Plant Cell Rep (2008) 27:1861–1868
DOI 10.1007/s00299-008-0608-8

GENETICS AND GENOMICS

# Cloning and characterization of the SnRK2 gene family from *Zea mays*

**Junling Huai · Meng Wang · Junguang He · Jun Zheng · Zhigang Dong · Hongkun Lv · Jinfeng Zhao · Guoying Wang**

Received: 27 May 2008 / Revised: 29 July 2008 / Accepted: 29 August 2008 / Published online: 17 September 2008


**Abstract** The SnRK2 gene family is a group of plant-specific protein kinases that has been implicated in ABA and abiotic stress signaling. We found 11 *SnRK2s* in maize, assigned names from *ZmSnRK2.1* to *ZmSnRK2.11* and cloned ten of them. By analyzing the gene structure of all the *SnRK2s* from *Arabidopsis*, rice, and maize, we found seven exons that were conserved in length among most of the *SnRK2s*. Although the C-terminus was divergent, we found seven conserved motifs. Of these, motif 1 was common to all of the *SnRK2* genes. Based on phylogenetic analysis using the kinase domain and motif 1, the SnRK2s were divided into three groups. Motifs 4 and 5 were found specifically in group I, and many genes of this group have been confirmed to be induced by ABA. This result suggests that these two motifs mediate the ABA response. The expression patterns of *ZmSnRK2* genes were characterized by using quantitative real-time RCR, which revealed that *ZmSnRK2* genes were induced by one or more abiotic stress treatments and therefore may play important roles in maize stress responses.

**Keywords** Maize · ZmSnRK2 · Abiotic stress · Protein kinase · Gene structure

J. Huai and M. Wang have contributed equally to this paper.

Communicated by H. S. Judelson.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00299-008-0608-8) contains supplementary material, which is available to authorized users.

J. Huai · J. He · Z. Dong · J. Zhao
State Key Laboratory of Agrobiotecnology,
China Agricultural University, 100094 Beijing, China

M. Wang
Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences, 100101 Beijing, China

J. Zheng · H. Lv · G. Wang (✉)
Institute of Crop Sciences,
Chinese Academy of Agricultural Sciences,
100081 Beijing, China
e-mail: gywang@caas.net.cn

## Introduction

In eukaryotes, protein kinases and protein phosphorylation events play key roles in growth and development. Phosphorylation activities regulate processes such as cell division, metabolism, intracellular signal transduction, and many others. In plants, there are many genes that encode putative protein kinases, some of which are unique to plants. The calcium-dependent protein kinase (CDPK) family is one of the largest kinase families in plants. The kinases related to the CDPK and SnRK (SNF1-related kinase) in *Arabidopsis* comprise the CDPK-SnRK superfamily (Hrabak et al. 2003). This superfamily consists of seven types of serine-threonine protein kinases found in vascular and nonvascular plants, green algae, and certain protozoa (ciliates and apicomplexans) (Hrabak 2000; Hrabak et al. 2003). According to the sequence similarity and gene structure, the SnRK subfamily can be further divided into three subgroups, SnRK1, SnRK2, and SnRK3.

There is increasing evidence that SnRK2 genes play important roles in abiotic stress response in plants (Li et al. 2000; Mustilli et al. 2002; Yoshida et al. 2002; Umezawa et al. 2004). The first described SnRK2 gene (PKABA1) was cloned from wheat, and was induced by ABA exposure and dehydration (Anderberg and Walker-Simmons 1992). Subsequently, PKABA1 was found to act as a key factor in

the suppression of GA-inducible gene expression in the aleurone layers of barley (Gomez-Cadenas et al. 1999). Furthermore, the SnRK2-type protein kinase AAPK was found to be activated by ABA in guard cells and involved in the regulation of stomatal closure in *Vicia faba* (Li et al. 2000).

