RESEARCH NOTE

Characterization of genomic sequence of a drought-resistant gene *TaSnRK2.7* in wheat species

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[Zhang H. Y., Li W., Mao X. G. and Jing R. L. 2015 Characterization of genomic sequence of a drought-resistant gene *TaSnRK2.7* in wheat species. *J. Genet.* **94**, 299–304]

Introduction

Hexaploid wheat (*Triticum aestivum* L.) is one of the world's most important crop plants. It is originated from hybridization between tetraploid emmer *T. turgidum* (genome AABB) and diploid *Aegilops tauschii* (genome DD) ~8000 years ago (Dubcovsky and Dvorak 2007). Drought, salinity and extreme temperature greatly influence on wheat growth and production. The objectives of the researchers were to elucidate the stress signaling pathway and to improve tolerance against abiotic stress in wheat. In addition, wheat ($2n = 6x = 42$, genome AABBDD) is an allohexaploid, with a large genome size and relatively few polymorphisms. Although polyploidy brings about an increase in genetic information and novel system(s) of gene regulation, high levels of duplication create difficulties in determining the ancestral origin of the genome. Wheat species provide model systems to study the genomics of polyploid plants, as most progenitors have been determined and the genetic relationships among wheat species have been extensively characterized (Tsunewaki 1993).

SnRK2 (sucrose nonfermenting 1-related protein kinase) has been identified in many plants that play a key role against abiotic stress and developmental processes in plants. Individual members of SnRK2 have acquired different regulatory properties under stress signaling (Boudsocq *et al.* 2007; Huai *et al.* 2008; Mao *et al.* 2010; Tian *et al.* 2013; Xu *et al.* 2013). Furthermore, SnRK2 kinases are also involved in the regulation of carbon metabolism and energy status in their respective systems, similar to the function of sucrose nonfermenting 1 (SNF1) in yeast, which is activated in response to low cellular glucose levels by directly modulating the phosphorylation state of a number of metabolic enzymes (Zhang *et al.* 2010; Zheng *et al.* 2010).

In our recent study, a SnRK2 subclass member, i.e. *TaSnRK2.7* responding to PEG, NaCl and cold stresses, was identified in *T. aestivum*. Earlier studies showed that the overexpression of *TaSnRK2.7* lead to enhanced multi-stress tolerance in *Arabidopsis* (Zhang *et al.* 2011a). In addition, the genetic diversity of *TaSnRK2.7-a* and *TaSnRK2.7-b* showed a heterogeneous distribution pattern of nucleotide diversity along the genomic sequence, whereas no single-nucleotide polymorphisms (SNP) was associated with stress resistance (Zhang *et al*. 2011b, c). Although, evidence revealed that *TaSnRK2.7* plays an important role in abiotic stress signaling, knowledge of the genomic sequences is fragmentary and the molecular mechanism of the activation is still enigmatic. In this report, *TaSnRK2.7* genomic sequences were characterized in common wheat and related species.

Materials and methods

Plant materials

Common wheat cultivar 'Chinese Spring' was used for isolation and gene structure analysis of *TaSnRK2.7* genomic sequences. A set of nulli-tetrasomic (NT) lines in 'Chinese Spring' was used for chromosomal location. Fifteen accessions of wheat species were used in the evolutionary analysis (table 1). Five wheat species included 'Chinese Spring' (*T. aestivum*, AABBDD), DM50 (*T. dicoccum*, AABB), UR201 (*T. urartu*, AA), Y2001 (*Ae. Speltoides*, SS, putative B genome donor species) and Y85 (*Ae. Tauschii*, DD) were used in Southern hybridization, transcript level analysis and sugar content determination.

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Keywords. *TaSnRK2.7*; genomic sequence; evolution; stress; *Triticum aestivum.*

Table 1. Plant materials used in the evolutionary analysis of *TaSnRK2.7*.

