DOI: 10.1007/s10535-017-0719-6 BIOLOGIA PLANTARUM 61 (4): 601-610, 2017

Cloning and characterization of four *TpSnRK2s* from dwarf Polish wheat

Y. JIANG[1], Y. WANG[2], Z. HUANG[1], H. KANG[2], L. SHA[2], X. FAN[2], H. ZHANG[2], S. GAO[3], Y. ZHOU[2], and X. YU[1]*

College of Landscape Architecture, Sichuan Agricultural University, Wenjiang 611130, Sichuan, P.R. China[1]
Triticeae Research Institute, Sichuan Agricultural University, Wenjiang 611130, Sichuan, P.R. China[2]
Landscape Architecture Institute, Sichuan Agricultural University, Wenjiang 611130, Sichuan, P.R. China[3]

Abstract

Protein phosphorylation/dephosphorylation is a major signalling event induced by abiotic stresses in plants. Sucrose nonfermenting 1-related protein kinase 2 (SnRK2) plays important roles in response to osmotic stress. In the present study, four *SnRK2*s, *TpSnRK2.1/3/7/8*, were cloned and characterized from *Triticum polonicum* L. (dwarf Polish wheat, DPW, AABB). All of these were individually located on 2AL, 1AL, 2AL, and 5BL. Two spliced isoforms of *TpSnRK2.8* (*TpSnRK2.8a* and *TpSnRK2.8b*) were observed. *TpSnRK2.1* and *TpSnRK2.3* were classified into the group II; *TpSnRK2.7* was classified into the group I; and *TpSnRK2.8a/b* were classified into the group III. Expression patterns revealed that *TpSnRK2.1* responded to cold, NaCl, polyethylene glycol (PEG), and abscisic acid (ABA) in both roots and leaves; *TpSnRK2.3* was strongly regulated by cold, NaCl, and ABA in both roots and leaves, and by PEG in roots; *TpSnRK2.7* was induced by NaCl and PEG in roots, but was not activated by ABA; and *TpSnRK2.8*s were significantly activated by cold, NaCl, PEG, and ABA in both roots and leaves. From the above results, we inferred that *TpSnRK2.1/3/8* may participate in the responses to environmental stresses in ABA-dependent signal transduction pathway but *TpSnRK2.7* is possibly involved in responses to environmental stresses in a non-ABA-dependent manner. They play important roles in specific tissues under different stresses.

Additional key words: abscisic acid, cold stress, gene expression, polyethylene glycol, salt stress, *Triticum polonicum.*

Introduction

Extreme temperatures, drought, and salinity negatively affect the growth and production of crops (Silva and Gerós 2009). Under these stresses, plants have established various signalling pathways to recognize stress signals and transmit them to different cellular compartments (Fujita *et al.* 2006, 2013, Coello *et al.* 2011). Signalling pathways consist of some protein kinases and phosphatases, such as AMP-activated protein kinases (AMPKs) and most of sucrose nonfermenting 1-related protein kinases (SnRKs) (Hrabak *et al.* 2003, Kulik *et al.* 2011) which play crucial roles in linking stress signalling and metabolism (Harmon 2003, Halford and Hey 2009). According to the similarity of amino acid sequences and domain structures, plant SnRKs are grouped into three subfamilies: SnRK1, SnRK2, and SnRK3 (Halford and Hardie 1998, Hrabak *et al.* 2003). The SnRK1s are involved in the regulation of global metabolism and energy status of plants (Polge and Thomas 2006, Shukla and Mattoo 2008). The SnRK3s are plant-specific kinases, which may function as cross-talk nodes in complex signalling networks and interact with calcineurin B-like (CBL) calcium-binding proteins (Kimura *et al.* 2006, Lin *et al.* 2009, Du *et al.* 2011, Luan *et al.* 2011). Previous study indicated that *SnRK3*s and *SnRK2*s may originate from the duplication of *SnRK1*s and then diverge rapidly to fulfill new roles that enable plants to develop networks linking abiotic stresses signalling, calcium signalling, and abscisic acid (ABA)

Submitted 8 July 2016, *last revision* 24 October 2016, *accepted* 2 November 2016.
Abbreviations: AA - amino acid; ABA - abscisic acid; AMPKs - AMP-activated protein kinases; DPW - dwarf Polish wheat; ORF - open reading frame; PEG - polyethylene glycol; qPCR - quantitative PCR; SnRK - sucrose nonfermenting 1-related protein kinase; UTR - untranslated region.
Acknowledgments: The authors thank the National Natural Science Foundation of China (Nos. 31671688 and 31470305), the Bureau of Science and Technology, and the Bureau of Education of Sichuan Province, China.
* Corresponding author; fax: (+86) 028 8629 0880; e-mail: xiaofangyu@sicau.edu.cn

signalling with metabolic responses (Halford and Hey 2009).

