

## Short Communication

## Environmental stresses activate a tomato SNF1-related protein kinase 2 homolog, *SlSnRK2C*

Takashi Yuasa<sup>1,\*</sup>, Yoko Tomikubo<sup>2</sup>, Takashi Yamauchi<sup>2</sup>, Akira Inoue<sup>3</sup>, Mari Iwaya-Inoue<sup>1</sup>

<sup>1</sup> Department of Plant Resources, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; <sup>2</sup> Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan; <sup>3</sup> School of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

\* E-mail: [yuasa@agr.kyushu-u.ac.jp](mailto:yuasa@agr.kyushu-u.ac.jp) Tel: +81-92-642-2819 Fax: +81-92-642-2826

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**Abstract** We isolated an SNF1-related kinase 2 homolog (*SlSnRK2C*) from tomato (*Solanum lycopersicum* L. cv. Micro-Tom) which has significant similarity to *AtSRK2C* (72% amino acid identity). We profiled expression levels and biochemical properties of *SlSnRK2C*. In contrast to *AtSRK2C* which phosphorylates histone H1 and is mainly expressed in root chip, *SlSnRK2C* phosphorylates myelin basic protein but not histone H1 and *SlSnRK2C* mRNA is expressed in leaves, flowers and fruits but not in roots. Immunoblot by anti-*SlSnRK2C*-COOH terminus specific antiserum shows that *SlSnRK2C* is expressed remarkably in developing fruits, and weakly in flowers, mature green fruits and shoots, but marginally in mature red fruits or roots. *SlSnRK2C* is stimulated strongly by chilling and NaCl stress. These observations suggest that *SlSnRK2C* is possibly involved in fruit development and stress signaling in response to salt and chilling stress.

**Key words:** Salt stress, SnRK, *Solanum lycopersicum* L.

Environmental stresses—drought and salinity in particular—are major factors limiting the growth and production of crops. The development and improvement of stress tolerance of crops are primary targets for plant molecular and genetic breeding. It is well known that a set of genes underlying the metabolism of osmolytes, ion transporters and chaperone-like heat shock proteins are up-regulated under salt stress, drought and ABA treatment (Shinozaki and Yamaguchi-Shinozaki 2000). In eukaryotes, protein phosphorylation/dephosphorylation plays a pivotal role in the signal cascade involved in stress tolerance and adaptation (Brewster et al. 1993; MAPK Group 1999). Many studies have indicated the involvement of stress signaling cascades composed of second messengers (Knight and Knight 2001), phospholipids (Munnik et al. 2000; Takahashi et al. 2001), protein kinases (Jonak et al. 1996) and phosphatases (Leung et al. 1994). In higher plants (Li and Assmann 1996; Mori and Muto 1997; Hoyos and Zhang 2000) and green algae (Yuasa and Muto 1996), several protein kinases with relative molecular masses of 35 to 50 kDa have been identified by *in-gel* kinase assay as stress-signaling protein kinases

that are specifically stimulated by salt stress, drought or ABA. MAP kinases are activated by various abiotic stresses, including osmotic stress, in plants (Droillard et al. 2000; Ichimura et al. 2000). In addition to MAP kinases, recent genetic and biochemical studies of plant stress-activated protein kinases have revealed that SNF1-related protein kinases (SnRKs) play essential roles in osmotic stress signaling in higher plants (Boudsocq and Lauriere 2005); *Vicia faba* SnRK2 ABA-activated and Ca<sup>2+</sup>-independent protein kinase (AAPK) appeared to be activated by ABA in guard cells (Li et al. 2000), *Arabidopsis thaliana* SRK2E (*AtSRK2E*) and SRK2C (*AtSRK2C*) are activated by ABA (Yoshida et al. 2002) and by drought and NaCl (Umezawa et al. 2004), respectively, and the *Oryza sativa* SnRK2s of OsSAPK1–10 are activated by salt stress (Kobayashi et al. 2004).

Osmotic stress and salinity stresses have been applied to improve the tomato fruit quality with high sugar contents and organic acids (Mitchell et al. 1991). We are concerned about molecular mechanisms involving salt sensing signal and sugar metabolism of tomato fruits

Abbreviations: GST, glutathione-S transferase; MAP kinase, mitogen-activated protein kinase; MBP, maltose-binding protein; MMS, 10 mM MES-KOH (pH 5.5) containing 10 mM MgSO<sub>4</sub> and 2% sucrose; PCR, polymerase chain reaction; SnRK, SNF1-related kinase; TBS, Tris-buffered saline. The nucleotide sequence reported in this paper has been submitted to DDBJ under accession number AB275884 (*LeSRK2C*, a former name of *SlSnRK2C*).

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under osmotic stress and focused on functions of an osmotic stress-activated SnRK2 homolog in tomato as a possible key molecule to improve the fruit quality.

In the present study, we have isolated a tomato SnRK2 homolog, SISnRK2C, from tomato leaves and profiled the expression levels in tomato plants and the activation of ectopically expressed GST-SISnRK2C in response to various stresses by agroinfiltration and immunoprecipitation in *Nicotiana benthamiana* leaves.