Name/accession	Species	Genome
W7984	*T. aestivum*	AABBDD
Opata 85	*T. aestivum*	AABBDD
Chinese Spring	*T. aestivum*	AABBDD
PS9	*T. persicum*	AABB
PO9	*T. polonicum*	AABB
DM50	*T. dicoccum*	AABB
UR201	*T. urartu*	AA
UR204	*T. urartu*	AA
UR208	*T. urartu*	AA
Y2001	*Ae. speltoides*	BB
Y2017	*Ae. speltoides*	BB
Y2041	*Ae. speltoides*	BB
Y85	*Ae. tauschii*	DD
Ae40	*Ae. tauschii*	DD
Ae46	*Ae. tauschii*	DD

Water stress experiments

Wheat seeds were sterilized for 10 min in 75% (v/v) ethanol and washed with sterilized water. From each accession, 20 germinating seeds were cultured in a growth chamber (20°C, 12 h light/12 h dark cycle). Two-leaf seedlings (nine days old) were stressed in PEG-6000 (−0.5 MPa) solutions which had been shown to constitute significant stress in pilot experiments. Untreated seedlings (control) continued to be cultured with double distilled water. Leaf samples were taken at 6 h after treatments, frozen immediately with liquid nitrogen and then stored at −80°C for RNA isolation and total soluble sugar analysis. The experiments were triplicated.

Southern blotting, real-time quantitative PCR and total soluble sugar analysis

These assays were performed as described previously (Zhang *et al.* 2011a). Sequence variants were validated in Southern blotting assay by using the specific fragment of *TaSnRK2.7-c* as a probe.

Genomic sequence analysis

Genomic DNA was extracted from leaf samples with the CTAB extraction method. According to *TaSnRK2.7* cDNA sequence (Zhang *et al.* 2011a), a pair of primers (5′-CCCAATCTTCGCCTCTGCC-3′ and 5′-TTTCCGCAATG CTAGCTTAATCAG-3′) flanking the ORF were used for genomic sequence studies. To further prove the chromosomal origin of different sequence variants, genome-specific primers were designed based on the DNA variations (see figure 1 and table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). DNA sequencing and sequence analysis were performed as described previously (Zhang *et al*. 2011b, c). To analyse the molecular evolution of *TaSnRK2.7*, sequences were aligned with ClustalW software, are then were used to construct a phylogenetic tree by using the neighbor-joining algorithm in programs of the PHYLIP package.

BLAST analysis

To validate the copy number and chromosomal location, *TaSnRK2.7* genomic sequences were used as queries to screen the wheat genome sequencing database using the newly developed rapid ENA sequence similarity search service (http://www.ebi.ac.uk/ena/search/) and the NCBI Sequence Read Archive (SRA) database. A significant match was declared when the queried sequences showed at least 95% nucleotide identity with an *e* value equal to 0. Database searches were performed by using BLAST. Sequence alignment and similarity analysis were conducted by multiple sequence alignment programs in DNASTAR.