To date, more than 50 *SnRK2*s have been identified in different plants, such as 10 *AtSnRK2*s from *Arabidopsis* (Yoshida *et al.* 2002, Boudsocq *et al.* 2004, Saha *et al.* 2014), 10 *OsSAPK*s from rice (Xu *et al.* 2013, Saha *et al.* 2014), 11 *ZmSnRK2*s from maize (Huai *et al.* 2008), four *SnRK2*s from soybean (Monks *et al.* 2001), and 10 *ShSnRK*s from sorghum (Li *et al.* 2010). These *SnRK2*s are divided into three groups. The kinases of group I are not activated by ABA; the members of group II are not activated or activated very weakly by ABA (depending on plant species); and the members of group III are strongly activated by ABA (Kulik *et al.* 2011). Meanwhile, *SnRK2*s respond to salt, cold, drought, and ABA (Boudsocq *et al.* 2004, 2007, Kobayashi *et al.* 2004, 2005), and also participate in the stomatal closure, seed germination (Li *et al.* 2000, Boudsocq and Lauriere 2005, Nakashima *et al.* 2009), root growth and archi-tectural maintenance (Boudsocq *et al.* 2004, Fujii *et al.* 2007), heavy metal uptake (McLoughlin *et al.* 2012) and sugar metabolism (Zhang *et al.* 2011). Therefore, increasing studies have been focused on this family.

In wheat, six (*PKABA1*, *TaSnRK2.3*, *TaSnRK2.4*, *TaSnRK2.7*, *TaSnRK2.8*, and *TaSnRK2.9*) and four (*TpSnRK2.2*, *TpSnRK2.5*, *TpSnRK2.10*, and *TpSnRK2.11*) *SnRK2s* were cloned from hexaploid and tetraploid wheat, respectively. Their transcripts are regulated by different stresses, such as salicylic acid (SA), mannitol, polyethylene glycol (PEG 6000; simulated drought), NaCl, cold, and ABA (Anderberg *et al.* 1992, Xu *et al.* 2009, Mao *et al.* 2010, Zhang *et al.* 2010, 2011, Tian *et al.* 2013, Wang *et al.* 2015). Overexpression of *TaSnRK2s* in *Arabidopsis* significantly enhances the tolerance to drought, salt, and freezing, suggesting these genes could be potentially utilized to improve abiotic stress tolerance in crops (Mao *et al.* 2010, Zhang *et al.* 2010, 2011, Tian *et al.* 2013). However, the information on these genes in tetraploid wheat, especially in dwarf Polish wheat (AABB, 2n=4x=28, *Triticum polonicum* L.) which has low genetic similarity to *T. durum* and *T. turgidum* (Stepien *et al.* 2007, Wang *et al.* 2013, Michalcová *et al.* 2014), is not sufficient. *TpSnRK2.5* cloned from Chinese dwarf Polish wheat (DPW) has a 106 bp nucleotide deletion resulting in a premature termination (Wang *et al.* 2015). However, the deletion exists in all tetraploid wheat (except for *T. dicoccon* and *T. dicoccoides*), but not in hexaploid wheat (unpublished data). Therefore, it attracted our interest to investigate other *TpSnRK2s* in DPW.

Materials and methods

Plants and treatments: DPW is a spontaneous mutant dwarf accession of *Triticum polonicum* L. originally collected from Tulufan, Xinjiang, China, and maintained at Triticeae Research Institute, Sichuan Agricultural University, Sichuan, China. The seeds of DPW were sterilized with 5 % (m/v) NaClO for 5 min, and then rinsed with double-distilled water (ddH_2O) three times. After germinating in glass Petri dishes with ddH_2O at room temperature for 5 d, the seedlings were grown in ddH_2O for further 5 d, and then cultured in nutrient solution (Hoagland's modified basal salt mixture, *MP Biomedicals*, CA, USA) in a growth chamber under a 16-h photoperiod, an irradiance of 150 μmol m^{-2} s^{-1} (fluorescent lamps), a constant temperature of 20 °C, and a relative humidity of 75 %.

Plants at three-leaves stage were treated with 10 mM PEG 6000, 300 mM NaCl, 4 °C (cold), or 50 μM ABA. After 4, 8, 12, and 24 h, leaf and root samples were randomly collected, frozen in liquid nitrogen, and stored at -80 °C.

Total RNA extraction and cDNA synthesis: Total RNA was extracted using the *E.Z.N.A.*® total RNA kit II (*Omega*, Shanghai, China) according to user manual. Genomic DNA was digested using the RNase-free DNase kit (*Omega*). Content of RNA in all samples was measured by a *NanoDrop-2000* spectrophotometer (*NanoDrop Technologies*, Wilmington, USA). All RNA samples were stored at -80 °C. The first strand cDNA was synthesized using *M-MLV* first strand cDNA synthesis kit (*Omega*) according to user manual.

Cloning *TpSnRK2s*: Four *TpSnRK2s* primers were designed by *Beacon Designer 7.9* (http://beacon-designer.software.informer.com/7.9/) according to the reference sequences from RNA-Seq (Table 1 Suppl.). The total PCR reaction mixture (25 mm^3) included 9.5 mm^3 of ddH_2O, 2.5 mm^3 of 10× *Ex-Taq* buffer, 2 mm^3 of 25 mM $MgCl_2$, 2 mm^3 of dNTP mixture, 4 mm^3 of DNA, 1 mm^3 of *Ex-Taq* (5 U mm^{-3}, *TaKaRa*, Dalian, China) and 2 mm^3 of each forward and reverse primers (10 μM). The PCR conditions were following: pre-denaturation at 94 °C for 2 min, 35 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min 30 s), and a final extension at 72 °C for 10 min. The PCR products were separated on 1.5 % (m/v) agarose gels and visualized with the *Gel Doc*^{TM} *XR*$^{+}$ with *Image Lab*^{TM} software (*Bio-Rad*, Hercules, CA, USA). The PCR products were purified using DNA gel extraction kit, cloned into the pMD19-T vector (*TaKaRa*), and finally sequenced at *Sangon Biotech* (Shanghai, China).