A dwarf tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seeds were incubated in 6-cm-square rockwool under continuous light (80W m<sup>-2</sup>). Four-week-old seedlings were subjected to stress treatments, and RNA was prepared from them. *Nicotiana benthamiana* plants were maintained at 25°C with a 16-h photoperiod and 8-h dark period. Six- to eight-week-old plants with 4 to 6 fully expanded leaves were subject to agroinfiltration.

Contig14422 was identified as a tomato SnRK2 by BLAST search using *Arabidopsis* SnRK2 sequences (Yoshida et al. 2002) as search terms against the *Solanum lycopersicum* EST library database at the Kazusa DNA Research Institute (MiBASE). Total RNA of tomato plants was isolated by the SDS/phenol/LiCl method. Single-stranded cDNA was synthesized with total RNA prepared from whole 4-week-old tomato plants by *NotI*-*XhoI*-oligo dT primer (5'-GTGCTCGAGTGCGGCCG-CAAGCTTTTTTTTTTTTTTTT-3') and M-MLV reverse transcriptase (TakaRa, Tokyo, Japan). The partial cDNA fragment of tomato SnRK2 homolog was amplified by PCR with ExTaq DNA polymerase (TakaRa) and a set of primers (SISnRK2-5': 5'-TATGGATCCAAAATGGAA-AGATATGAAATTCAGAAA-3', SISnRK2-3': 5'-GCT-GTTAACAAAATAATCACTTGCTTTAGATCC-3') according to a partial sequence coding SnRK2, EST contig14422, in MiBASE (<http://www.kazusa.or.jp/jsol/microtom/indexj.html>). To isolate the full-length tomato SnRK2 cDNA, we used 3'-RACE method to clone the 3'-extension sequence of contig14422 with a set of nested primers (SISnRK2 (nest1)-5': 5'-AAGAGGATA-ACGATCGAAGAAATAAAG-3', SISnRK2C (nest2)-5': 5'-CATCCATGGTTCTTAAAGAAGCTTGCC-3') and *XhoI*-*NotI*-oligo dT. A pCR-SISnRK2-COOH terminus (pCR-SISnRK2-CT) was constructed by cloning the resultant PCR fragments into a pCR II TOPO TA cloning kit (Invitrogen). An *NcoI*-*NotI* fragment of pCR-SISnRK2-CT was inserted into pCR-partial SISnRK2 to generate pCR-full-length SISnRK2C (pCR-SISnRK2C FL). cDNA fragments were sequenced by an ABI Prism 310 DNA sequencer with a Big Dye Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems).

RT-PCR was performed with total RNA from leaves and other organs of tomato plants by M-MLV reverse transcriptase and ExTaq DNA polymerase according to the manufacturer's manuals with gene-specific primers designed to target the 3' region of *SISnRK2C* cDNA (this

study); *LeHSP17.6* (U72396) (Kadyrzhanova et al. 1998); *LeRac1* (contig15492), a tomato homologue of *Arac1* (At2g17800); and *LeCOR413* (Contig7260), a tomato homologue of *Atcor413-pm1* (At3g50830). Sequences used for RT-PCR were follows: SISnRK2C forward, 5'-CATCCATGGTTCTTAAAGAAGCTTGCC-3' and reverse 5'-TTATCTAAGAAAAGTATCACTCAT-3'; *LeHSP17.6*, forward 5'-GCCGGATCCAAAATGG-ATTTGAGGTTGTTGGGTATCGAT-3' and reverse 5'-CCGAATCAAGCAACTTTACACCTCAATTGTTTTGG-3'; *LeRac1*, forward 5'-ATAGGATCCAAAATGAG-CGCATCCAGGTTTCATCAAGTGT-3' and reverse 5'-A-ACAAGCTTCTACAAAATTGAGCAGGCCCTTTGG-GACTT-3'; *LeCOR413*, forward 5'-ATGGGTAGGATG-GATTATTTGGCTATG-3' and reverse 5'-TCAGACGG-CTCGAAGAACCAGAGC-3'. To synthesize 1st stranded cDNAs, a total 20 µL of reverse transcription reaction mixture containing 1 µg of total RNA, 2.5 µM oligo-dT<sub>15-18</sub>, each 0.5 mM sNTPs, 40 unit of cloned RNase inhibitor and 200 unit of M-MLV reverse transcriptase (TaKaRa) in the standard buffer condition was incubated at 42°C for 60 min after annealing. Amplification was carried out in the program Temp Control System PC-816 (ASTECH) with each 20 µL of PCR reaction mixture containing 1 µL of the 1st strand cDNA sample, each 200 µM dNTPs, each 400 nM 5'- and 3'-gene specific primers, and 1 unit of ExTaq DNA polymerase (TaKaRa) in the standard PCR reaction buffer under following conditions: initial denaturizing at 94°C for 2 min; followed by 3-step cycling of denaturing at 94°C for 20 sec, annealing at 58°C for 20 sec, extension at 72°C for 40 sec; final extension at 72°C for 5 min. The cycle numbers for each gene were: 27 or 30 for *SISnRK2C*; and 30 for *LeHSP17.6*, *LeRac1*, and *LeCOR413*. The identity of these RT-PCR products was confirmed by sequencing.