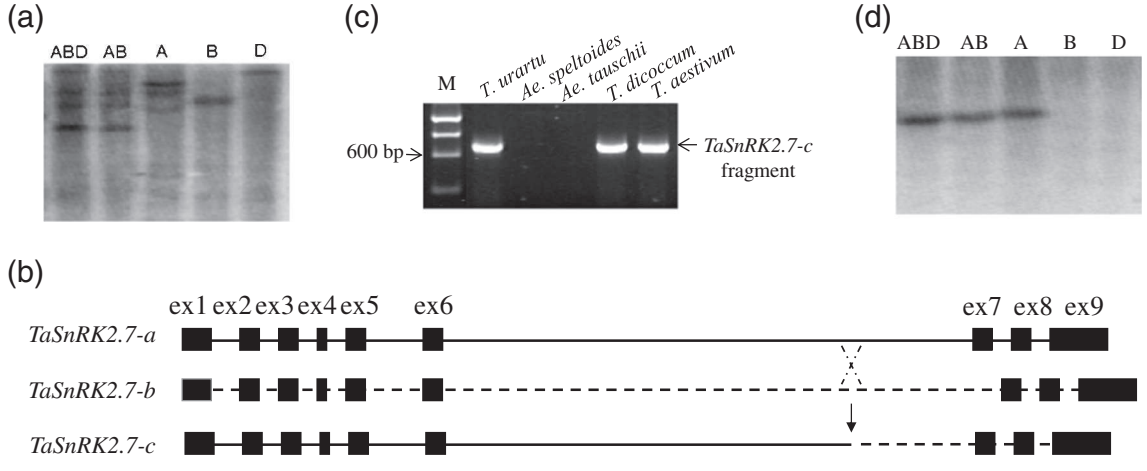

Figure 1. Gene structure and genotyping analysis of *TaSnRK2.7* in A genomes. (a) Southern blotting analysis of *TaSnRK2.7*. (b) Schematic diagram of *TaSnRK2.7* in A genome diploids. Filled rectangular boxes represent exons, horizontal lines and dashed lines indicate the positions of introns. *TaSnRK2.7-c* exhibited similar sequence with *TaSnRK2.7-a* in 5′ sequence, and *TaSnRK2.7-b* in 3′ sequence. (c) PCR-based identification of *TaSnRK2.7-c*. (d) Southern blotting analysis of *TaSnRK2.7-c*. The ABD genome DNA of hexaploid wheat, AB genome DNA of tetraploid wheat, A genome DNA of *T. urartu*, B genome DNA of *Ae. speltoides* and D genome DNA of *Ae. tauschii* were digested with *Nco*I, respectively.

Results and discussion

Gene structure and genotyping of TaSnRK2.7

Southern blotting analysis were performed by using *Eco*RV, *Hin*dIII and *Nco*I in our previous study (Zhang *et al.* 2011a). The hybridization patterns of DNA digested with *Eco*RV and *Hin*dIII reveal that *TaSnRK2.7* is most likely to exist as multi-copy genes in the genomes of hexaploid wheat. However, four and three bands were separately found in ABD and A genomes digested with *Nco*I. In this study, to further analyze the copy number, Southern blotting analysis were carried out by using hexaploid wheat, related tetraploid and diploid species. As shown in figure 1a, four hybridized bands were evident in ABD and AB genomes digested with *Nco*I. The A genome contained three bands in the blot, while a single band is present in B and D genomes. This analysis indicated that *TaSnRK2.7* had at least four copies in the polyploid wheat, three copies in the A genome, and one copy in the B and D genomes. Southern hybridization results reflected different banding patterns between diploid and polyploid wheat and similar hybridization patterns between hexaploid and tetraploid wheats. It suggested that the rapid genomic changes had occurred after polyploidization and no major variations occurred in *TaSnRK2.7* gene during further evolution of the polyploids.

To obtain genomic sequences of *TaSnRK2.7*, primer pairs flanking the open reading frame (ORF) sequence were designed. The comparison of the sequencing results showed that among the genomic sequences of *TaSnRK2.7*, five sequence variants (designated *TaSnRK2.7-a*, *TaSnRK2.7-b*, *TaSnRK2.7-c*, *TaSnRK2.7-d* and *TaSnRK2.7-e*) existed in hexaploid wheat, four (*TaSnRK2.7-a*, *TaSnRK2.7-b*, *TaSnRK2.7-c* and *TaSnRK2.7-d*) in tetraploid wheat, three (*TaSnRK2.7-a*, *TaSnRK2.7-b* and *TaSnRK2.7-c*) in A genome diploids, and only one in B (*TaSnRK2.7-d*) and D (*TaSnRK2.7-e*) diploids. These sequence variants exhibited similar coding regions and gene structures consisted of nine exons and eight introns respectively, however the differences mainly occurred in introns (figure 1 in electronic supplementary material). Gene structure is consistent with *TaSnRK2.7* counterparts in rice, maize and *Arabidopsis* (Hrabak *et al.* 2003; Huai *et al.* 2008). Notably, sequence alignments of the three sequence variants in A diploids showed that *TaSnRK2.7-c* exhibited similar sequence with *TaSnRK2.7-a* in 5′ sequence, and *TaSnRK2.7-b* in 3′ sequence, suggesting that *TaSnRK2.7-c* might be generated by sequence exchange and recombination between *TaSnRK2.7-a* and *TaSnRK2.7-b* at the longest intron (figure 1b). To determine the existence of *TaSnRK2.7-c in vivo*, specific primers were designed on the basis of DNA variations. The specific fragment of *TaSnRK2.7-c* was used as a probe in Southern blotting analysis, which showed a single band in ABD, AB and A genomes. Sequence variant-specific PCR, sequencing and Southern blotting analysis confirmed that *TaSnRK2.7-c* was present in A genomes, while absent in PCR-generated chimera (figure 1, c&d). This finding was an indicative of sequence exchange between gene duplications which might occur in the evolutionary process of A diploids or even earlier. Similar phenomenon was also reported in other studies (Joshi and Nayak 2013; Leister 2004). It is evident that sequence exchange between divergent sequences are rare while exchange between similar genes appear frequently (Baumgarten *et al.* 2003; Meyers *et al.* 2003).

