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Cloning and characterization of four novel *SnRK2* genes from *Triticum polonicum*

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Abstract

SnRK2 are plant-specific serine/threonine kinases that are involved in plant responses to abiotic stresses. In this study, four novel genes *SnRK2s:TpSnRK2.11, TpSnRK2.2, TpSnRK2.5*, and *TpSnRK2.10* from dwarf Polish wheat (*Triticum polonicum* L.) were characterized and classified into three groups. *TpSnRK2.5* and *TpSnRK2.11* were members of group 1; *TpSnRK2.2* was member of group 2; *TpSnRK2.10* belonged to group 3. The expression of *TpSNRK2.2* was strongly regulated by polyethylene glycol (PEG), NaCl, and cold in roots and leaves, as well as by ABA in leaves. The transcript of *TpSnRK2.5* was intensely induced by all the treatments in roots and leaves. The distinct expression patterns of *TpSnRK2.10* indicate that this gene was very sensitive to ABA and NaCl, less sensitive to cold and PEG. The transcript of *TpSnRK2.11* was activated significantly by PEG, NaCl, and cold, but weakly by ABA. Our results indicate that these four genes were probably involved in wheat responses to different abiotic stresses in different tissues.

Additional key words: abiotic stress, abscisic acid, cold, dwarf wheat, gene expression, NaCl, PEG.

Introduction

Plants have developed various mechanisms to sense the changes of environment to resist abiotic stresses, such as cold, drought, and salinity. Some protein kinases and phosphatases as key components of some signalling pathways have been found to be involved in recognizing and transmitting stress signals (Hong *et al.* 1997, Droillard *et al.* 2002). Sucrose non-fermenting 1 (SNF1) from yeast (*Saccharomyces cerevisiae*) and AMP-activated protein kinases (AMPKs) from mammals, named as SNF1/AMPK family, are sensors controlling cellular homeostasis by regulating metabolic processes in response to different stresses (Celenza and Carlson 1986, Hardie *et al*. 1989, Polge and Thomas 2006, Hardie 2007, 2011). Plant SNF1-related kinases (SnRKs), homologous to the SNF1/AMPK family, have been found in many species to act as central switches in abiotic stress signalling (Boudsocq *et al*. 2004, Chevalier and Walker 2005, Fujii *et al*. 2007, Dong *et al*. 2012). Increasing evidence indicates that SnRKs may be crucial in linking stress signalling and metabolism (Halford and Hey 2009, Hey *et al*. 2010).

Based on their sequence similarity and domain structure, SnRKs were divided into three subfamilies: SnRK1, SnRK2, and SnRK3 (Hrabak *et al*. 2003). SnRK1s of various plants, which have a 48 % amino acid sequence identity to SNF1 and AMPK, are involved in the global regulation of carbon and nitrogen metabolism (Halford *et al*. 2003, 2004, Halford and Hey 2009). SnRK2s and SnRK3s are plant-specific kinases that function mainly in stress signalling. Although their

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Abbreviations: ABA - abscisic acid; AMPKs - AMP-activated protein kinases; DPW - dwarf Polish wheat; PEG - polyethylene glycol; RT-qPCR - real-time quantitative polymerase chain reaction; SNF1 - sucrose non-fermenting 1; SnRK - SNF1-related protein kinase; UTR - untranslated region.
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sequences are little similar to SnRK1s, SnRK2s and SnRK3s may originate from the gene duplication of SnRK1s and then diverge rapidly during plant evolution to fulfil new roles that enable plants to develop networks that link stress and abscisic acid (ABA) signalling with metabolic signalling (Halford and Hey 2009). Some *SnRK2s* and *SnRK3s* genes have been identified and characterized at a molecular level (Hrabak *et al*. 2003, Boudosq *et al*. 2004, Fujii *et al*. 2007, Halford and Hey 2009, Xu *et al*. 2013, Saha *et al*. 2014).

Since the first *SnRK2* gene (*PKABA1*) was identified in wheat (Anderberg and Walker-Simmons 1992), more than 50 *SnRK2s* were cloned and characterized in various plants (Holappa *et al*. 2005, Huai *et al*. 2008, Li *et al*. 2010, Mao *et al*. 2010, Zhang *et al*. 2010, 2011). Ten *SNRK2s* were identified in *Arabidopsis* (Boudsocq *et al*. 2004, Saha *et al*. 2014), rice (Xu *et al*. 2013, Saha *et al*. 2014), and *Sorghum* (Li *et al*. 2010), 11 *SnRK2s* in *Zea mays* (Huai *et al*. 2008), and 4 in soybean (Monks *et al*. 2001). The expression of these genes is regulated differently by ABA, NaCl, polyethylene glycol (PEG), and a low temperature in different plant organs. A functional analysis indicated that some *SnRK2s* genes regulate the stomatal closure, seed germination, root growth and architectural maintenance, heavy metal uptake, and cell membrane stability (Fujii *et al*. 2007, Mao *et al*. 2010, Kulik *et al*. 2012, McLoughlin *et al*. 2012).