For expression of maltose-binding protein-fused (MBP) SISnRK2C in *E. coli*. A *BamHI*-*XhoI* fragment of full-length *SISnRK2C* was inserted into *BamHI*-*SalI* sites of pMalc (New England Biolabs). A *BamHI*-*ScaI* fragment of pMalc-SISnRK2C was inserted into *BamHI*-*SmaI* sites of a glutathione S-transferase(GST)-tagged expression vector, pBE2113-GST (Yuasa et al. 2005), to generate pBE2113-GST-SISnRK2C. Then the *XhoI*-*SpeI* fragment was subcloned into *XhoI*-*SpeI* sites in pTA7002, a DEX-inducible vector (Aoyama and Chua 1997). pTA7002-GST-SISnRK2C is used for transient production of GST-tagged polypeptides in higher plants by agroinfiltration with *Agrobacterium tumefaciens* EHA105.

MBP-SISnRK2C was purified from extracts of *E. coli* containing pMal-SISnRK2C with in a column of amylose resin according to the manufacturer's instruction manual. The purified MBP-SISnRK2C was dialysed overnight at 4°C in 1×TBS containing 0.5 mM EDTA, 0.1% Triton X-100, 0.1% β-mercaptoethanol and 30%

glycerol and then stored at  $-40^{\circ}\text{C}$ .

To raise a specific antibody against a COOH-terminus polypeptide (CT) of *SISnRK2C* in rabbit, pCR *SISnRK2C*-CT was digested with *Bam*HI and *Xho*I and then the resultant DNA fragment was inserted into *Bam*HI–*Sall*I sites of pQE9 (Qiagen Inc., Valencia, CA, USA) to construct pQE-*SISnRK2C*-CT. His(6-tagged *SISnRK2C*-CT (256–345 aa) (Hisx6-*SISnRK2C*-CT) was produced in BL21DE3 *E. coli* cells. His $\times$ 6-*SISnRK2C*-CT were purified from extracts of *E. coli* containing pQE-*SISnRK2C*-CT with an Ni-NTA His-Bind Resin (Qiagen) column according to the manufacturer's instruction manual. Rabbit antiserum was raised against His $\times$ 6-*SISnRK2C*-CT (0.5 mg protein). We confirmed by immunoblot analysis that the anti-*SISnRK2C*-CT antiserum cross-reacted with endogenous polypeptides with relative molecular masses of 35 kDa in tomato immature fruit extracts (Figure 2C) and with GST-*SISnRK2C* produced in *N. benthamiana* (Figure 3B, lower).

Agroinfiltration assay was performed as previously described (Yuasa *et al.* 2005) with minor modifications.  $\text{OD}_{600}$  of *A. tumefaciens* EHA105 suspension containing agrobinary plasmids was adjusted to 0.5 in MMS (10 mM MES-KOH (pH 5.5), 10 mM  $\text{MgSO}_4$ , 2% sucrose and 0.25 mM acetosyringone). The *Agrobacterium* suspension was infiltrated through a 1-mL syringe into *N. benthamiana* leaves pin-holed with a #23 needle, and then plants were covered with transparent plastic bags and incubated at  $25^{\circ}\text{C}$  under a 16-h photoperiod and 8 h dark for 24 h. The leaves were sprayed with  $25\ \mu\text{M}$  dexamethazone, then 24 h later were subjected to stress treatments. Stress-treated leaves were frozen in liquid  $\text{N}_2$  and then stored at  $-80^{\circ}\text{C}$ .

Frozen leaves were homogenized on an ice-cooled motor in an equal weight of lysis buffer containing 20 mM HEPES-NaOH, 50 mM  $\text{Na}_2$ - $\beta$ -glycerophosphate (pH 7.6), 5 mM EDTA, 5 mM EGTA, 30 mM NaF, 5 mM  $\text{Na}_3\text{VO}_4$ , 10% glycerol, 1% Triton X-100, 0.1%  $\beta$ -mercaptoethanol, 1 mM PMSF, 5 mM *n*-aminocaproic acid, 1 mM benzimidazole and 1 mM Na-bisulphite. The resultant homogenates were centrifuged at  $20,000\times g$  for 20 min at  $4^{\circ}\text{C}$ . Protein concentrations in samples were measured by Bio Rad protein assay kit (BioRad) with bovine serum albumin as standards. Then, the supernatant (containing 5 mg protein) was mixed with  $0.5\ \mu\text{L}$  anti-GST-antibody (G7781)(Sigma) and  $30\ \mu\text{L}$  50% (v/v) protein G-Sepharose CL-4B bead (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The suspensions were rotated for 2 h at  $4^{\circ}\text{C}$ . The immune-complex beads were precipitated by centrifugation at  $10,000\times g$  for 1 min at  $4^{\circ}\text{C}$ , then beads were washed in 1 mL of washing buffer containing 25 mM Tris·HCl (pH 7.4) 1 M NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Triton X-100 and 0.1%  $\beta$ -mercaptoethanol. After 3 more

washes, the immuno-decorated beads were equilibrated in *in vitro* protein kinase assay buffer as described below.

