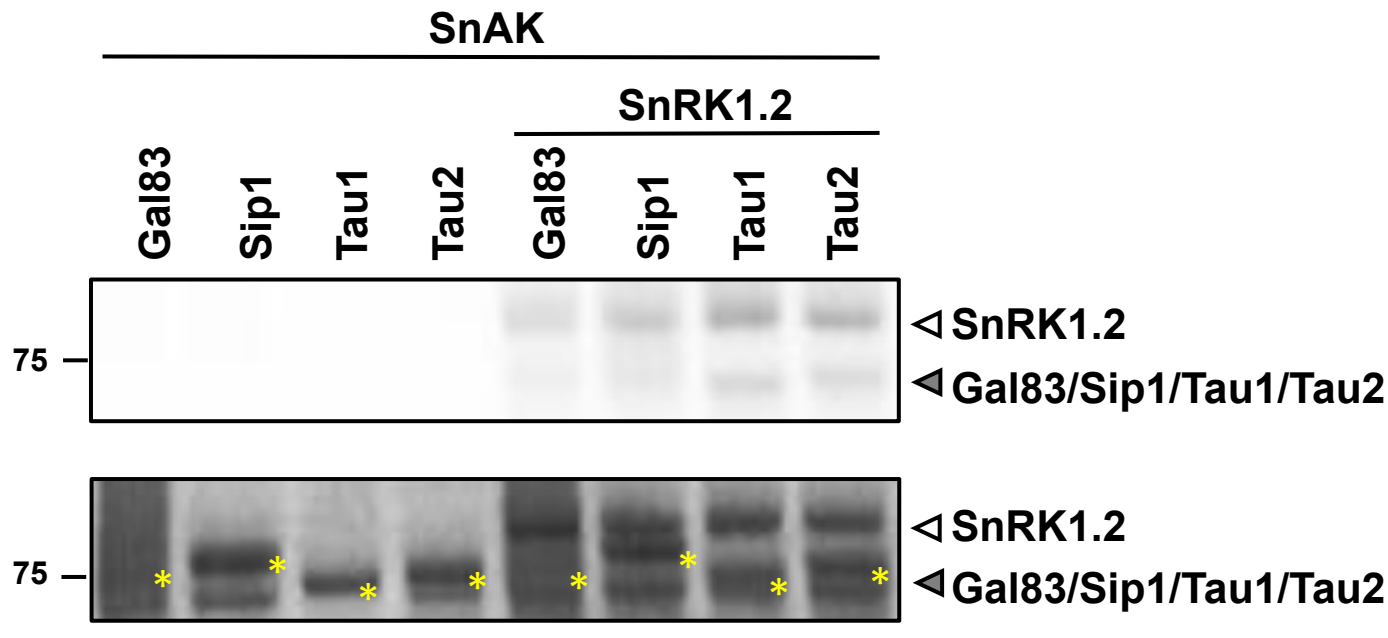
Percent Identity Matrix

	*Sl*SnAK	*At*SnAK1	*At*SnAK2
*Sl*SnAK	100.00	62.25	64.56
*At*SnAK1	62.25	100.00	79.95
*At*SnAK2	64.56	79.95	100.00

Supplemental Fig. 7. Protein sequence alignment of tomato SnAK and *Arabidopsis* SnAK1 and SnAK2 using Clustal Omega. *Sl, Solanum lycopersicum*; *At, Arabidopsis.thaliana.*



Supplemental Fig. 8. *Sl*SnAK phosphorylates *Sl*SnRK1.1 and *Sl*SnRK1.2. In both (A) and (B) top panel, phosphorimage; bottom panel, Coomassie blue-stained gel. Images are representatives from three independent assays. The indicated combinations of proteins were incubated with γ-[^{32}P]ATP in an *in vitro* kinase assay at 30°C for 15 min, with exposure to the phosphorimager screen for the time indicated below. (A) *Sl*SnAK phosphorylation of *Sl*SnRK1.1. Wild-type *Sl*SnRK1.1 (4μg), kinase-active *Sl*SnRK1.1^{T175D} (4μg), kinase-inactive *Sl*SnRK1.1^{K48Q} (4μg), and *Sl*SnAK (1μg) were used in the indicated combinations and the gel was exposed to the phosphorimager screen for 18 hrs. (B) *Sl*SnAK activation of *Sl*SnRK1.2. Wild-type *Sl*SnRK1.2 (4μg), kinase-active *Sl*SnRK1.2^{T173E} (4μg), kinase-inactive *Sl*SnRK1.2^{K45Q} (4μg), and *Sl*SnAK (1μg) were used in indicated combinations and the gel was exposed to the phosphorimager screen for 48 hrs.



Supplemental Fig. 9. Phosphorylation of β-subunits by *Sl*SnAK is below detection level of β-subunit phosphorylation by *Sl*SnAK activated *Sl*SnRK1.2. *Sl*SnAK (1μg) and *Sl*SnRK1.2 (4μg) were pre-activated with non-radioactive ATP at 30°C for 15 min. Different β-subunits (4μg) as indicated were phosphorated by *Sl*SnAK alone or *Sl*SnAK activated *Sl*SnRK1.2 in the presence of γ-[^{32}P]ATP in an *in vitro* kinase assay at 30°C for 30 min, the gel was exposed to the phosphorimager screen for 48 hrs. Top panel, phosphorimage; bottom panel, Coomassie blue-stained gel. *Sl*SnAK was run off the gel in order to impart enough separation between *Sl*SnRK1.2 and the β-subunits. Yellow asterisks indicate the protein bands corresponding to the phosphorated β-subunits in the top panel. The images shown are representative of three independent assays.