To date, six *SnRK2s* (*TaW55*, TaSnRK2.9, *TaSnRK2.3*, *TaSnRK2.4*, *TaSnRK2.7*, and *TaSnRK2.8*) have been cloned and characterized in common wheat (2n=6x=42, AABBDD), a part of them were induced by an ABA and dehydration stresses (Anderberg and Walker-Simmons 1992, Mao *et al*. 2010, Zhang *et al*. 2010, 2011, Tian *et al*. 2013). *TtPK1* from *Triticum tauschii* (2n=2x=14, DD) was also reported in diploid wheat (Holappa *et al*. 2005). Therefore, additional *SnRK2s* might exist in *Triticum* spp. Nevertheless, no information on *SNRK2s* in tetraploid wheat has been reported.

Since the overexpression of the four genes, *TaSnRK2.3*, *TaSnRK2.4*, *TaSNRK2.7*, and *TaSnRK2.8*, in *Arabidopsis* significantly increased its tolerance to drought, salinity, and cold (Mao *et al*. 2010, Zhang *et al*. 2010, 2011, Tian *et al*. 2013), SnRK2s may play similar roles in *T. polonicum*. The objective of this study was to clone *SnRK*2 genes in dwarf Polish wheat (DPW) and study their expression patterns under different environmental stresses.

Materials and methods

Dwarf Polish wheat (DPW, *Triticum polonicum* L.) is a spontaneous mutant dwarf accession of *T. polonicum*, originally collected from Tulufan, Xinjiang, China, and maintained at the Triticeae Research Institute, the Sichuan Agricultural University, Sichuan, China. Based on the RNA-Seq data of DPW (unpublished data), 15 functionally-annotated *SnRK2* were found and they were unigenes. Their full putative amino acid sequences and some *SnRK2s* from other plant species were used to perform a phylogenetic analysis. Eight *TpSnRK2s* were identified and four of them were similar to *TaSnRK2.3*, *TaSnRK2.4*, *TaSnRK2.7*, and *TaSnRK2.8* (Mao *et al*. 2010, Zhang *et al*. 2010, 2011, Tian *et al*. 2013), respectively. The other four, *TpSnRK2s*:*TpSnRK2.2*, *TpSnRK2.5*, *TpSnRK2.10*, and *TpSnRK2.11,* were novel genes (Fig. 1).

Seeds of DPW were sterilized with 5 % (m/v) NaClO for 5 min and washed with double distilled water three times. The sterilized seeds were germinated on a double distilled water soaked filter paper in glass Petri plates at room temperature for 5 d. The seedlings were then grown in distilled water for 5 d and then in the nutrient solution (Hoagland's modified basal salt mixture, *MP Biomedicals*, CA, USA) in a growth chamber under a 16-h photoperiod, an irradiance of 180 μmol m^{-2} s^{-1}, a temperature of 25 °C, and a relative humidity of 75 %. Three-leaf seedlings were subjected to following treatments: NaCl (300 mM), ABA (50 μM), PEG-6000 (10 mM), or cold (4 °C). Untreated seedlings were used as controls. Leaf and root samples from 5 - 8 plants were collected after 4, 8, 12, and 24 h (two biological replicates), snap frozen in liquid nitrogen and stored at -80 °C.

A total RNA was extracted using *E.Z.N.A.® Total RNA Kit II* (*Omega*, Shanghai, China) according to the user's manual, and a RNase-free DNase set (*Omega*) was used to digest DNA. The RNA concentration was measured twice on a *NanoDrop-2000* spectrophotometer (*NanoDrop Technologies*, Wilmington, USA). A total RNA (2 μg) was used to the synthesis of cDNA using a *M-MLV* first strand cDNA synthesis kit (*Omega*) following the user's manual. All cDNA samples were diluted to 2 ng mm^{-3} (RNA) and stored at -20 °C until used in a real-time quantitative polymerase chain reaction (RT-qPCR).

The full-length cDNAs of *TpSnRK2.2*, *2.5*, *2.10*, and *2.11* were amplified using four pairs of primers (Table 1). All PCR products were cloned into pMD^{TM}19-T vector (*TaKaRa*, Dalian, China) and sequenced at *Sangon Biotech* (Shanghai, China).

The *Vector NTI* software (*Invitrogen*, Carlsbad**,** CA, USA) was used to align all DNA sequences and to analyze the homology of the amino acid sequences predicted by *ExPASy-Translate* (http://web.expasy.org/translate/). The signal sequence and transmembrane regions were predicted with *SignalP 4.1* server (http://genome.cbs.dtu.dk/services/SignalP/) and *TMpred*

(http://www.ch.embnet.org/software/TMPRED_form.html). The functional region and activity sites were scanned using *ScanProsite* tool (http://prosite.expasy.org/scanprosite/). Other motifs were identified according to Kulik *et al*. (2011). A phylogenetic tree was constructed by *MEGA v.5.05* software (http://www.megasoftware.net/) using the *Vector NTI* aligned sequences.