Sequence alignment and homology analyses were conducted by *Clustalw*. The open reading frames (ORFs) and deduced amino acid sequences were predicted using *BLAST* (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and *ExPASy* (http://www.expasy.org/), respectively. The functional regions and activity sites were scanned using

PHYRE[2] (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Isoelectric point (pI) was calculated using *Peptide Property Calculator* (https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi). Chromosome locations of the four genes were mapped using *BLAST* of *Gramene* with the genome of Chinese Spring (http://www.gramene.org/).

The neighbor-joining (NJ) algorithm tree was constructed by *MEGA5.05* software (http://www.megasoftware.net/) based on the *Clustalw* aligned sequences. Bootstrap analysis was performed using 1 000 replicates to evaluate the reliability of different phylogenetic groups.

Real time quantitative reverse transcription PCR: Primers were designed using the *Beacon Designer 7.9* (Table 2 Suppl). *Actin* (*ACT*, F: CCGATTGCTTGT TATCTGTT; R: GAGGAT GAAGACGAGAGTTT) was used as the reference gene to normalize relative expressions. Real time qPCR was performed on the *CFX-96*^{TM} real-time system (*Bio-Rad*, CA, USA) with 96-well plates. Each reaction had a total volume of 15 mm^3, including 6.3 mm^3 of cDNA equal to 12.6 ng of total RNA, 0.6 mm^3 (3 pmol mm^{-3}) of each forward and reverse primers, and 7.5 mm^3 of *iTaqTM* universal *SYBR® Green Supermix* (*Bio-Rad*). In template controls, 6.3 mm^3 of RNase free water replaced the cDNA. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min, followed by the generation of a dissociation curve by increasing temperature from 65 to 95 °C to check for specificity of amplification. The relative expressions were calculated using *Bio-Rad CFX* manager *v3.1* with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistics: The Dunnett's test ($\alpha = 0.05$) was conducted using the software of *SigmaPlot 12.0* (Duncan 1955).

Results

Four *TpSnRK2s*, *TpSnRK2.1*, *TpSnRK2.3*, *TpSnRK2.7*, and *TpSnRK2.8* (two isoforms named *TpSnRK2.8a* and *TpSnRK2.8b*), were cloned from DPW (Table 1 and Fig. 1). Their sequences were deposited into the National Center for Biotechnology Information (NCBI) with accession numbers: KR736349, KR736350, KR736351, KR736352, and KU961676, respectively. The four *TpSnRK2s* were mapped on chromosomes 2AL, 1AL, 2AL, and 5BL, respectively.

The cDNA sequence of *TpSnRK2.1* had 1 185 bp, which consisted of 27 bp 5'-untranslated region (5'-UTR), 1 026 bp open reading frame (ORF) and 132 bp 3'-UTR (Table 1). The ORF encoded 342 amino acids (AAs) (Table 3), which had a 98.83 % identity to TaW55a (DQ343300.1), 91.25 % to OsSAPK1 (AB125302.1), and 87.46 % to ZmSnRK1 (EU676033.1) (Fig. 1 Suppl.). TpSnRK2.1 had a conserved N-terminal domain and a complete C-terminal. The N-terminal domain contained one ATP binding site (AAs 9 - 33), one potential N-myristoylated site (AAs 110 - 115), one Ser/Thr protein kinase active site (AAs 118 - 131), one transmembrane spanning region in the catalytic domain (AAs 182 - 219) and a conserved threonine residue (AAs 146 - 165). At the C-terminal domain, a stretch of acidic amino acid residues (AAs 318 - 337) which was abundant in aspartic acid was found (Fig. 1). The secondary structure prediction revealed that TpSnRK2.1 protein had 15 α-helices and 11 β-pleated sheets.

The cDNA sequence of *TpSnRK2.3* was 1 377 bp, which contained a 177 bp of the 5'-UTR, a 1 026 bp of the ORF and a 171 bp of the 3'-UTR (Table 1). The *TpSnRK2.3* encoded 342 AAs, which had a 86.26 % identity to OsSAPK3 (JF733761.1), 84.21 % to ZmSnRK2.3 (NM001143024) and 97.37 % to TaSnRK2.3 (Fig. 1 Suppl.). TpSnRK2.3 also had two main domains: N-terminal and C-terminal regions. The highly conserved N-terminal catalytic domain contained an ATP-binding site (AAs 8 - 35), one potential N-myristoylated site (AAs 110 - 115), one Ser/Thr protein kinase active-site (AAs 119 - 132), one conserved threonine residue (AAs 146 - 167), and one transmembrane spanning region (AAs 183 - 221). The signal peptide of one potential transmembrane helix was not detected. The secondary structure prediction revealed

Table 1. The characteristics of four *TpSnRK2*s. Untranslated region (UTR) and open reading frame (ORF) were predicted using *ExPASy*. Proteins were characterized by length, Mr and pI using *Peptide Property Calculator* online tool.

Name	Length [bp]	5'-UTR [bp]	OFR [bp]	3'-UTR [bp]	Length [AA]	Mr [kDa]	pI
TpSnRK2.1	1185	27	1026	132	342	38.8	5.78
TpSnRK2.3	1377	177	1026	171	342	38.7	5.40
TpSnRK2.7	1389	204	1071	114	357	40.9	5.24
TpSnRK2.8a	1224	90	645	489	215	24.5	7.89
TpSnRK2.8b	1149	120	882	147	294	33.5	4.56

that TpSnRK2.3 formed 15 α-helices and 11 β-pleated sheets. The relatively short C-terminal domain was abundant in glutamic acid (Fig. 1), and was predicted to be a coiled coil.