The first two SnRK2s in *Arabidopsis*, *ASK1,* and *ASK2*, were cloned in 1993 (Park et al. 1993). Until now, 10 SnRK2 genes have been identified and renamed from *SnRK2.1* through *SnRK2.10* (Halford and Hardie 1998; Hrabak et al. 2003). A number of experiments have indicated that SnRK2s may be activated by ABA or hyperosmotic stress, and phosphorylate the downstream ABA-responsive element binding factors (ABFs), although the activation mechanisms appear to be different for each SnRK2 (Boudsocq et al. 2004; Boudsocq et al. 2007; Kobayashi et al. 2005). SnRK2.6 (SRK2E or OST1) was activated by ABA, and mutations in this gene resulted in a wilted phenotype, mainly because of the loss of stomatal closure in response to a rapid humidity decrease (Mustilli et al. 2002; Yoshida et al. 2002). Overexpression of *SRK2C* (SnRK2.8) has been shown to result in improved drought tolerance in transgenic *Arabidopsis* (Umezawa et al. 2004). Recently, SnRK2.2 and SnRK2.3 were reported to play key roles in ABA signaling in *Arabidopsis* (Fujii et al. 2007). Ten SnRK2 s (SAPKs) reported in rice were also found to be activated by hyperosmotic stress, and three of them were also activated by ABA (Kobayashi et al. 2004).

To better understand the function of SnRK2 genes in maize, we conducted a genome-wide survey of the PlantGDB and related databases using the SnRK2 sequences from *Arabidopsis* and rice. We found 11 putative SnRK2 sequences in maize and assigned names from ZmSnRK2.1 through ZmSnRK2.11. Except for *ZmSnRK2.9*, all of the *ZmSnRK2* genes were amplified by RT-PCR. The gene structure and evolution of the SnRK2s from *Arabidopsis*, rice, and maize were analyzed and their expression profiles under various stress treatments were characterized by quantitative real-time PCR analysis.

## Materials and methods

### In silico analysis

The SnRK2 sequences (including cDNA, genome sequence, promoter sequence) of *Arabidopsis* and rice were obtained from the TAIR (http://www.arabidopsis.org) and TIGR rice databases (http://www.tigr.org/tdb/e2k1/osa1). To identify the SnRK2 genes in maize, we subjected the sequences from *Arabidopsis* and rice to a tblastn search against the PlantGDB database (http://www.plantgdb.org). We found 11 SnRK2 cDNA sequences and designated them as *ZmSnRK2.1* through *ZmSnRK2.11*. According to the cDNA sequences, we designed specific primers to amplify the full-length cDNAs by PCR. Sequencing of the products confirmed that we had obtained 10 SnRK2s. Concurrently, we used the protein sequences of ZmSnRK2 genes to perform local blastp searches against the NON-TE_TRANSLATIONS database downloaded from the Maize Genome Browser (http://www.maizesequence.org) to obtain the genomic and promoter sequences.

### Gene structure analysis

After obtaining all the SnRK2 genomic and ORF sequences of *Arabidopsis*, rice, and maize, gene structure predictions were generated using the online web server Spidey (an mRNA-to-genomic alignment program http://www.ncbi.nlm.nih.gov/spidey/). The results were manually adjusted according to the GT-AG rule.

### Identification of C-terminal conserved motifs

We identified the C-terminal conserved motifs of the SnRK2s using MEME (http://meme.sdsc.edu/meme/meme.html) with the motif length set at 6–80, motif sites 2–200, and e-value $<1e^{-10}$.

### Phylogenetic analysis

The conserved kinase domain and motif 1 in the C-terminus of SnRK2s from *Arabidopsis*, rice, and maize were aligned using ClustalX1.83 with default parameters (Dinkins et al. 2002). The result was manually adjusted with Jalview (2.07) (Clamp et al. 2004). The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA (3.1) (Kumar et al. 2004; Saitou and Nei 1987). Bootstrap analysis was performed using 1,000 replicates in MEGA (3.1) to evaluate the reliability of different phylogenetic groups. The tree obtained was viewed using TREEVIEW software (Page 1996).

### Promoter analysis

The 2 kb region upstream of the translation start site of each gene was considered to be the putative promoter region. We searched for abiotic stress-associated elements (ABRE, DRE/CRT and LTRE) in the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al. 1999).