*In vitro* kinase assay with immunoprecipitates or MBP-*SISnRK2C* as follows. The reaction mixture contained 25 mM Tris·HCl (pH 7.5), 10 mM  $\text{MgSO}_4$ , 2 mM EGTA 0.1%, 10 mM  $\text{Na}_2$ - $\beta$ -glycerophosphate, 2 mM  $\text{Na}_3$ -orthovanadate  $\beta$ -mercaptoethanol, 0.2 mg  $\text{mL}^{-1}$  of myelin basic protein, histone or casein (Sigma), and 1 mM ATP in a total volume of  $50\ \mu\text{L}$ . The reactions were performed at  $30^{\circ}\text{C}$  for 180 min and were then terminated by addition of SDS-PAGE sample buffer. After electrophoresis, acrylamide gels were stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) according to the instruction manuals, and then phosphorylated polypeptides in gel were visualized by an imaging analyzer FluorChem (Alpha Innotech) with 354 nm excitation/595 nm emission (AlphaInnotech).

Immunoblot was carried out with anti-*SISnRK2C*-CT antiserum (1/2,000 dilution (v/v)) in TBS-milk containing 0.05% Tween 20 (TBS–milk–Tween 20) and horseradish peroxidase(HRP)–conjugated protein G (GE Healthcare Bio-Sciences) (1/10,000 dilution (v/v)) in TBS–milk–Tween 20. The immuno-decorated protein bands were visualized by ECL Plus kit (GE Healthcare Bio-Sciences) and FluorChem.

By searching an EST database of MiBASE assembled from our Micro-Tom ESTs and public available tomato ESTs, we identified contig14422 as encoding a kinase domain closely related to SnRK2-class protein kinases (Yoshida *et al.* 2002). The full-length tomato SnRK2 cDNA was obtained by PCR and 3'-RACE from a tomato 'Micro-Tom' cDNA library and primers based on contig14422. The resultant cDNA, named *SISnRK2C* (of which the former name is *LeSRK2C*, accession number: AB275884), was 1.6 kb in length and had an ORF of 1038 bp, encoding a predicted protein of 345 amino acids with a relative molecular weight of 39.3 kDa and a putative pI of 5.5. The SnRK2-class kinases are composed of two parts, a highly conserved N-terminal kinase domain similar to that of SNF1/AMPK, and a divergent COOH-terminus containing putative regulatory regions rich in acidic and hydrophobic amino acids. The predicted protein and the parameters of the *SISnRK2C* cDNA are highly similar (72% amino acid identity) to those of *SRK2C/SnRK2.8/OSK1.4* (relative molecular mass of 38.25 kDa and putative pI of 5.38) of *Arabidopsis* SnRK2 subclass II (Mustilli *et al.* 2002; Hrabak *et al.* 2003; Umezawa *et al.* 2004), OsSAPK1 (Kobayashi *et al.* 2004) and PKABA1 of wheat (Anderberg and Walker-Simmons 1992). A phylogenetic tree showed that SnRK2 subfamily kinases can be subdivided into three subclasses (Hrabak *et al.* 2003; Kobayashi *et al.* 2004). *SISnRK2C* can be placed in the SnRK2 subclass III with AtSRK2C, OsSAPK1 and PKABA1 (Figure 1B). The COOH terminus of