The cDNA sequence of *TpSnRK2.7* was 1 389 bp, which included a 204 bp of the 5'-UTR, a 1 071 bp of the ORF and a 114 bp of the 3'-UTR (Table 1). The deduced polypeptide of TpSnRK2.7 had 357 AAs and had an identity of 92.48 % to OsSAPK7 (AB125308.1), 92.48 % to ZmSnRK2.7 (EU676039.1) and 98.88 % to TaSnRK2.7 (Fig. 1 Suppl.). Similar to TpSnRK2.1 and TpSnRK2.3, TpSnRK2.7 had a highly conserved N-terminal catalytic domain and an intact C-terminal domain. In the N-terminal catalytic domain, one ATP-binding site (AAs 10 - 33), one potential N-myristoylated site (AAs109 - 114), one Ser/Thr protein kinase active-site (AAs 119 - 131) and one transmembrane spanning region (AAs 183 - 220) were found, but no signal peptide was detected. The secondary structure prediction revealed that TaSnRK2.7 formed 17 α-helixes and 10 β-pleated sheets. Similar to TpSnRK2.3, the C-terminal domain of TpSnRK2.7 was also abundant in glutamic acid (Fig. 1).

Two isoforms of *TpSnRK2.8* were amplified from treated samples. *TpSnRK2.8a* consisted of a 90 bp of the 5'-UTR, a 645 bp of the ORF and a 489 bp of the 3'-UTR; *TpSnRK2.8b* included a 120 bp of the 5'-UTR, a 882 bp of the ORF and a 147 bp of the 3'-UTR (Table 1). Aligning to *PhSnRK2.8* cloned from the *Psathyrostachys huashanica* (unpublished data) which is a widely relative species of DPW, *TpSnRK2.8a* had 235 bp nucleotides deletion which resulted in partial of N-terminus catalytic domain deletion (Fig. 2), and a nucleotide deletion was observed, resulting in a premature termination during translation (Fig. 3). C-terminal domain of *TpSnRK2.8a* was deficient (Fig. 1). Meanwhile, 266 bp nucleotides deletion was detected in *TpSnRK2.8b*, leading to the deletion of the ATP binding site in N-terminus catalytic domain. TpSnRK2.8a with 215 AAs had 54.45 % identity

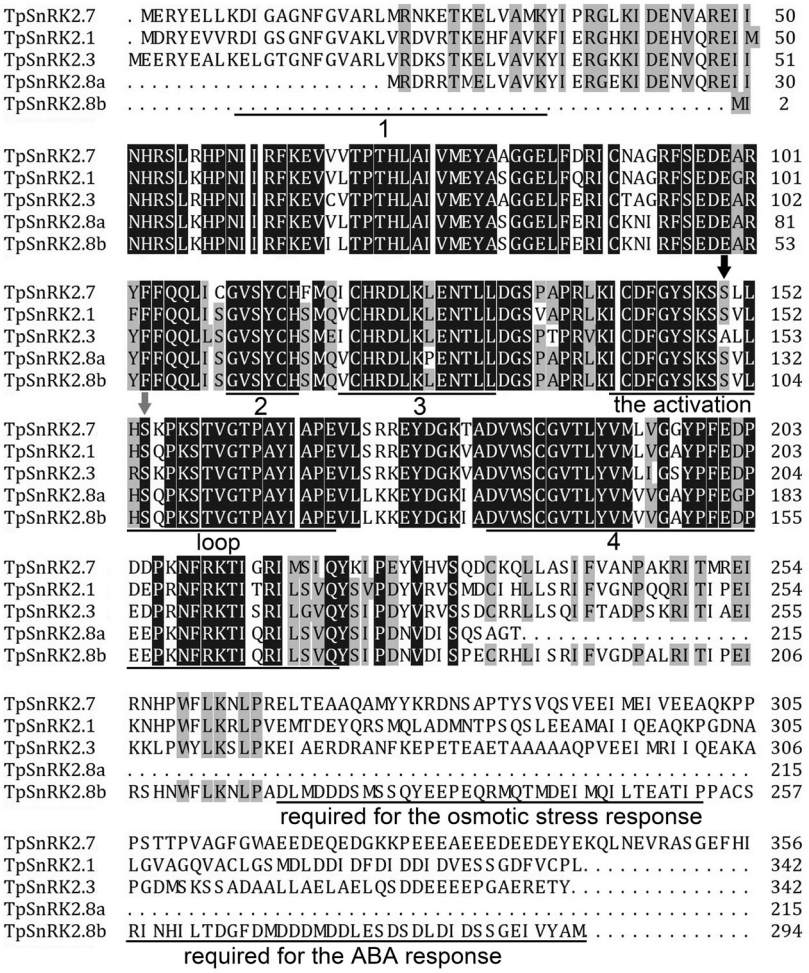

Fig. 1. The sequences alignment of five TpSnRK2s. *1* - ATP binding region, *2* - potential N-myristoylation site, *3* - serine/threonine protein kinases activity site, *4* - potential transmembrane spanning region. The other *lines* represent the activation loop, the region required for the osmotic stress response, and the region required for the ABA response. The *black* and *gray* arrows indicate two reversible phosphorylated serine residues.