### Plant materials and growth conditions

Seeds of maize inbred line Han21 were directly sown in vermiculite saturated with water in salver. The seeds were

grown for 9 days under a 16 h light/8 h dark cycle at 26°C. For NaCl and ABA treatments, the roots of seedlings were submerged into a water solution of 250 mm NaCl or 100 μm ABA for 1, 3, 6, and 12 h, respectively. For cold and heat treatments, the young seedlings were put into 4 or 42°C conditions for periods of 1, 3, 6, and 12 h.

### Quantitative real-time PCR analysis

RNA was isolated from maize samples using the TRIzol reagent (Invitrogen) according to the standard protocol. The quality and quantity of every RNA sample was assessed by agarose gel electophoresis. The cDNA was synthesized from the total RNA using the M-MuLV reverse transcriptase (New England BioLabs) according to the manufacturer's instructions. The specific sequences of the primers used for real-time PCR are listed in Table S1. The real-time PCR was conducted with SYBR *Premix Ex Taq*$^{TM}$ (Takara) and carried out using a Biorad system. The reaction procedures were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 15 s. Tubulin (accession number AY103544) was used as the internal control. Relative fold expression changes were calculated using the relative $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgrn 2001).

## Results

### Identification of ZmSnRK2 genes

Through tblastn searching, we obtained 11 SnRK2 sequences from maize that were designated *ZmSnRK2.1* to *ZmSnRK2.11*. We then conducted RT-PCR to confirm 10 *ZmSnRK2*s; the exception was *ZmSnRK2.9* (Table 1, Table S1). We concluded that there are at least 10 SnRK2s existing in the maize genome.

In *Arabidopsis*, SnRK2 genes are found on all chromosomes (Table S2), which explains why the distribution of this family shows no chromosomal bias. We searched for segmental duplications of SnRK2 genes in *Arabidopsis* using the TIGR database (http://www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml) and found two pairs of genes (*AtSnRK2.1* and *AtSnRK2.5*, *AtSnRK2.2* and *AtSnRK2.3*). This result is different from that of Hrabak et al. (2003) where *SnRK2.2/2.3* and *SnRK2.4/2.10* were identified as segmental duplication pairs. The rice SnRK2 genes were found to be located on chromosomes 1, 2, 3, 4, 7, 10, and 12. Two pairs of genes (*SAPK1/SAPK2* and *SAPK4/SAPK5*) were identified as segmental duplication pairs in the TIGR Segmental Genome Duplication of Rice database (http://rice.plantbiology.msu.edu/segmental_dup/index.shtml). The ZmSnRK2 genes were also found on all chromosomes except chromosomes 8 and 9 (the locus of ZmSnRK2.1 is unknown; data not shown).

### Gene structures of SnRK2 from maize, *Arabidopsis,* and rice

Analysis of the exon-intron structures of SnRK2 genes can provide important information regarding the evolution of this gene family. We determined the distribution of the predicted exon-intron structures using the coding regions of all the SnRK2 genes from maize, *Arabidopsis,* and rice (Fig. 1). Most of the SnRK2s exhibited a highly conserved distribution of exons and introns, with all members having nine exons except *AtSnRK2.6* (ten exons), *AtSnRK2.8* (six exons), *SAPK5* (four exons), *SAPK10* (seven exons),

**Table 1** SnRK2 genes in maize and some characteristics of them

| Name | Accession number | Locus | ORF (bp) | Protein | | |
|---|---|---|---|---|---|---|
| | | | | Length (aa) | Mol. wt. (kd) | pI |
| ZmSnRK2.1 | EU676033 | AC199054.2 | 1029 | 342 | 38.7 | 5.06 |
| ZmSnRK2.2 | EU676034 | AC186803.4 | 1020 | 339 | 38.5 | 5.26 |
| ZmSnRK2.3 | EU676035 | AC196411.3 | 1002 | 333 | 37.8 | 5.32 |
| ZmSnRK2.4 | EU676036 | AC195225.3 | 1086 | 361 | 42.2 | 5.88 |
| ZmSnRK2.5 | EU676037 | AC190828.2 | 1092 | 363 | 41.8 | 6.30 |
| ZmSnRK2.6 | EU676038 | AC201983.3 | 1095 | 364 | 41.8 | 5.74 |
| ZmSnRK2.7 | EU676039 | AC191130.2 | 1071 | 356 | 40.6 | 6.08 |
| ZmSnRK2.8 | EU676040 | AC206916.1 | 1101 | 366 | 41.3 | 4.62 |
| ZmSnRK2.9 | | AC208970.1 | 1095 | 364 | 40.8 | 4.61 |
| ZmSnRK2.10 | EU676041 | AC185635.4 | 1089 | 362 | 40.8 | 4.65 |
| ZmSnRK2.11 | EU676042 | AC196642.4 | 1080 | 359 | 41.5 | 5.61 |