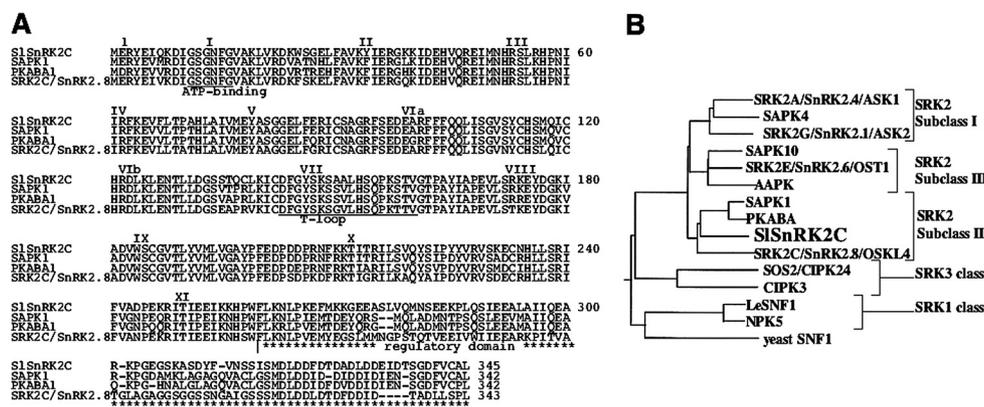


Figure 1. Deduced amino acid sequence of SiSnRK2C and alignment with related sequences and phylogenetic analysis. (A) Deduced amino acid sequence of SiSnRK2C. The deduced amino acid sequence of SiSnRK2C (of which former name is LeSRK2C, accession number: AB275884) was aligned with the sequences from SAPK1 (AB125302) of *Oryza sativa*, and PKABA (BAB61735) of *Hordeum vulgare* and SnRK2C/SnRK2.8/OSKL4 (At1g78290) of *Arabidopsis thaliana*. The numbers on the right indicate the amino acid positions. Gaps, indicated by double line (=), were introduced to maximize alignment. The boundaries of the kinase domain and the putative regulatory domain at the carboxyl terminus were indicated (|). (B) Phylogenetic tree of SiSnRK2 and related proteins. Sequences used for phylogenetic analysis were as follows: SRK2A/SnRK2.4/ASK1 (At1g10940), SRK2E/SnRK2.6/OST1 (At4g33950), SRK2G/SnRK2.1/ASK2 (At5g08590), CIPK3 (At2g26980) and SOS2/CIPK24 (At5g35410) of *A. thaliana*, SAPK4 (AB125305) and SAPK10 (AB125311) of *Oryza sativa*, LeSNF1 (AF143743) of *Lycopersicon esculentum*, NPK5 (D26602) of *Nicotiana tabacum*, AAPK (AF186020) of *Vicia faba* and SNF1 (M13971) of *Saccharomyces cerevisiae*. Amino acid sequences were aligned using the CLUSTALW program (<http://align.genome.jp/>), and the corresponding tree was built.

SiSnRK2C shows that the acidic patches are rich in Asp, as are other proteins of SnRK2 subclass III, and that the COOH-end sequence is very similar to those of OsSAPK1, PKABA1 and AtSRK2C (Figure 1A).

Expression pattern of *SiSnRK2C* mRNA was analyzed by RT-PCR with total RNA prepared from various organs of tomato plants. Signals of *SiSnRK2C* and *LeHSP17.6* were detected in mature and young leaves and in flowers and young and ripening fruits, but not in stems or root (Figure 2A), whereas *LeRac1* was expressed in all those organs. This tissue distribution of *SiSnRK2C* in tomato is not consistent with an observation that *AtSRK2C* is expressed abundantly in roots and weakly in leaves and siliques of *A. thaliana* (Umezawa et al. 2004). AtSRK2C is a root-specific protein kinase in *Arabidopsis* and may function as a signaling molecule of water and nutrient status in soil. In *A. thaliana*, other SnRK2s are expressed in vegetative tissues, as is *SiSnRK2C* in tomato. Kobayashi et al. (2004) reported that *SAPK1*, *SAPK2* and *SAPK3*, rice orthologues of *AtSRK2C* belonging to SnRK2 subclass II, were expressed in blades and sheaths of rice seedlings as well as in the roots. *SRK2E/OST1* is specifically expressed in guard cells but not in mesophyll cells, whereas the mRNA was detected in various tissues, including leaves, stems, flowers and roots (Mustilli et al. 2002).

Effect of various environmental stresses on mRNA levels of *SiSnRK2C* in leaves was analyzed. There was no, or only marginal, alteration of *SiSnRK2C* expression levels in RT-PCR analysis when tomato plants were subjected to NaCl, drought, ABA and chilling (Figure 2B). In fact, *SiSnRK2C* appeared to be constitutively expressed under those treatments. On the other hand,

*OsSAPK1* is induced by ABA, NaCl and mannitol (Kobayashi et al. 2004). *PKABA* in barley is upregulated by ABA and is involved in maintaining seed dormancy (Gomez-Cadenas et al. 1999).

Immunoblot by anti-SiSnRK2C-CT antiserum detected endogenous SiSnRK2C at significant levels in flowers (FL) and developing young fruits, immature fruit (IM) at 10DAF and immature green (IG) at 20DAF, and weakly in mature green fruits (MG) at 30DAF and leaf/stem (LS) but at marginal levels in mature orange (OR) at 40DAF, mature red fruits (MR) at 50DAF and roots (Figure 2C), whereas *SiSnRK2C* mRNA was expressed in mature and young leaves, flower and young and ripening fruits, but not in root or stem (Figure 2B). Two immuno-reacting signals with relative molecular masses of 35 kDa and 32 kDa were detected significantly in FL and weakly in LS, while single immunoreactive signals at 35 kDa were detected in developing fruits, IM and IG, and weakly in MG (Figure 2C). The immuno-detected 32-kDa polypeptides may result from degradation of the 35-kDa SiSnRK2C polypeptides. The difference of organ-specific expression levels between RT-PCR and immunodetection by anti-SiSnRK2C-antibody may be due to the difference in stabilities between *SiSnRK2C* mRNA and the endogenous SiSnRK2C polypeptides, or to degradation during protein extraction from tissues. Alternatively, mechanisms of degradation or stabilization of proteins possibly regulates the protein level of SiSnRK2C during development, because SiSnRK2C mRNA was essentially constitutively expressed in various organs. The immunoblot data suggests that SiSnRK2C functions in fruit specific development in addition to stress signaling in photosynthetic tissue.