Recently, the genomes of bread wheat *T. urartu* and *Ae. Tauschii* have been sequenced and assembled using a whole-genome shotgun strategy (Brenchley *et al.* 2012; Jia *et al.* 2013; Ling *et al.* 2013). The sequencing data are available at the MIPS Wheat Genome Database (http://mips.helmholtz-muenchen.de/plant/wheat/uk454survey/index.jsp). Further, Illumina and 454 sequence reads could be obtained from the NCBI SRA for diploid and tetraploid wheat. Through BLAST analysis of genome sequence, five copies of *TaSnRK2.7* were identified, of which three copies (*TaSnRK2.7-a*, *TaSnRK2.7-b* and *TaSnRK2.7-c*) were present in the series of the A genome of hexaploid wheat (location: IWGSS_CSS_2AL_scaff_6406711) and the *T. urartu* (A) genome (location: scaffold18266), and *TaSnRK2.7-d* was localized in the B genome of hexaploid wheat (location: IWGSS_CSS_2BL_scaff_8062458). Moreover, *TaSnRK2.7-e* was also identified in the D genome of hexaploid wheat (location: IWGSS_CSS_2DL_scaff_9891739) and the *Ae. tauschii* (D) genome (location: scaffold99829). The Southern blotting and genotyping analysis of *TaSnRK2.7* findings support and thus confirm the results that five sequence variants of *TaSnRK2.7* existed in the genomes of polyploid wheat: three in A genome and one each in B and D genomes, and each sequence variant was present in a single copy. Homology searches also indicated that *TaSnRK2.7-c* existed in A genomes, and did not appear in PCR-generated chimeras.

Evolutionary analysis

TaSnRK2.7 sequences were used for the construction of phylogenetic tree to find the origin and evolutionary aspect of *TaSnRK2.7* in hexaploid wheat and their relatives. As shown in figure 2a, all these sequences could be divided into five distinct groups: three sequence variants (*TaSnRK2.7-a*, *TaSnRK2.7-b* and *TaSnRK2.7-c*) were identified in polyploidy wheat and A diploids (clades I–III), *TaSnRK2.7-d* sequence variant was in polyploidy wheat and B diploids (clade IV) and *TaSnRK2.7-e* was present in hexaploid wheat and D diploids (clade V). Evolutionary analysis showed that three *TaSnRK2.7* variants (*TaSnRK2.7-a*, *TaSnRK2.7-b* and *TaSnRK2.7-c*) originated from A diploids; B and D diploids were individually the genomic donors of *TaSnRK2.7-d* and *TaSnRK2.7-e*. Moreover, one sequence variant was simultaneously present in hexaploid wheat and relatives, implying the sequence variants of *TaSnRK2.7* occurred before polyploidization.

To further prove the chromosome origin of different sequence variants, genome-specific primers based on DNA variations were designed. The genomic DNAs of hexaploid