RT-qPCR was performed in 96-well plates with *CFX-96*™ system (*Bio-Rad*, Hercules, CA, USA). Each reaction contained 6.3 mm^3 of cDNA equal to 12.6 ng of total RNA, 0.6 mm^3 (3 nmol cm^{-3}) of each forward and reverse primers (Table 1), and 7.5 mm^3 of *iTaq*™ universal *SYBR*® *Green Supermix* (*Bio-Rad*), in a total volume of 15 mm^3. A no template control contained 6.3 mm^3 of an RNase free water. All reactions were carried out in triplicate for each cDNA sample, for three technical replicates. *Actin* (*ACT*) (F: CCGATTGCTTGTTATCTGTT; R: GAGGATGAAGACGAGAGTTT) was used as a reference gene. To reduce a plate-to-plate variation, each plate was also run with all five genes (*ACT*, *TpSnRK2.2*, *2.5*, *2.10*, and *2.11*) with one sample (a 4-h control root) as an internal calibrator. PCR reaction 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 starting from 65 to 95 °C to check for the specificity of amplification. The size of the PCR products was verified on 1.5 % (m/v) agarose gel electrophoresis after the RT-qPCR analysis. The ΔΔCt method (Livak and Schmittgen 2001) was used to evaluate the quantities of each amplified product using *Bio-Rad CFX* manager *v. 1.6.541.1028*. The Dunnett's test (α = 0.05) was conducted for the evaluation of significance of mean values.

Table 1. The information about eight *TpSnRK2* genes. TS - transcriptome sequencing (these sequences come from RNA-Seq); Tian *et al*. (2013), Mao *et al*. (2013), Zhang *et al*. (2011), and Zhang *et al*. (2010) reported four *SnRK2s* which are similar to *TpSnRK2.3*, *TpSnRK2.3*, *2.4*, *2.7*, and *2.8*, respectively; others are from the present experiments.

Name	Accession number	ORF [bp]	Length (AA)	CDS amplified primer	RT-qPCR primer
TpSnRK2.2	KF688097	1026	341	F: GTTCGGTGCTTGAATCTTGA	F: GGCACAAGATTGATGAGAAT
				R: AGAAGGATGAACACATTACAA	R: AGCAGCGTATTCCATAACTA
TpSnRK2.3	TS	1029	342		
TpSnRK2.4	TS	1092	363		
TpSnRK2.5	KF688098	873	290	F: CCACTCATCTTCATCATCAG	F: CATCATCAGCGGCAGCAA
				R: ATGTGGTGAGAAGAGATTGA	R: GGAGGAGAACAGAGGAAGAAGA
TpSnRK2.7	TS	1074	357		
TpSnRK2.8	TS	1101	366		
TpSnRK2.10	KF688099	1086	361	F: CTCATCATCATCAGCTAACT	F: GGAGAAGATTGACGAGAAC
				R: CGGTCAACTTATAGAAGAACT	R: TTGAAGCGGATGATGTTG
TpSnRK2.11	KF688100	1083	360	F: ATGGACAAGTACGAGGAG	F: GCAAGCAGACTACTACAAGA
				R: CAAAGCATACGTCCTACCAAAAC	R: TTCCTCATCATCCGATAACTC

Results

The full length cDNA of *TpSnRK2.2* (KF688097) was amplified. It consisted of 130 bp of a 5'-untranslated region (5'-UTR), 1 026 bp of an open reading frame (ORF), and 64 bp of 3'-UTR. Using a *BLAST* search of the *NCBI* database, the cDNA had a 96 % identity to *TtPK1* (AY714526) with three insertions at the 5'-UTR (Fig. 2). The ORF encoded the polypeptide of 341 amino acids (AAs) (Table 1). The deduced amino acid sequence of TpSnRK2.2 had a 99 % identity to TtPK1 (AAW49219), 90.9 % to OsSARK2 (JF733760), and 89 % to ZmSnRK2.2 (ACG50006) (Fig. 1), respectively. The *Scansite* analysis indicates that TpSnRK2.2 had two domains at N- and C-terminal regions (Fig. 3). Similarly to other SnRK2s, the N-terminal catalytic domain (AAs 4 - 260) was highly conserved, containing an ATP-binding site (AAs 10 - 33), a serine/threonine protein kinases active site (AAs 119 - 131), a potential N-myristoylation site (AAs 109 - 114), an activation loop (AAs 142 - 169) and a potential transmembrane helix (AAs 180 - 198), but no signal peptide. A region required for an osmotic stress response (AAs 267 - 301) was, however, detected in a relatively short C-terminal domain that is abundant in Asp (D) (Kulik *et al*. 2011).

The full length cDNA of *TpSnRK2.5* (KF688098) included 148 bp of 5'-UTR, 872 bp of ORF, and 231 bp of 3'-UTR. Aligning to a homologous sequence, *TpSnRK2.5.1* from RNA-seq of tall *T. polonicum* (unpublished data), a 106 bp nucleotide deletion occurred at 987 bp in *TpSnRK2.5* (Fig. 4) resulting in a premature termination during translation. Therefore, the ORF

encoded the polypeptide of only 290 AAs (Table 1) which had a 97 % identity to BdSAPK2.5 (XP_003580888), 93 % to OsSARK5 (JF733763), and 92 % to ZmSnRK2.5 (EU676037) (Fig. 1), respectively. The *Scansite* analysis indicates that TpSnRK2.5 had a complete N-terminal region and an incomplete C-terminal region (Fig. 3). Other motifs in the N-terminal region were the same as in TpSnRK2.2.