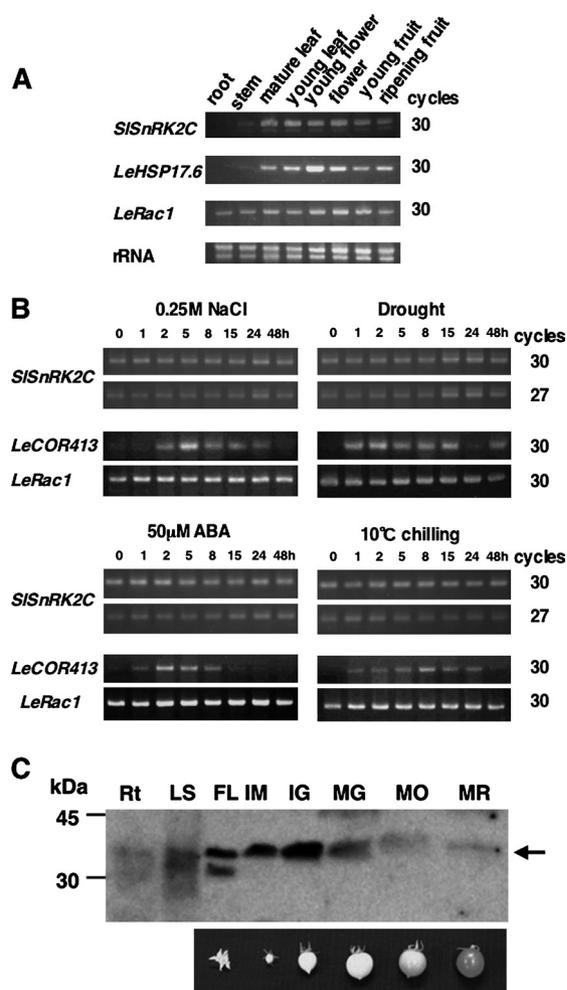


Figure 2. Expression of *SISnRK2C*. (A) RT-PCR analysis of *SISnRK2C* mRNA accumulation in tomato root, stem, mature leaf, young leaf, young flower, mature flower, young fruit and mature fruit. The cycling numbers for each gene were 30 in RT-PCR. (B) RT-PCR analysis of *SISnRK2C* mRNA accumulation in tomato leaves in response to various environmental stress treatments. The cycling numbers in RT-PCR were 25 and 30 for *SISnRK2C*; and 30 for *LeCOR413* and *LeRac1*, as indicated. (C) Accumulation of *SISnRK2C* in tomato fruits at various growth stages. Immunoblot was carried out with soluble fractions of tomato organs by anti-*SISnRK2C*-CT specific antiserum and enhanced chemiluminescence. 50 µg protein per lane from tomato root (Rt), leaf/stem (LS), flower (FL) and fruits of indicated developmental stages; immature fruit (IM) at 10 days after flowering (DAF), immature green (IG) at 20DAF, mature green (MG) at 30DAF, mature orange (MO) at 40DAF and mature red (MR) at 50DAF were subjected to SDS-PAGE and immunoblot. An immunoreactive signal at 35 kDa according to *SISnRK2C* polypeptide is indicated by an arrow (←). Tomato flower and developing fruits were shown in a lower panel of panel C. Immuno-decorated signals of endogenous *SISnRK2C* polypeptides on PVDF membrane were visualized by ECL Plus kit with FluorChem.

Several studies using recombinant proteins reported that SnRK2s expressed in *E. coli* cells show no activity (Li et al. 2000; Johnson et al. 2002; Mustilli et al. 2002; Kelner et al. 2004). We successfully purified a maltose binding protein tagged recombinant *SISnRK2C* protein in active form and detected its kinase activity in *in vitro*