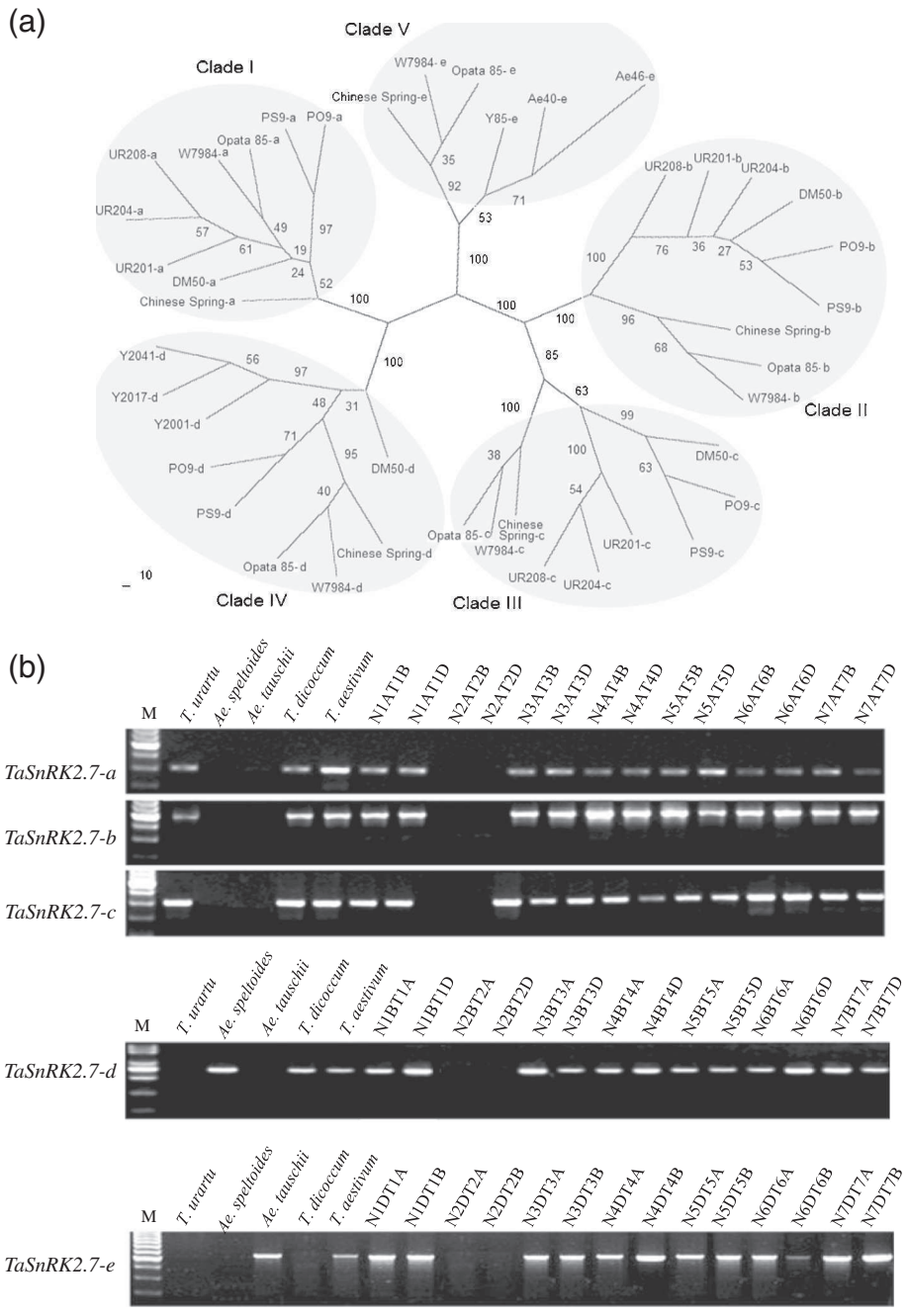

Figure 2. Evolutionary analysis of *TaSnRK2.7* in wheat. (a) Phylogenetic analysis of *TaSnRK2.7* genomic sequences from hexaploid, tetraploid and diploid wheat. The phylogenetic tree was constructed using PHYLIP 3.68 package; bootstrap values are provided in percentage. (b) PCR-based chromosome location of the *TaSnRK2.7*. Absence of a band indicates that the specific sequence is located on the corresponding null genome or chromosome.

wheat, related relatives and NT lines were amplified by using genome-specific primers. These findings have been supported in homology searches, *TaSnRK2.7-a*, *TaSnRK2.7-b* and *TaSnRK2.7-c* were located on chromosome 2A. *TaSnRK2.7-d* and *TaSnRK2.7-e* were individually existed on chromosomes 2B and 2D (figure 2b). In our previous study, *TaSnRK2.7-b* was mapped on chromosome 2AL flanked by *WMC179.4* and *WMC401*, which were colocated in the same or adjacent chromosome intervals with QTLs for phosphorus utilization efficiency and accumulation efficiency of stem water-soluble carbohydrates (Zhang *et al.* 2011c).

Expression patterns of TaSnRK2.7 in wheat species

The expression patterns of *TaSnRK2.7* in different wheat species were identified under normal and water deficit conditions. As shown in figure 3, the expression levels of

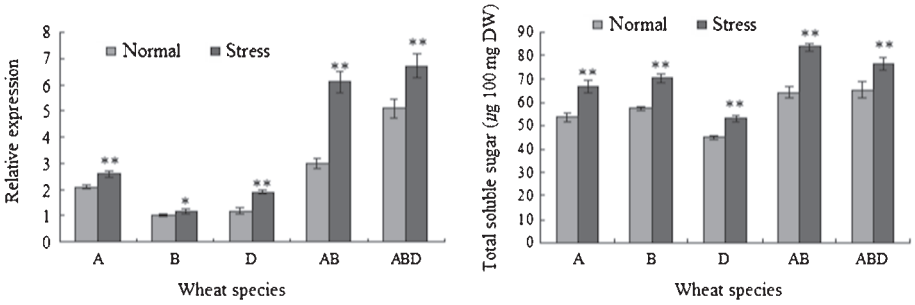

Figure 3. Expression patterns of *TaSnRK2.7* and total soluble sugars under normal and stress conditions. The $2^{-\Delta\Delta CT}$ method was used to measure the relative expression level of target gene. The transcripts and total soluble sugars in nonstressed seedling leaves were used as the control. Values are mean of three samples ±S.E. *Significantly different from the control with *F*-test (*P <0.05, **P <0.01).