to OsSAPK7 (AB125309.1), 53.01 % to ZmSnRK2.3 (EU676040.1), and 56.56 % to TaSnRK2.8 (KR611569). TpSnRK2.8b with 294 AAs had 75.20 % identity to OsSAPK7, 58.77 % to ZmSnRK2.3, and 80.05 % to TaSnRK2.8. TpSnRK2.8a had just only the N-terminus catalytic domain, which contained an incomplete ATP binding site (AAs 1-13), one potential N-myristoylation site (GVSYCH, AAs 90 - 95), a protein kinase activating signature (AAs 98 - 111), and one potential transmembrane spanning region (AAs 152 - 181). In the N-terminus catalytic domain of TpSnRK2.8b, the ATP binding site was deleted. The C-terminal domain of TpSnRK2.8b was abundant in aspartic acid (Fig. 1). Interestingly, we had also found a complete sequence of *TpSnRK2.8* from non-tested samples. To check whether other wheat samples produce the two mRNA species, we performed PCR using primers encompassing the deletions (Fig. 4). Interestingly, another spliced isoform of *TpSnRK2.8* with a complete sequence was found in non-tested samples from 12 tetraploid wheats.

Fig. 2. The 5'-UTR sequences alignment of TpSnRK2.8a/b and PhSnRK2.8 from *Psathyrostachys huashanica*.

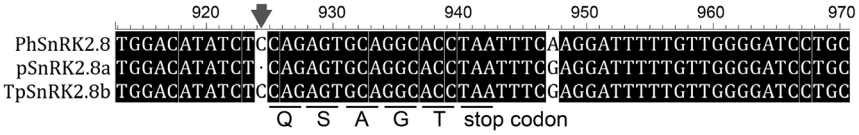

Fig. 3. The sequences alignment of TpSnRK2.8a and PhSnRK2.8. The *gray arrow* labels a nucleotide deletion, and the *lines* represent the codons and the stop codon, respectively.

The five TpSnRK2s were classified into three groups (Fig. 5). TpSnRK2.7 with its three counterparts, OsSAPK7, ZmSnRK2.7, and TaSnRK2.7, were classified into the group I. TpSnRK2.1 with its three counterparts (OsSAPK1, ZmSnRK2.1 and TaW55a) and TpSnRK2.3 with its three counterparts (OsSAPK3, ZmSnRK2.3, and TaSnRK2.3), were grouped into the group II. TpSnRK2.8a and TpSnRK2.8b with their four counterparts OsSAPK8, ZmSnRK2.8, TaSnRK2.8, and PhSnRK2.8 were classified into the group III.

The various expression patterns of *TpSnRK2.1* were observed under different stresses (Fig. 6). In controls, the expressions of *TpSnRK2.1* in leaves were higher than those in roots. Compared with the control, ABA significantly enhanced the expressions at all sampling times in roots whereas in leaves, significantly decreased the expressions at 4 h, then increased at 8 h and 24 h (Fig. 6). NaCl significantly up-regulated the expressions at all sampling times in both roots and leaves, with peak at 12 h in roots and 24 h in leaves (Fig. 6). Cold significantly increased the expressions after 12 and 24 h in roots; in leaves, cold significantly increased the expressions after 8 and 12 h, and then decreased after 24 h (Fig. 6). The PEG significantly increased the expressions after 4 h in roots, but significantly reduced the expressions after 8 h in leaves (Fig. 6).

In controls, the expressions of *TpSnRK2.3* in roots were higher than those in leaves. Compared with controls, the expressions of *TpSnRK2.3* under ABA stress were significantly up-regulated in all sampling times in both roots and leaves (Fig. 6). Under NaCl stress, the expressions of *TpSnRK2.3* were significantly up-

regulated after 4 and 8 h in roots, and after 12 and 24 h in leaves (Fig. 6). Under cold stress, the expressions of *TpSnRK2.3* in roots were significantly down-regulated after 4 and 8 h, then the expressions were obviously up-regulated after 24 h; in leaves, the expressions were significantly up-regulated in all sampling times (Fig. 6). Under PEG stress, the expressions of *TpSnRK2.3* in roots were significantly up-regulated after 4 and 8 h; in leaves, *TpSnRK2.3* was not affected by PEG stress (Fig. 6).

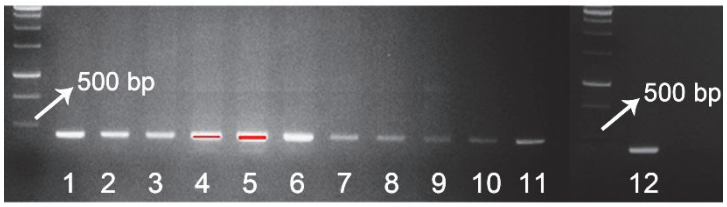

Fig. 4. The complete SnRK2.8 transcript isoforms coexist in tetraploid wheat without testing. 1 - *Triticum paleo-colchicum* Men., 2 - *Triticum turgidum* L. var. *dicoccoides* (Koern.) Bowden, 3 - *Triticum polonicum* L., 4 - *Triticum dicoccum* Schrank, 5 - *Triticum turgidum* L. var. *durum* (Desf.) Yan. ex P.C., 6 - *Triticum turanicum* Jakubz., 7 - *Triticum persicum* Zhuk., 8 - *Triticum turgidum* L., 9 - *Triticum timopheevi* L., 10 - *Triticum araraticum* Jakubz., 11 - *Triticum paleo-colchicum* Men., 12 - *Triticum* subsp. *ispahanicum* Heslot. PCR control on another spliced cDNA showing a DNA fragment at 476 bp in un-tested samples. The size markers show a DNA fragment at 500 bp.