The full length cDNA of *TpSnRK2.10* (KF688099) consisted of 153 bp of 5'-UTR, 1 086 bp of ORF and 45 bp of 3'-UTR. The deduced polypeptide of *TpSnRK2.10* consisted of 361 AAs and had a 96 % identity to OsSAPK10 (JF733768) and 93 % to ZmSnRK2.10 (NM_001137396) (Fig. 1), respectively. Similarly to TpSnRK2.2, TpSnRK2.10 had a highly conserved N-terminal catalytic domain (AAs 23 - 279) and an intact C-terminal domain (Fig. 3). In the N-terminal catalytic domain, an ATP-binding site (AAs 29 - 52), a serine/threonine protein kinases activity site (AAs 138 - 150), a potential N-myristoylation site (AAs 129 - 134), an activation loop (AAs 161 - 188), and a potential transmembrane helix (AAs 201 - 217) were found, but no signal peptide was detected. The relatively short C-terminal domain that was abundant in Asp (D) consisted of a region required for an osmotic stress response (AAs 286 - 320) and of a region required for an ABA response (AAs 321 - 361).

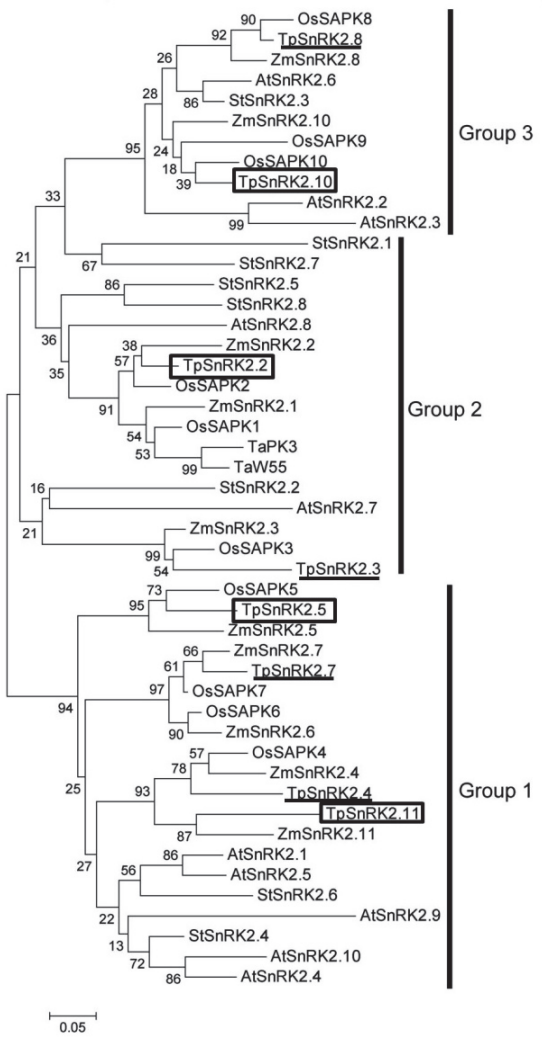

Fig. 1. Phylogenetic relationships of eight TpSnRK2s and other plant SnRK2s. Group 1: ABA-independent kinases; group 2: ABA-independent or weakly dependent kinases; group 3: ABA-dependent kinases. All amino acid sequences were aligned with the software *Vector NTI* and the phylogenetic tree was constructed with *Mega v. 5.05* using the neighbour-joining method. The *boxes* indicate the four novel genes, the *lines* indicate that the sequences of these genes come from the RNA-sequence.

The full length cDNA of *TpSnRK2.11* (KF688100) was amplified. It consisted of 1083 bp of ORF and 81 bp of 3'-UTR. The deduced polypeptide was 360 AAs and had a 83 % identity to ZmSnRK2.11 (EU676042) (Fig. 1). The *Scansite* analysis indicate that TpSnRK2.11 had two domains at its N- and C-terminal regions. The N-terminal catalytic domain (AAs 4 - 260) was highly conserved and included an ATP-binding site (AAs 10 - 33), a serine/threonine protein kinases activity site (AAs 119 - 131), an N-myristoylation site (AAs 109 - 114), an activation loop (AAs 142 - 169), and a potential transmembrane helix (AAs 180 - 198) (Fig. 3), but no signal peptide was detected. A region required for an osmotic stress response (AAs 267 - 301) existed at the C-terminal domain that was abundant in Glu (E).

To reveal relationships among the eight TpSnRK2s and other SnRK2s of *Arabidopsis*, rice, maize, and potato, a phylogenetic tree was constructed (Fig. 1). All the TpSnRK2s were classified into three groups. TpSnRK2.2 was clustered with ZmSnRK2.2 and OsSAPK2; TpSnRK2.3 was clustered with ZmSnRK2.3 and OsSAPK3; TpSnRK2.2 and TpSnRK2.3 are members of the group 2 kinases which are either not activated or activated very weakly by ABA. TpSnRK2.4 was clustered with ZmSnRK2.4 and OsSAPK4; TpSnRK2.5 was clustered with ZmSnRK2.5 and OsSAPK5; TpSnRK2.7 was clustered with ZmSnRK2.7 and OsSAPK7; TpSnRK2.11 was clustered with ZmSnRK2.11; TpSnRK2.4, TpSnRK2.5, TpSnRK2.7, and TpSnRK2.11 were members of the group 1 kinases that were not activated by ABA. TpSnRK2.8 was clustered with ZmSnRK2.8 and OsSAPK8; TpSnRK2.10 was clustered with OsSAPK10; TpSnRK2.8 and TpSnRK2.10 belong to the group 3 kinases that were strongly activated by ABA.