TaSnRK2.7 increased significantly under PEG treatments. In accordance with copy numbers analysis, *TaSnRK2.7* was expressed remarkably in hexaploid wheat, weakly in tetraploid wheat and marginally in diploid species under normal conditions. The results suggested that more gene duplications may induce higher transcription levels under normal conditions. However, this expression pattern was not observed under stress conditions. It was found that transcription levels of *TaSnRK2.7* in B diploids (one *TaSnRK2.7* duplication) were higher than that in A diploids (three *TaSnRK2.7* duplications). This result suggests that may be some other stress-responsive regulatory mechanisms are involved which are independent of the number of *TaSnRK2.7* duplications to regulate the transcription levels of *TaSnRK2.7* in stress responses.

Despite the fact that the SnRK2 family is related to the evolutionarily conserved Snf1/AMPK/SnRK1 kinases which are active in metabolic functions and energy sensing, little is known about the functions of SnRK2 kinases in carbohydrate metabolism. Recently, the AtSnRK2.6 protein was found to mediate the regulation of sucrose metabolism and plant growth in *Arabidopsis* (Zheng *et al.* 2010). In our previous study, the overexpression of wheat *SnRK2s* in *Arabidopsis* resulted in high total soluble sugars and enhanced tolerance to stresses (Zhang *et al.* 2010; Zhang *et al.* 2011c; Tian *et al.* 2013) It was speculated that soluble sugar accumulation may lower the osmotic potential in the plant sap, maintaining regular cell turgor and avoiding damage to cell membranes that would enhance osmotic stress. In the present study, *TaSnRK2.7* transcripts and total soluble sugars increased significantly under PEG treatments (figure 3), indicating that *TaSnRK2.7* and sugar metabolism is playing a role in stress tolerance. However, no significant association between transcription levels of *TaSnRK2.7* and sugar contents under normal and stress conditions were noted (data not shown). It was proposed that environmental stresses were associated with extensive physiological, morphological and molecular changes. Soluble sugar accumulation in the assay was related to the whole gene family expression rather than a single gene.

Conclusions

Southern blotting and genotyping analysis suggested that five sequence variants of *TaSnRK2.7* genomic sequences (*TaSnRK2.7-a*, *TaSnRK2.7-b*, *TaSnRK2.7-c*, *TaSnRK2.7-d* and *TaSnRK2.7-e*) existed in hexaploid wheat, three (*TaSnRK2.7-a, TaSnRK2.7-b* and *TaSnRK2.7-c*) in A genome diploids, and only one in B (*TaSnRK2.7-d*) and D (*TaSnRK2.7-e*) diploids. Notably, *TaSnRK2.7-c* may be generated by sequence exchange between *TaSnRK2.7-a* and *TaSnRK2.7-b* at the sixth intron. This finding was validated by sequence variant-specific PCR and Southern blotting analysis. Evolutionary analysis indicated that *TaSnRK2.7-a*, *TaSnRK2.7-b* and *TaSnRK2.7-c* originated from A, B and D diploids, were individually the genomic donors of *TaSnRK2.7-d* and *TaSnRK2.7-e.* Consistent with copy numbers and sequence variants analysis, *TaSnRK2.7* expressed remarkably in hexaploid wheat, weakly in tetraploid wheat and marginally in diploid species under normal conditions. *TaSnRK2.7* transcripts and total soluble sugars increased significantly under different stresses whereas no significant association between gene transcripts and sugar contents was detected in the present study.

Acknowledgements

This work was supported by the National Natural Science Foundation (31271720) and the National High-tech R&D Programme (2012AA10A308) of China.

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Received 14 September 2014, in revised form 21 November 2014; accepted 12 December 2014
Unedited version published online: 18 December 2014
Final version published online: 19 May 2015