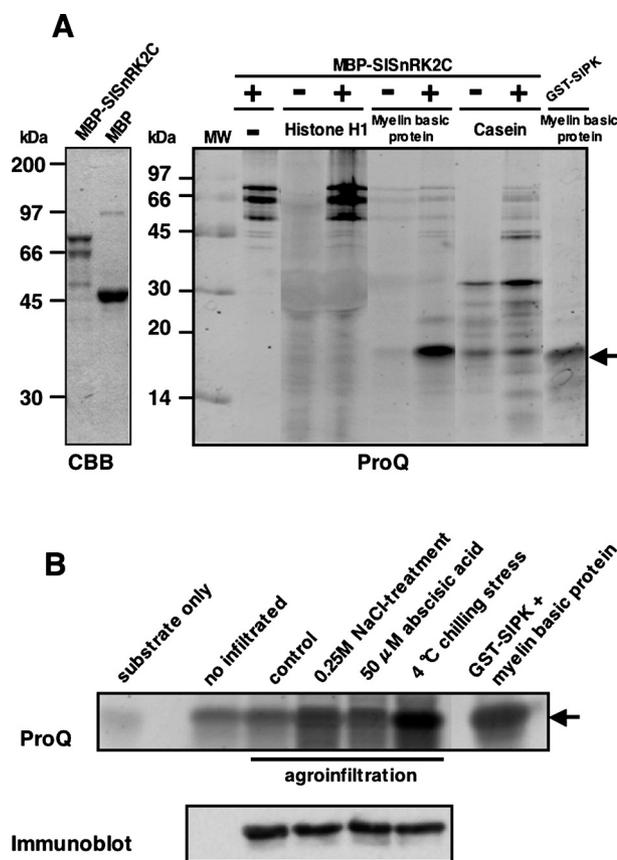


Figure 3. Protein kinase activity of recombinant maltose-binding-protein (MBP)-fused *SISnRK2C* and GST-*SISnRK2C* transiently expressed in *N. benthamiana* leaves by agroinfiltration. (A) Substrate specificity of recombinant MBP-*SISnRK2C* purified from *E. coli*. Purified MBP-*SISnRK2C* and MBP in SDS-PAGE gel (10%) are shown by Coomassie Brilliant Blue staining (left). Phosphoproteins in SDS-PAGE gel (15%) are shown by Pro-Q staining (right). Arrow indicates phosphorylated myelin basic protein. (B) Protein kinase activities of GST-*SISnRK2C* expressed in *N. benthamiana* leaves treated with various environmental stresses. *N. benthamiana* leaves were agroinfiltrated with *Agrobacterium* suspension containing pTA7002-GST and then GST-*SISnRK2C* was induced with 25 µM dexamethazone at 24 h after agroinfiltration. Stress treatments and immunoprecipitation were carried out as described in the text. The purified proteins on the immune-complex beads were assayed by *in vitro* protein kinase assay using myelin basic protein as substrate. After SDS-PAGE, phosphoproteins were stained with Pro-Q Diamond (Molecular Probes) and visualized by fluorescence imager (FluorChem, AlphaInnotech). Arrow indicates Pro-Q-stained signals of phosphorylated myelin basic protein. Immunoblot: GST-*SISnRK2C* polypeptide in leaf extracts was immuno-detected with anti-*SISnRK2C*-CT-specific antiserum and HRP-conjugated protein G was carried out with ECL Plus kit and FluorChem.

kinase assay (Figure 3A). We also detected *in vitro* kinase activity of GST-tagged *SISnRK2C* expressed in *E. coli* (data not shown). The inconsistent results in kinase activity of the recombinant SnRK2s among several groups may be due to the stability or folding conditions of recombinant SnRK2s expressed in *E. coli* cells or the specific properties of molecular species of SnRK2s. We examined whether *SISnRK2C* can phosphorylate generic substrates, histone H1, myelin

basic protein and dephosphorylated casein. MBP-SISnRK2C preferentially phosphorylated myelin basic protein, rather than histone H1 and casein (Figure 3A, right). The presence of free  $\text{Ca}^{2+}$  in the assay did not stimulate kinase activity of SISnRK2C (data not shown).

Extensive biochemical studies on protein kinases indicate that phosphorylation of activation loop (T-loop) in protein kinases is often necessary for kinase activity and regulation of the proteins (Johnson et al. 1996; Adams 2003). It was reported that multiple serine and threonine residues in SnRK2s are autophosphorylated and that the autophosphorylation of a specific serine residue in the activation T-loop of SnRK2s is critical for the activation mechanisms (Kobayashi et al. 2004; Belin et al. 2006). When MBP-SISnRK2C was autophosphorylated in the absence of substrate, three phosphorylated polypeptides were detected (Figure 3A, right) with molecular masses with almost same sizes of 85, 66 and 55 kDa as those of purified polypeptides shown in Coomassie Brilliant Blue stained gel (left). It is not clear whether SISnRK2C itself or MBP was phosphorylated in the assay. *In vitro* kinase assay also showed that SISnRK2C is significantly autophosphorylated in the presence of histone H1 but not or marginally in the presence of myelin basic protein or casein (Figure 3A, right). The observation of significant autophosphorylation of SISnRK2C in the presence of histone H1 is consistent with autophosphorylation of OSRK1 (Chae et al. 2006) and OST1/AtSnRK2E (Belin et al. 2006) while OSRK1 and OST1 phosphorylates both histone H1. Failure of phosphorylation of histone H1 by the recombinant SISnRK2C suggests that SISnRK2C has distinct substrate specificity from that of OST1/AtSRK2E and OSRK1.

In the next, we examined whether transiently expressed SISnRK2C in agroinfiltrated leaves was stimulated in response to various stresses. When GST-SISnRK2C was expressed in agroinfiltrated leaves, GST-SISnRK2C polypeptide was detected by immunoblot (Figure 3B, lower). The leaves were treated with 0.25 M NaCl, 50  $\mu\text{M}$  ABA for 20 min or chilling (4°C) for 60 min. To analyze the specific activity of GST-SISnRK2C expressed in *N. benthamiana* leaves, we purified GST-SISnRK2C in the extracts of the leaves with anti-GST antibody and protein G-Sepharose beads and then analyzed it by *in vitro* kinase assay using myelin basic protein. GST-SISnRK2C was significantly activated by 0.25 M NaCl and chilling, as indicated by the signals of phosphorylation of myelin basic protein, and weakly activated by ABA (Figure 3B, upper panel). These results indicate that SISnRK2C is a stress-activated protein kinase in tomato, which is stimulated under NaCl stress and chilling stress.