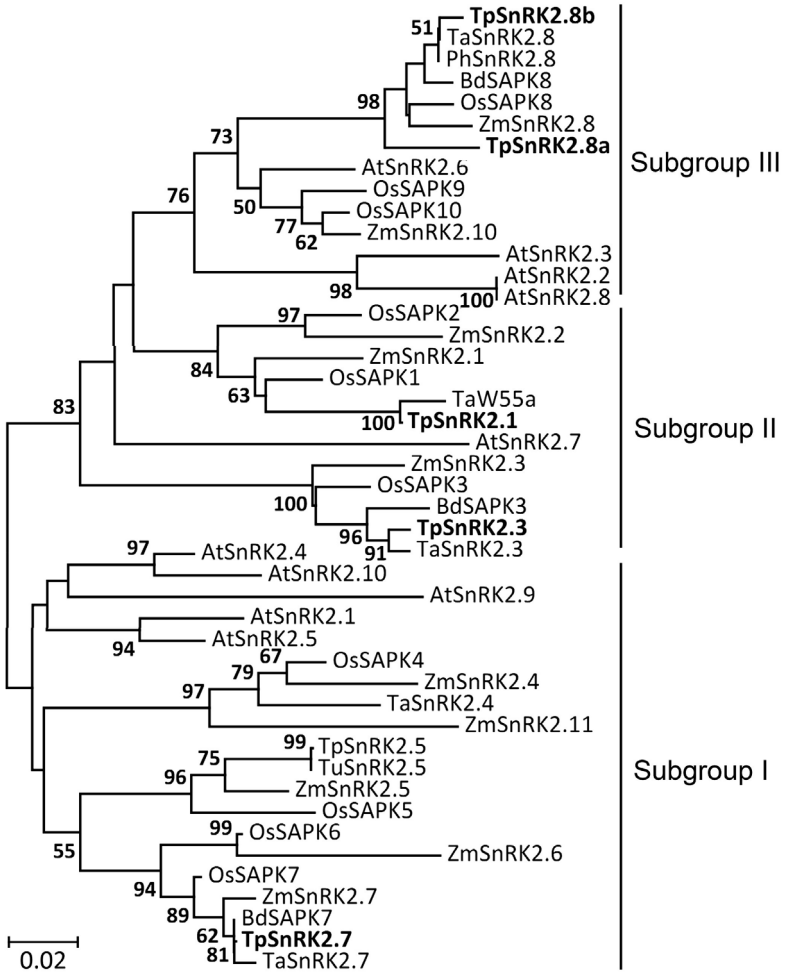

Fig. 5. Phylogenetic tree of TpSnRK2s and other SnRK2s from other plant species. All amino acid sequences were aligned with the *Clustalw* and the phylogenetic tree was constructed with *MEGA 6.0* using the neighbour-joining method. Numbers in *bold* at nodes are bootstrap values $P \geq 50$ %. *Bar* at left bottom indicates scale value. Three distinct subclasess were presented in *vertical line*. TpSnRK2s were highlighted *in bold*.

The expressions of *TpSnRK2.7* in controls were similar in roots and leaves. Compared with controls, the expression of *TpSnRK2.7* was not induced by ABA stress in both roots and leaves (Fig. 6). NaCl significantly enhanced the expression at 8 and 12 h in roots and at 8, 12, and 24 h in leaves. Cold stress slightly decreased the expressions in roots at 24 h; in leaves, the expressions of *TpSnRK2.7* were not affected by cold stress (Fig. 6). PEG significantly increased the expressions in roots at 8 h, significantly decreased at 12 h, and then significantly increased at 24 h; in leaves, the expressions were significantly down-regulated at 4 h, but significantly up-regulated at other sampling times (Fig. 6).

In controls, the expressions of *TpSnRK2.8* in leaves were slightly higher than in roots. Compared with the control, ABA and PEG decreased the expressions of *TpSnRK2.8* in roots at 4, 8, and 12 h, and then increased at 24 h; in leaves, ABA and PEG significantly decreased the expressions at 8 and 24 h and slightly increased at 12 h (Fig. 6). Under NaCl stress, the expressions of *TpSnRK2.8* were down-regulated at all sampling times in roots and leaves, except at 24 h in leaves (Fig. 6). Under cold stress, the expressions of *TpSnRK2.8* in roots were significantly up-regulated at 8 and 12 h; in leaves, the expressions were significantly up-regulated at 4 and 12 h, and then decreased at 24 h (Fig. 6).