In the control, the expression of *TpSnRK2.2* in roots was higher than in leaves (Fig. 5*A*). Compared with the control, the expression of *TpSnRK2.2* in roots was strongly regulated by ABA (Fig. 5*B*), PEG (Fig. 5*C*), and NaCl (Fig. 5*D*). The expression pattern of *TpSnRK2.2* in roots under the cold stress was similar as in the control,

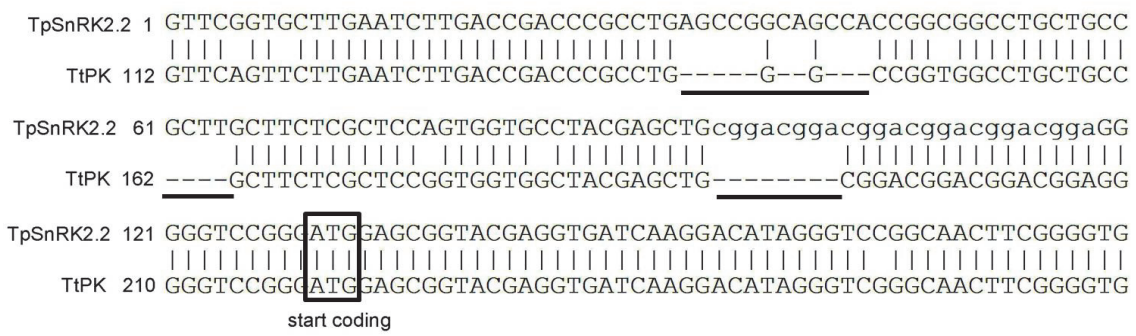

Fig. 2. The 5'-UTR sequence alignment of *TpSnRK2.2* and *TtPK1* from *T. tauschii*. The three *lines* represent three insertions; the *box* represents the start codon.