Tomato fruit development is well understood from a hormonal and developmental regulatory changes in sugar

and cell wall metabolism and pigment synthesis by analyzing metabolites and transcript levels (Carrari et al. 2006). In plants, SnRKs and CDPKs also play an important role in sugar metabolism by directly phosphorylating the key enzymes (Rolland et al. 2002; Boudsocq and Lauriere 2005). Anguenot et al. (2006) reported that activity and intracellular localization of sucrose synthase are regulated by CDPK in growing fruits of tomato. A SNF1-like gene, together with genes encoding enzymes in primary sugar metabolism (ADP-glucose pyrophosphorylase and sucrose synthase), was shown to be expressed asymmetrically in tomato specific tissues (Pien et al. 2001). The high expression levels of SISnRK2C in flower, IM and IG fruits prompts us to speculate that SISnRK2C may play important roles on stress signaling and/or metabolisms in flower and growing fruit.

SISnRK2C shows high similarity to those of OsSAPK1 and AtSRK2C. But in spite of the significant homology, our immunoblot and biochemical assays indicate that SISnRK2C has distinct features from AtSRK2C and OsSAPK1 as follows; 1) SISnRK2C is highly expressed in immature fruit and flower, and weakly in leaves; 2) *SISnRK2C* mRNA in tomato leaf is not induced by various stresses; 3) SISnRK2C prefers to phosphorylate myelin basic protein rather than histone or casein; 4) SISnRK2C heterologously expressed in *N. benthamiana* is activated significantly by chilling and NaCl, but weakly by ABA. The osmotic- and chilling-stress-dependent enzymatic activation of SISnRK2C indicates that SISnRK2C is possibly involved in an ABA-independent osmotic stress signaling pathway in tomato immature fruit, flower and photosynthetic organs, rather than in ABA-induced signaling such as stomatal closure.

Furihata et al. (2006) reported that phosphorylation of specific sites in AREB is essential for induction of a set of ABA-inducible genes, and that some SnRK2 molecules can phosphorylate AREB downstream of ABA perception in *Arabidopsis*. Kobayashi et al. (2005) reported that SAPK8, SAPK9 and SAPK10 in rice are activated by ABA and in turn phosphorylate TRAB1, an AREB-like transcriptional factor. Recently, Chae et al. (2007) also reported that rice SnRK2, OSRK1, is involved in ABA signaling pathway by *in vivo* phosphorylating rice AREB family proteins. Umezawa et al. (2004) have shown that overexpression of *AtSRK2C* in *Arabidopsis* resulted in drought tolerance and up-regulation of many stress-responsive genes, such as *RD29*, *COR15* and *DREB/CBF*. At present, it is not clear whether osmotic- or chilling-stress-activated SISnRK2C phosphorylates specific transcriptional factors involving stress-responsive genes in tomato fruit, flower and leaf. However, it is conceivable that SISnRK2C has important roles in fruit development such as accumulation of

sugars and ion up-take by modulating stress-specific transcription factors and/or transporters in response to hormonal signals and environmental stresses.

Recently, association of SnRKs with protein phosphatase 2C (PP2C), ABI1/ABI2, such as AtSRK2E with ABI1 and SOS2 with ABI2, has been demonstrated by yeast two-hybrid assay (Ohta et al. 2003; Yoshida et al. 2006). Functional dissection of the SnRKs revealed that the COOH terminus is required for specific interaction with the PP2Cs. In the case of SnRK2 subclass II, including SISnRK2C, AtSRK2C and OsSAPK1, extensive studies by construction of chimeric molecules between SAPK1 and SAPK2 and site-directed mutagenesis of SAPK1 showed that multiple serine and threonine residues are essential for kinase activation, and that divergence of the COOH terminus is responsible for interacting to specific upstream factors and selectivity in the switching profiles of activation in response to different stresses (Kobayashi et al. 2004). Concerning of regulation mechanism of SnRKs, it was reported that mRNA levels of *LeSNF1* in leaves of tomato seedlings was essentially constitutively expressed while its regulatory subunit, *LeSNF4*, is induced by ABA or dehydration (Bradford et al. 2003). If SISnRK2C interacts with such regulatory molecules, functioning at upstream of SISnRK2C, it is tempting to speculate that regulatory molecules of SISnRK2C are induced in response to environmental stresses and/or fruit developmental process, leading to activation of SISnRK2C.

To elucidate the functions of SISnRK2C in fruit development and stress tolerance and the regulatory mechanisms, we are going to examine whether SISnRK2C interacts with specific signal molecules *in vivo* by agroinfiltration and to develop transgenic tomato expressing modified *SISnRK2C*.

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