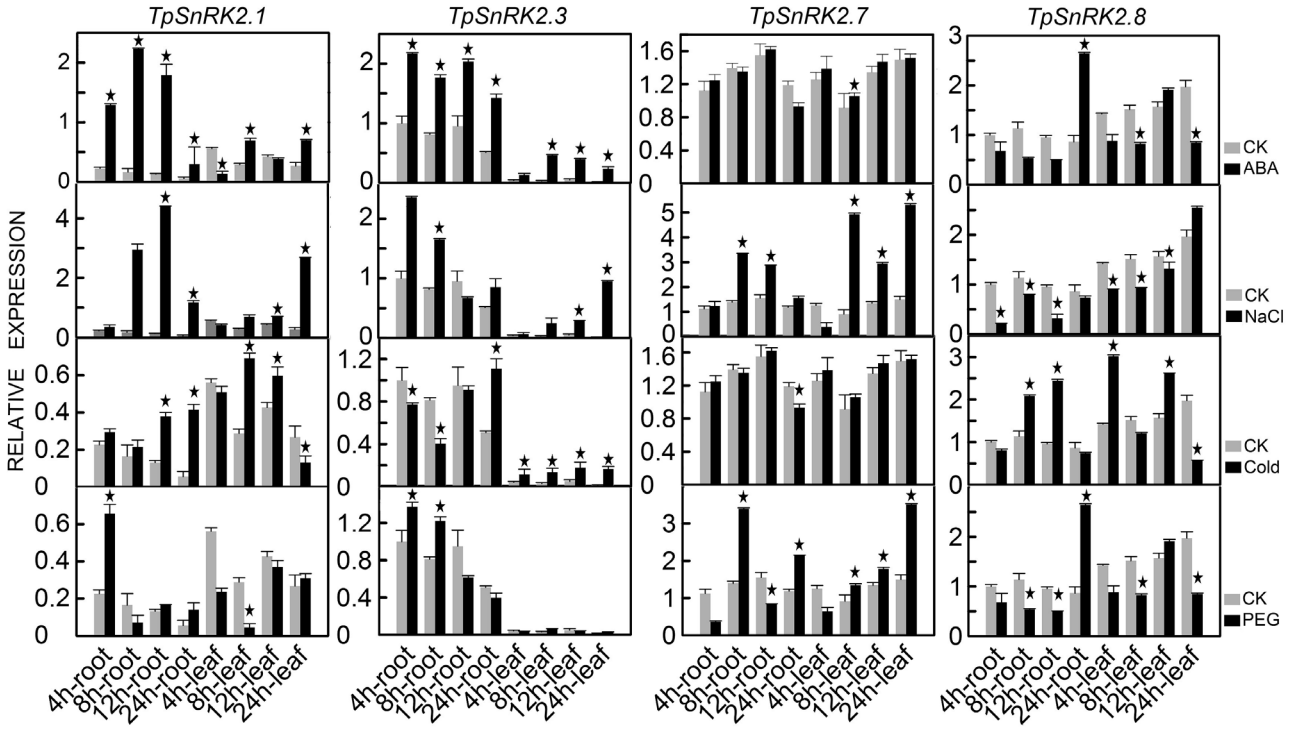

Fig. 6. Expression profiles of four *TpSnRK2s* in leaves and roots in response to various stresses. Means ± SEs of three biological replicates and three technical replicates. The *asterisks* represent significant differences between treatments and their corresponding controls. The data analysis and figures were done with *Sigma plot 12.0*.

Discussion

Since plant *SnRK2s* are hubs within a network of interacting signalling pathways (Halford and Hardie 1998, Halford and Hey 2009), they play important roles in responding to abiotic stresses, nutrient limitation, seed maturation, and germination (Fujita *et al.* 2009, Nakashima *et al.* 2009). Thus, *SnRK2s* can be potentially used for improvement of abiotic stress tolerance and yield enhancement (Piattoni *et al.* 2011). Recent studies have been focusing on their molecular/biochemical properties and functions (*e.g.*, Kulik *et al.* 2011). In the present study, four *SnRK2s*, *TpSnRK2.1*, *TpSnRK2.3*, *TpSnRK2.7*, and *TpSnRK2.8*, were cloned and characterized. Although *TpSnRK2.7* had 98.88 % identity to *TaSnRK2.7*, *TpSnRK2.1* had 98.83 % identity to *TaSnRK2.1*, and *TpSnRK2.3* had 97.37 % identity to *TaSnRK2.3*, *TpSnRK2.8* with two copies had 235 and 266 bp deletions, which had low identity to *TaSnRK2.8*. Additionally, *TpSnRK2.4* was not observed using the primer of *TaSnRK2.4*, suggesting the divergence between *TpSnRK2.4* and *TaSnRK2.4*, or the deletion of *TpSnRK2.4* in DPW. Therefore, all these results proved the low genetic similarity between DPW and *T. aestivum* (Wang *et al.* 2013, Michalcová *et al.* 2014).

Due to the deletions, the ATP binding site was not

observed in TpSnRK2.8b and an incomplete ATP binding site was found in TpSnRK2.8a (Fig. 1). On the other hand, N-myristoylation, serine/threonine protein kinases activity site and serine residue were observed in the five TpSnRKs, suggesting they might participate in regulating conformational stability and respond to hyperosmotic treatments (Resh 1999, Burza *et al.* 2006, Zheng *et al.* 2010). Characteristic D-rich patch of C-terminal domain was detected in TpSnRK2.1/8b and E-rich patch in TpSnRK2.3/7 (Fig. 1), suggesting that the four genes may regulate environmental responses and ABA activation (Halford and Hardie 1998, Boudsocq *et al.* 2004, Kobayashi *et al.* 2004, Kulik *et al.* 2011). Subcellular localization showed the presence of majority *TaSnRK2s* in the cell membrane, cytoplasm, and nucleus (Mao *et al.* 2010, Zhang *et al.* 2010, 2011, Tian *et al.* 2013), which indicates the potential sub-cellular location of *TpSnRK2s* in DPW.