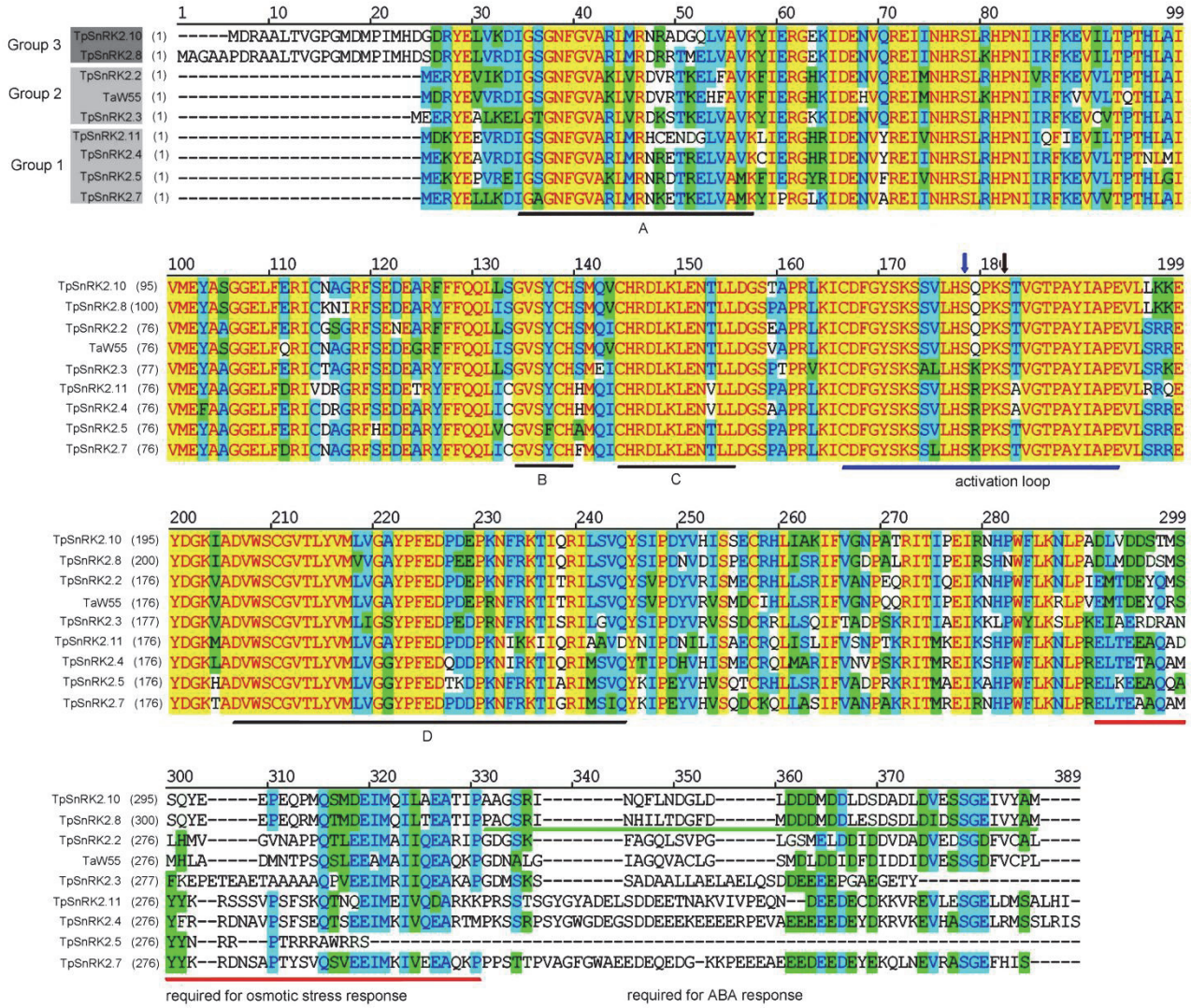

Fig. 3. The sequence alignment of eight TpSnRK2s and Taw55. *A* - ATP binding region, *B* - N-myristoylation site, *C* - serine/threonine protein kinases activity site, *D* - transmembrane helix site; the *blue line* represents the activation loop, the *red line* represents the region required for the osmotic stress response, the *green line* represents the region required for the ABA response, the *blue* and *black arrows* indicate two reversible phosphorylated serine residues.

but the expression was much lower (P = 0.002; Fig. 5*E*). In leaves, the expression of *TpSnRK2.2* was strongly regulated by PEG (Fig. 5*C*), NaCl (Fig. 5*D*), cold (Fig. 5*E*), and also slightly affected by ABA (Fig. 5*B*).

Since *TpSnRK2.5* encodes an incomplete polypeptide, it may lack a functional activity. Nevertheless, the expression of *TpSnRK2.5* in control leaves was higher than in control roots (Fig. 5*F*). In both roots and leaves, the expression was strongly regulated by ABA (Fig. 5*G*), PEG (Fig. 5*H*), NaCl (Fig. 5*I*), and cold (Fig. 5*J*).

The expression of *TpSnRK2.10* in control roots and leaves was similar (Fig. 5*K*,*O*). The transcriptions of *TpSnRK2.10* in roots and leaves were significantly activated by ABA (Fig. 5*L*), PEG (Fig. 5*M*), NaCl (Fig. 5*N*), and cold (Fig. 5*O*). The expression patterns of *TpSnRK2.10* revealed significant differences among these stresses. In comparison with the control, ABA significantly increased the expression of *TpSnRK2.10* in roots at 4 h (P = 0.031) and 8 h (P = 0.012), but

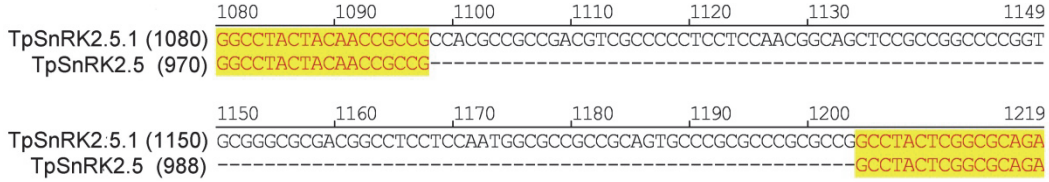

Fig. 4. The sequence alignment of *TpSnRK2.5* and *TpSnRK2.5.1* from a tall *T. polonicum*. A nucleotide deletion of 106 bp occurring at 987 bp in *TpSnRK2.5* is shown.