The locus of the ZmSnRK2s means the Bac number from maize genome browser database. ZmSnRK2.9 hasn't been submitted to the NCBI and the sequence was in the supplementary materials

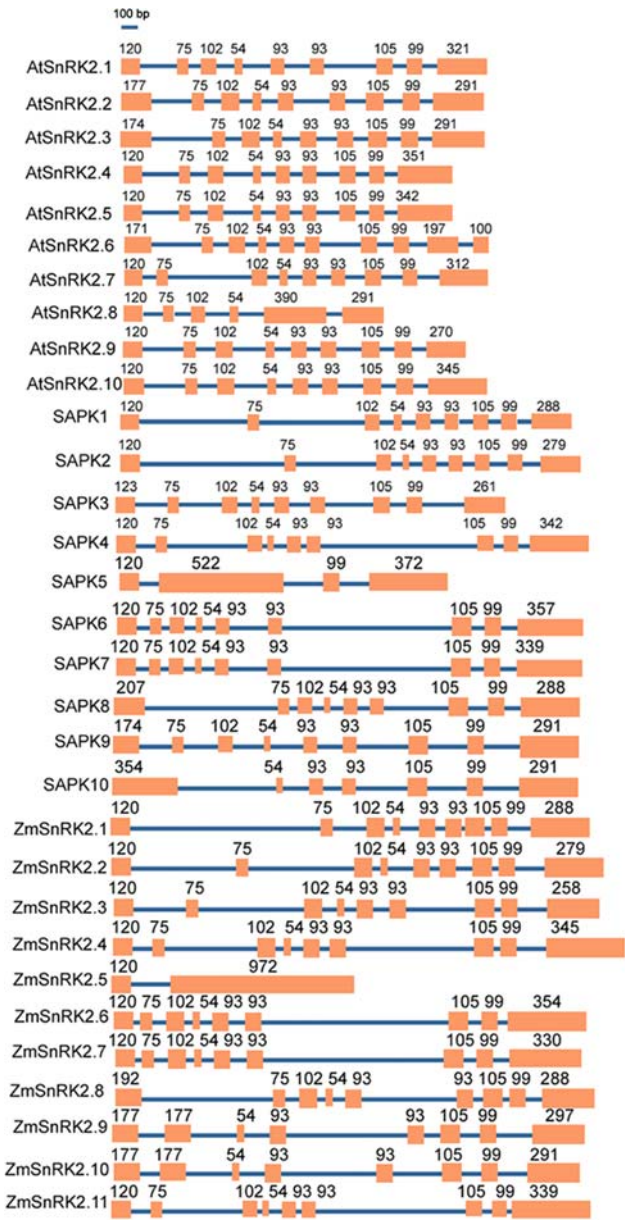


**Fig. 1** Gene structures of SnRK2 genes in *Arabidopsis*, rice and maize. Introns and exons are represented by *black lines* and *boxes* respectively. The *numbers above the exons* indicate the length of the exons