Under the control conditions, the expressions of *TpSnRK2.1* (Fig. 6) and *TpSnRK2.8* (Fig. 6) in leaves were higher than those in roots, and *TpSnRK2.3* expressions in roots were higher than in leaves (Fig. 6). Meanwhile, the expressions of *TpSnRK2.7* were similar in roots and leaves (Fig. 6). These results suggest that the different SnRK2s have different functions in different tissues (Mao *et al.* 2010, Zhang *et al.* 2010, 2011, Tian *et al.* 2013, Wang *et al.* 2015).

Numerous studies indicated that *SnRK2s* were involved in response to environmental stresses (Fujii *et al.* 2007, Coello *et al.* 2011, Kulik *et al.* 2012). The four *TpSnRK2s* showed obvious differences in the expression under PEG, NaCl, cold, and ABA treatments (Fig. 6). The expressions of *TpSnRK2.1* were similar to *PKABA1* that were up-regulated within dehydration, cold-temperature and salt stresses. Moreover, *PKABA1* participates in the interaction between ABA and GA, and its expressions were regulated by ABA (Holappa *et al.* 2005). In this study, the expressions of *TpSnRK2.1* were strongly regulated by ABA, suggesting *TpSnRK2.1* may also participate in interaction between ABA and GA. On the other hand, *TpSnRK2.1* was regulated by cold, which was different from *TaW55* that was not induced by cold, although it was also induced by ethylene and methyl jasmonate (Xu *et al.* 2009). Compared with other *SnRK2.1*s, the expression of *TpSnRK2.1* was different from *ZmSnRK2.1* that was only slightly induced by NaCl treatment (Huai *et al.* 2008), it was alike as *OsSAPK1* (Kobayashi *et al.* 2004).

TpSnRK2.3 was regulated by all treatments in roots and leaves, except for the induction by PEG in leaves. It was different from other homologues, such as *OsSAPK3* that was only regulated by PEG and ABA (Kobayashi *et al.* 2004) and *ZmSnRK2.3* only induced by NaCl and cold (Huai *et al.* 2008). *TpSnRK2.3* regulation had some similarities with *TaSnRK2.3* that was very sensitive to PEG and NaCl stresses. Their differences were seen under ABA stress; the expressions of *TaSnRK2.3* were weakly regulated, and *TpSnRK2.3* was strongly induced in leaves. Therefore, the involved molecular mechanisms of *TaSnRK2.3* and *TpSnRK2.3* might be different.

TpSnRK2.7 was induced by NaCl and PEG stresses in roots and leaves, and was not regulated by ABA and cold stresses. It was different from *OsSAPK7* and *ZmSnRK2.7* that were activated by ABA stress (Kobayashi *et al.* 2004, Huai *et al.* 2008). *TaSnRK2.7* was expressed highly in seedling roots, suggesting it might act as a fundamental signalling molecule of water and/or nutrient status in soil, by contrast to *TpSnRK2.7* (Zhang *et al.* 2011). The expression patterns of *TaSnRK2.7* and *TpSnRK2.7* were also inconsistent; *TaSnRK2.7* was significantly up-regulated under cold stress, while *TpSnRK2.7* was not regulated by cold stress. We suggest that *TpSnRK2.7* participates in ABA-independent signal transduction pathways. Further effort should be directed to deciphering the biological roles and functions of *TpSnRK2.7* in this pathway. Although amino acid sequence homologies were very similar, several single nucleotide poly-morphisms were observed. Due to the differences of genetic background and genetic divergence, the expressions of *TpSnRK*s were different from *TaSnRK*s, suggesting that *TpSnRK2*s may have different functions.

*TpSnRK2.8*s were significantly activated by cold, NaCl, PEG, and ABA (Fig. 6), which varied significantly from *OsSAPK8* (Kobayashi *et al.* 2004), *ZmSnRK2.8* (Huai *et al.* 2008), and even *TaSnRK2.8* (Zhang *et al.* 2010). Comparing the expression patterns of *TpSnRK2.8*s under other stresses, we found that NaCl down-regulated the expressions in all sampling dates, suggesting *TpSnRK2.8*s might play a negative regulation role in responding to salt stress. In addition, induction of *TaSnRK2.8* by PEG and ABA was more prolonged than induction by cold, suggesting that cold stress might be directly involved in *TpSnRK2.8*s induction, or that PEG and ABA stresses might induce *TpSnRK2.8*s indirectly.

In the present study, two spliced isoforms of *TpSnRK2.8* with 235 and 266 bp deletions were stabilized in the treated tissues. Interestingly, another *TpSnRK2.8* isoform with full sequences was observed in un-treated tissues. Meanwhile, the full *SnRK2.8* isoform existed in other tetraploid wheat (Fig. 6). These results suggest the alternative splicing resulted in the different isoform content between treated and un-treated tissues, similarly as *AtNRAMP6*. Previous study found the *AtNRAMP6* transcripts coexisted with a partially spliced isoform in all shoot cell types tested and a fully spliced isoform in un-treated samples. Although *AtNRAMP6* is still sensitive to cadmium, the misspliced isoform was non-functional in yeast assay (Cailliatte *et al.* 2009). All results suggest that *TpSnRK2.8*s were involved in responses to cold, PEG, NaCl and ABA, *TpSnRK2.8a* and *b* might be non-functional under these stresses and might cause a redundant function of *TpSnRK2.8s* in signal pathways.

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