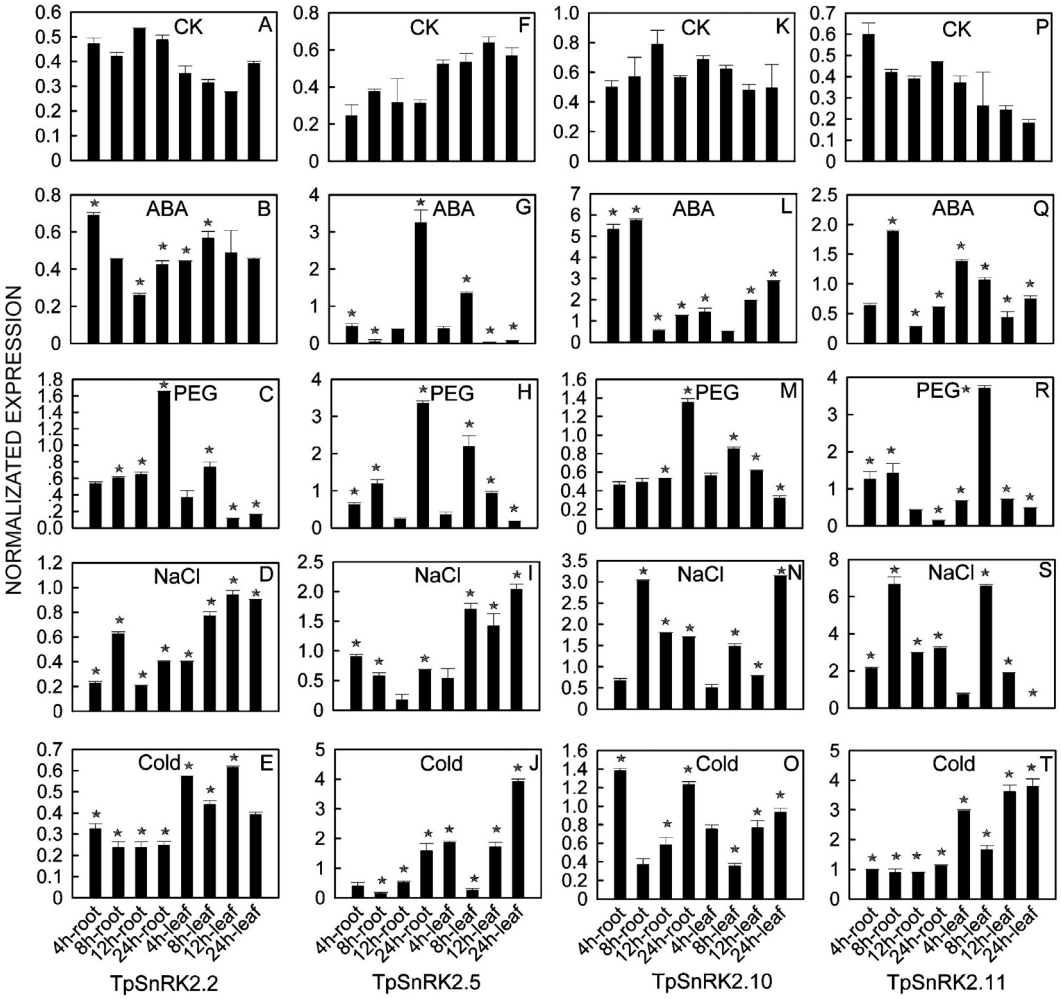

Fig. 5. Expression profiles of four novel *TpSnRK2s* in leaves and roots under different abiotic stresses. The *bars* represent standard errors of two biological replicates and three technical replicates. The *stars* represent significant differences between treatments and their corresponding controls. The data analysis and figures were done with *Sigma plot 12.0*.

decreased it at 12 h (P = 0.004) and then increased again at 24 h (P = 0.022). PEG significantly reduced its expression at 12 h (P = 0.013) and then increased it at 24 h (P = 0.015). NaCl significantly enhanced its expression at 8 h (P = 0.012), 12 h (P = 0.003), and 24 h (P = 0.023). The cold also significantly enhanced its expression at 4 h (P = 0.033) and 24 h (P = 0.029). In leaves, the expression of *TpSnRK2.10* was significantly increased by ABA and cold at 4 h (P = 0.020), 12 h (P = 0.016), and 24 h (P = 0.015), and reduced at 8 h. PEG significantly suppressed its expression at 4 h (P = 0.011) and 24 h (P = 0.012). NaCl significantly increased its expression at 12 h (P = 0.014) and 24 h (P = 0.003).

The expression of *TpSnRK2.11* in control roots was higher than in control leaves (Fig. 5*P*). *TpSnRK2.11* was strongly activated by ABA (Fig. 5*Q*), PEG (Fig. 5*R*), and NaCl (Fig. 5*S*) in both roots and leaves, and by the cold (Fig. 5*T*) in leaves. Compared with the control, ABA significantly up-regulated its expression at 8 h (P = 0.009) and 24 h (P = 0.012) in roots and at all sampling times (P < 0.05) in leaves. PEG significantly increased its expression at 4 h (P = 0.027) and 8 h (P = 0.024) in roots and at all times (P < 0.05) in leaves, but significantly reduced it at 24 h (P = 0.004) in roots. NaCl significantly enhanced its expression at all sampling times (P < 0.05) in both roots and leaves except for 24 h in leaves. The cold significantly increased the expression levels of *TpSnRK2.11* at all sampling times (P < 0.05) in both roots and leaves.

Discussion

Accumulating evidence indicates that SnRK2s are key components of the ABA signalling pathway that regulates many adaptive responses (Fujii *et al*. 2007, Coello *et al*. 2012, Nakashima and Yamaguchi-Shinozaki 2013) and played roles in other signalling pathways, *e.g*., in response to a heavy metal stress (Kulik *et al*. 2012). Therefore, many studies have been focusing on SnRK2s to understand their molecular/biochemical properties and functions. Based on our RNA-Seq data, eight homologous *SnRK2s*, *TpSnRK2.2*, *TpSnRK2.3*, *TpSnRK2.4*, *TpSnRK2.5*, *TpSnRK2.7*, *TpSnRK2.8*, *TpSnRK2.10*, and *TpSnRK2.11* were identified from tetraploid wheat, and four of them, *TpSnRK2.2*, *TpSnRK2.5*, *TpSnRK2.10*, and *TpSnRK2.11,* were novel genes. The deduced amino acid sequence of *TpSnRK2.2* had a 99 % identity to *TtPK1*. *TtPK1* was cloned from the D genome of *T. tauschii* (Holappa *et al*. 2005), whereas *TpSnR2.2* was cloned from the A or B genome of *T. polonicum*. Three insertions were found in 5'-UTR of *TpSnR2.2*. Several laboratories illustrated that SnRK2 activity is regulated by the reversible phosphorylation of two serine residues within the kinase activation loop (Belin *et al*. 2006, Burza *et al*. 2006, Boudsocq *et al*. 2007). Different phosphorylation mechanisms, however, might be involved in SnRK2 activation by hyperosmolarity and ABA (Boudsocq *et al*. 2007). In our study, an activation loop was detected in all four genes (Fig. 3) and each of the four genes was clustered into one of the three groups (Fig. 1). These results indicate that different phosphorylation mecha-nisms might exist among the four genes.