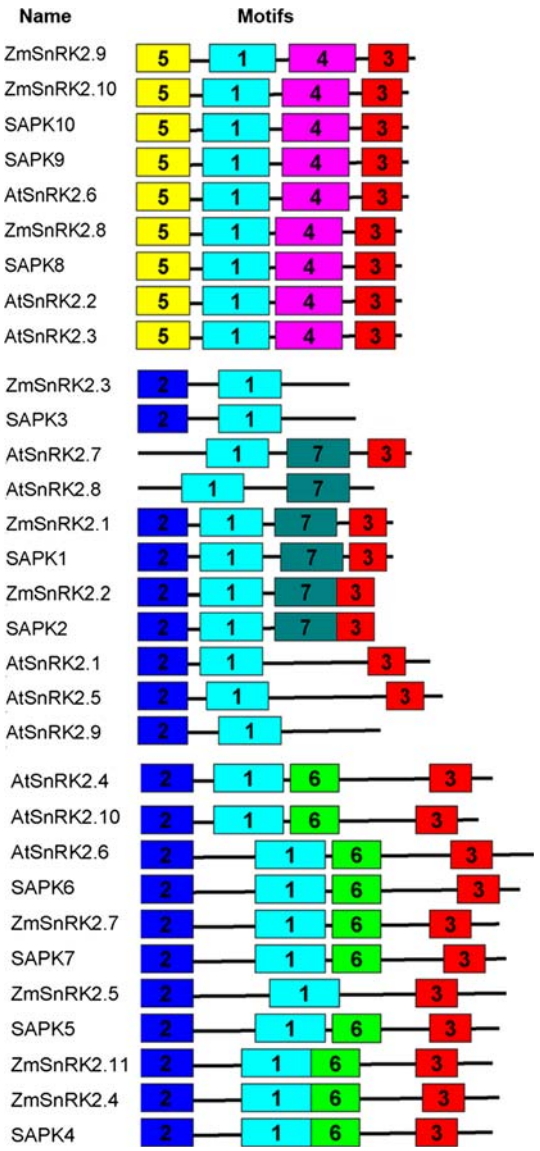


**Fig. 2** Distribution of the conserved motifs in the C-terminus of SnRK2 genes from *Arabidopsis*, rice, and maize identified using MEME tool. Each motif is represented by a number in the *colored box*. The *black lines* represent the non conserved sequences

*ZmSnRK2.5* (two exons), *ZmSnRK2.9* (eight exons), and *ZmSnRK2.10* (eight exons). The genes with nine exons have strictly conserved exon lengths. The lengths of the second through the eighth exons were 75, 102, 54, 93, 93, 105, and 99 (bp), respectively. Although the rest had variable exon lengths, we were able to identify several common features. For example, AtSnRK2.8 has six exons, but the length of the fifth exon (390 bp) was the addition of the fifth to the eighth conserved exons (the sum of 93, 93, 105, and 99) existed in the most of the genes. The most discrepant gene was *ZmSnRK2.5* with only two exons.

We also analyzed the intron phase of SnRK2 genes (Nicole et al. 2006). The introns of the SnRK2 genes all belonged to phase 0 except for the ninth intron of *AtSnRK2.6* (phase 2), which was the result of one additional intron insertion.

## Conserved motifs in the C-terminus

The C-terminal regions of *SnRK2s* were divergent, but we were able to identify several conserved motifs using the MEME motif research tool. A total of seven conserved motifs were found (Fig. 2 and Table S3). Motif 1 was found in all members of the SnRK2 family. Most of the

SnRK2 genes contain motif 3. Motif 2 is unique to twenty of the SnRK2 genes, motifs 4 and 5 are unique to nine, motif 6 is unique to ten, and motif 7 is unique to six of the *SnRK2s*. At the same time, some motifs were common to monocots or eudicots.

## Phylogenetic analysis

Because all of the SnRK2 proteins has a conserved kinase domain and motif 1 identified in the C-terminus, we performed a multiple alignment analysis using the amino acid sequences of these two parts. An unrooted phylogenetic tree was constructed using the neighbor-joining method based on the alignment. As shown in Fig. 3, all of the SnRK2 genes could be divided into three distinct groups. This is consistent with a previous report by Kobayashi et al. in 2004. The group I genes, which include *AtSnRK2.2*, *AtSnRK2.3*, *AtSnRK2.6*, *SAPK8*, *SAPK9,* and *SAPK10*, have been reported to be activated by ABA and to be involved in ABA signal transduction (Fujii et al. 2007; Kobayashi et al. 2005; Kobayashi et al. 2004; Mustilli et al. 2002). *AtSnRK2.8*, which has been reported to improve the drought tolerance of transgenic *Arabidopsis* and to participate in metabolic processes, falls into group II (Shin et al. 2007; Umezawa et al. 2004). *SAPK6* (OSRK2) is induced by dehydration and is included in group III. In the three groups, each of the ZmSnRK2 has a counterpart among the SAPKs, demonstrating the high similarity of this gene family in the two species during evolution. Since genes belonging to the same group may have similar functions, we can speculate regarding the functions of uncharacterized SnRK2 genes in maize according to the phylogenetic tree.

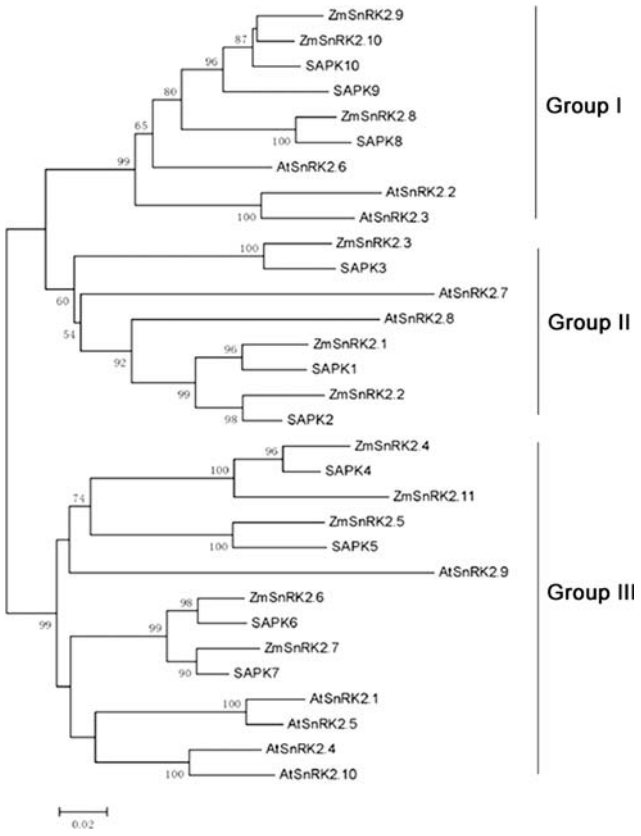


**Fig. 3** Phylogenetic analysis of SnRK2s from *Arabidopsis*, rice and maize using the kinase domain and motif 1 identified in the C-terminus

## Expression analysis of ZmSnRK2 genes

Real-time PCR was used to analyze the expression of ZmSnRK2 genes. Figure 4 shows the relative expression changes for the ZmSnRK2 genes under ABA, salt, cold, and heat stress treatments. The transcripts of *ZmSnRK2.2*, *ZmSnRK2.4*, *ZmSnRK2.5*, *ZmSnRK2.7,* and *ZmSnRK2.10* were induced by ABA treatment. *ZmSnRK2.3* and *ZmSnRK2.6* were strongly induced by NaCl treatment. Other genes (excluding *ZmSnRK2.4*) were slightly induced by NaCl treatment. Under cold treatment, *ZmSnRK2.3* and *ZmSnRK2.7* were induced intensely. Surprisingly, the expression of some genes, such as *ZmSnRK2.5*, *ZmSnRK2.6,* and *ZmSnRK2.9*, was inhibited when exposed to heat stress. The transcripts of the other genes were not upregulated during heat treatment, with the exception of *ZmSnRK2.2* and *ZmSnRK2.4*. The expression profile suggests that the ZmSnRK2 genes might be involved in the stress responses of maize.