In addition, a potential N-myristoylation site and a potential transmembrane helix were also identified in three of the four genes (except *TpSnRK2.5*, Fig. 3), the two motifs are essential for a protein function in mediating membrane associations and protein-protein interactions in plant responses to an environmental stress (Podel and Gribskov 2004). A relatively diverged C-terminal region is mainly responsible for a functional distinction of SnRK2s (Kobayashi *et al*. 2004). A region required for an osmotic stress response at the C-terminal, which characterizes all the SnRK2s (Kulik *et al*. 2012), was identified in TpSnRK2.2, TpSnRK2.10, and TpSnRK2.11 (Fig. 3), which suggest that the three genes might have a potential function in mediating an osmotic balance. A region required for an ABA response is specific to the ABA-dependent SnRK2s (group 3) and is responsible for a kinase activation by ABA and an interaction with ABI1 phosphatase (Yoshida *et al*. 2006, Vlad *et al*. 2009). In our study, TpSnRK2.10 had this motif which might be activated by ABA and might interact with ABI1 phosphatase (Yoshida *et al*. 2006, Vlad *et al*. 2009).

Different SnRK2s may have different functions in different tissues, such as *OST1* (*AtSnRK2.6*) controlling stomata closure in leaves (Mustilli *et al*. 2002). *AtSPK2C* (*AtSnRK2.8*) is a root-specific protein kinase (Umezawa *et al*. 2004), *TaSnRK2.8* and *TaSnRK2.7* are mainly expressed in seedling roots (Zhang *et al*. 2010, 2011), and *TaSnRK2.4* and *TaSnRK2.3* are strongly expressed in booting spindles (Mao *et al*. 2010, Tian *et al*. 2013). In our study, *TpSnRK2.2* and *TpSnRK2.11* revealed a higher expression in control roots than in control leaves, the expression of *TpSnRK2.10* was similar in both roots and leaves, and the expression of *TpSnRK2.5* was higher in leaves than in roots (Fig. 5) suggesting that the four genes played different roles in different tissues.

As the key components of the ABA signalling pathway, SnRK2s were involved in response to environmental stresses (Fujii *et al*. 2007, Coello *et al*. 2012, Kulik *et al*. 2012, Nakashima and Yamaguchi-Shinozaki 2013). Evidences have shown that the expression of *SnRK2s* is differently regulated by ABA, PEG, NaCl, and cold in rice (Kobayashi *et al*. 2004),

maize (Huai *et al*. 2008), and wheat (Anderberg and Walker-Simmons 1992, Mao *et al*. 2010, Zhang *et al*. 2010, 2011, Tian *et al*. 2013). In the present study, the different expressions of the four genes under the ABA, PEG, NaCl, and cold stresses were observed (Fig. 5). The expression of *TpSnRK2.2* was strongly regulated by ABA, PEG, and NaCl in roots, and by PEG, NaCl, and the cold in leaves (Fig. 5*A*-*E*), which is different from *OsSAPK2* that was significantly up-regulated by NaCl and PEG in leaf blades and weakly in sheaths but not in other organs (Kobayashi *et al*. 2004), and *ZmSnRK2.2* that is induced by ABA but not by NaCl and cold (Huai *et al*. 2008). The transcript of *TpSnRK2.5* was intensely induced by all the treatments in roots and leaves (Fig. 5*F*-*G*), which differed from *OsSAPK5* that is only induced by PEG (Kobayashi *et al*. 2004), and from *ZmSnRK2.5* that is only induced by ABA (Huai *et al*. 2008). A different expression pattern of *TpSnRK2.10* indicated that this gene was very sensitive to ABA (Fig. 5L), NaCl (Fig. 5*N*), cold (Fig. 5*O*), and PEG (Fig. 5*M*). In contrast, its homologous gene *OsSAPK10* was down regulated by ABA and PEG in rice roots (Kobayashi *et al*. 2004), and *ZmSnRK2.10* is only up regulated by ABA (Huai *et al*. 2008). The transcript of *TpSnRK2.11* was significantly activated by PEG, NaCl, and cold, and weakly by ABA (Fig. 5*P*-*T*). These results are also different from *TaSnRK2s* (*PKABA1*, *TaSnRK2.3*, *TaSnRK2.4*, *TaSnRK2.7*, and *TaSnRK2.4*) whose expressions are induced by PEG, NaCl, and cold, and up-regulated by ABA with the exception of *TaSnRK2.7* (Anderberg and Walker-Simmons 1992, Mao *et al*. 2010, Zhang *et al*. 2010, 2011, Tian *et al*. 2013).

Conclusions

In the present study, four novel genes, *TpSnRK2.2*, *TpSnRK2.5*, *TpSnRK2.10*, and *TpSnRK2.11* were cloned and characterized in *T. polonicum*. The expression studies have shown that *TpSNRK2.2* was strongly regulated by PEG, NaCl, and cold in roots and leaves; *TpSnRK2.5* was intensely induced by all the treatments both in roots and leaves; *TpSnRK2.10* was very sensitive to ABA and NaCl, less sensitive to cold and PEG; *TpSnRK2.11* was activated significantly by PEG, NaCl, and cold, and weakly by ABA. These results indicate that the four genes were involved in the intricate responses of DPW to multi-environmental stresses, and played different roles in different tissues under different stresses.

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