We also analyzed the presence of cis elements in the promoter regions of the ZmSnRK2 genes. We took the 2 kb region upstream of the start codon as the putative promoter region and searched for stress responsive elements including ABA responsive element (ABRE), dehydration-responsive element (DRE/CRT), and low temperature responsive

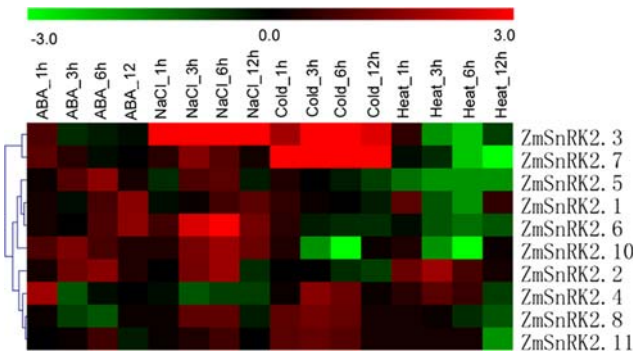


**Fig. 4** Expression analysis of ZmSnRK2 genes responding to abiotic stresses. The data from real-time PCR is represented using the hierarchical cluster display. *Green* color represents low level expression and *red* shows high level expression. The *color bar* demonstrates $\log_2$ expression values

element (LTRE) in the PLACE database (Higo et al. 1998; Jiang et al. 1996; Kizis and Pagès 2002; Nakashima et al. 2006; Ono et al. 1996; Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 1994) (Table 2). We hypothesized that genes induced by a specific stress treatment might have corresponding cis elements in their promoter regions. However, genes without such cis elements might also be induced by such stress. The *ZmSnRK2.2*, *ZmSnRK2.4*, *ZmSnRK2.5*, and *ZmSnRK2.10*, which were induced by ABA treatment, contain several ABREs in their promoter regions. None of the ZmSnRK2 genes contains the heat-shock element (HSE) in their putative promoter region (data not shown) but *ZmSnRK2.2* and *ZmSnRK2.4* could be induced by heat stress (Fig. 4).

## Discussion

### The evolution of the SnRK2

We searched the *Physcomitrella patens* database (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html) and found four SnRK2 genes (supplementary materials). We analyzed their gene structures and found that all four SnRK2s from moss have nine exons. The lengths of the second to the eighth exons were 75, 102, 54, 93, 93 105, and 99 (bp), respectively. This is in agreement with what has been found in most of the SnRK2 s from higher plants, suggesting that the conserved gene structure formed from moss. Later, most of the SnRK2s in higher plants maintained the conserved structure, with only a minority losing several introns (AtSnRK2.6 get one intron). The conservation of this gene structure suggests that it might be common feature of this gene family in plants, and it also supports the idea that this gene family plays important roles in plant growth and development.

During evolution, most genes expand by duplication and differentiation. At present, three classes of duplication events have been reported; namely genomic tandem duplication, segmental duplication, and background duplication (Adams and Wendel 2005; Kim et al. 2006; Moore and Purugganan 2005; Yu et al. 2005). The expansion of the SnRK2s family from rice and *Arabidopsis* might be the result of the polyploidy events. In our study, we identified two segmental duplication pairs from *Arabidopsis* and rice each. We were unable to obtain specific duplication results for maize because the maize genome sequencing is not finished at this time. However, we speculated that the highly conserved structure of the SnRK2 genes in maize might be the result of the same evolutionary process. After expansion, most of the genes maintained their original gene structure found in moss while others lost a few introns.

**Table 2** *Cis* elements existed in the 2 kb upstream region of ZmSnRK2 genes

| Name | ABRE | DRE/CRT | LTRE |
|---|---|---|---|
| ZmSnRK2.1 | 3 | 0 | 0 |
| ZmSnRK2.2 | 3 | 1 | 1 |
| ZmSnRK2.3 | 6 | 8 | 5 |
| ZmSnRK2.4 | 6 | 5 | 4 |
| ZmSnRK2.5 | 10 | 3 | 2 |
| ZmSnRK2.6 | 2 | 1 | 2 |
| ZmSnRK2.7 | 0 | 1 | 0 |
| ZmSnRK2.8 | 2 | 0 | 1 |
| ZmSnRK2.9 | 7 | 5 | 6 |
| ZmSnRK2.10 | 7 | 1 | 1 |
| ZmSnRK2.11 | 2 | 1 | 3 |

The sequence of ABRE elements include ACGTG, MACGYGB, TACGTGTC, YACGTGGC, and CCACGTGG

The sequence of DRE/CRT element include RCCGAC, ACCGAC, ACCGAGA, and GTCGAC

The sequences of LTRE element include CCGAC, CCGAAA, ACCGACA, and CCGAC

### Two novel motifs in the C-terminus might contribute to the ABA response

The SnRK2 genes from *Arabidopsis* have been divided into two groups in previous reports (Boudsocq et al. 2004). Kobayashi et al. (2004) divided the SnRK2 s from *Arabidopsis* and rice into three groups according to the amino acid sequence of the kinase domain. In our study, the SnRK2 genes from *Arabidopsis*, rice, and maize could be divided into three groups based on the amino acid sequences of the kinase domain and motif 1, which is consistent with the grouping of *Arabidopsis* and rice.

In group I, all the members contain motifs 1, 3, 4, and 5 in the C-terminus, with motifs 4 and 5 unique to them. In addition, *AtSnRK2.2*, *AtSnRK2.3*, *AtSnRK2.6*, SAPK8, SAPK9, and *SAPK10* have been confirmed to be activated by ABA and function in ABA signal transduction by phosphorylating ABFs (Boudsocq et al. 2004; Fujii et al. 2007; Kobayashi et al. 2005; Kobayashi et al. 2004; Mustilli et al. 2002; Yoshida et al. 2006). SAPK2 cannot be induced by ABA, but the chimeric kinase SAPK2-8, which contains the SAPK2 kinase domain and the SAPK8 C-terminus could be activated by ABA. Conversely, the chimeric kinase SAPK8-2, which contains the SAPK8 kinase domain and the SAPK2 C-terminus was not activated by ABA. It is likely that the C-terminal region mainly contributes to functional distinction (Kobayashi et al. 2004), but the replacement was carried out using the entire C-terminal region. According to the specific division, we speculate that motifs 4 and 5 might participate in the ABA response. *ZmSnRK2.8*, *ZmSnRK2.9*, and *ZmSnRK2.10*, which contain the same C-terminal motifs as

other members in group I, may have similar functions. Other SnRK2s, such as *AtSnRK2.8* containing motif 7 and SAPK6 containing motif 6, have different functions that may arise from differences in the C-terminus (Meissner and Michael 1997; Shin et al. 2007; Umezawa et al. 2004).

### The expression profiles of the SnRK2s from maize, *Arabidopsis* and rice

We also analyzed gene expression patterns for *Arabidopsis* and rice. The AtSnRK2 and SAPK gene expression data were obtained from the BAR database (http://bar.utoronto.ca/) and GEO (http://www.ncbi.nlm.nih.gov/geo/). As shown in Fig. S1 and Table S5, the expression of *AtSnRK2* and SAPK genes was induced by various stress conditions. For instance, the expression of *AtSnRK2.6* was strongly induced by ABA based on microarray data, and plays an important role in ABA signal transduction in *Arabidopsis* (Belin et al. 2006; Mustilli et al. 2002; Yoshida et al. 2006). The expression of ZmSnRK2 genes is induced by various stress treatments, which may suggest their potential roles in stress responses.

Genes that have a particular stress responsive element might not be induced by such stress, while the absence of a specific element may not mean it was "un-induced". For example, there is no LTRE in the promoter region of *ZmSnRK2.7*, although it was strongly induced by cold stress. There are several LTREs in most of the SAPKs, but the SAPK genes are not induced by low temperature. These observations suggest that there are other unrecognized mechanisms involved in the regulation of these genes by temperature.

## Conclusion

Although the functions of some SnRK2s from *Arabidopsis* and rice have been clearly demonstrated, the functions of many others remain unclear. Currently, there are no reports concerning SnRK2s in maize. In our research, we found 11 SnRK2s in the maize genome and identified ten of them by RT-PCR. We performed a genome-wide analysis of the SnRK2 gene family in maize, *Arabidopsis,* and rice, including gene structure, evolution, and expression. We also found two new motifs in the C-terminus, which may contribute to the ABA response. According to the phylogenetic grouping, we can speculate the possible functions of the unknown SnRK2s specifically in maize.

**Acknowledgments** This work was supported by the National High-tech Program of China (2006AA10Z103) and the Natural Science Foundation of China (30730063).

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