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REVIEW

The SnRK2 family in pepper (*Capsicum annuum* L.): genome-wide identification and expression analyses during fruit development and under abiotic stress

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Abstract

Plant-specific *SnRK2* (sucrose nonfermenting-1-related protein kinase 2) genes play crucial roles in the coordination of plant growth and development and responses to stress. However, comprehensive studies have not been performed for this gene family in pepper (*Capsicum annuum*), a very important *Solanaceous* vegetable worldwide. To fully understand the status of SnRK2s in chili pepper, a total of 9 putative *SnRK2* genes (named *CaSnRK2.1-2.9*) were identified in pepper in the present study. These genes were located on 7 different chromosomes and classified into three subfamilies based on the phylogenetic tree. Their conserved motif compositions and exon-intron structures were systematically analyzed, and the results strongly supported the classification. Furthermore, a total of 81 putative *cis*-elements were found in the promoter regions, and the *cis*-elements related to hormone and stress signaling were abundant. Finally, the *CaSnRK2* gene expression profiles among different tissues, especially developing fruit tissue, and under various abiotic stresses were investigated to identify tissue-specific or stress-responsive candidates. This study was the first to comprehensively investigate the *SnRK2* family in pepper, and the results provide important clues for further functional analyses of fruit development and abiotic stress responses.

Keywords Pepper (*Capsicum annuum* L.) · SnRK2 family · Fruit development · Abiotic stress · Gene expression

Introduction

Harsh environments greatly limit plant growth and crop yields. To adapt to changing environments, plants have formed a series of different defense-related metabolic mechanisms. Protein kinases and protein phosphorylation/dephosphorylation play important roles in signal transduction pathways with respect to the recognition and transmission of stress signals to different parts of cells. Members of the SnRK (or SNF1-related protein kinase) family are specific types of serine/threonine protein kinases that exist widely in plants and function significantly in a host of processes, including growth and development, defense against various stresses, and hormone-mediated signaling (Wang et al. 2018; Zhao et al. 2018; Lin et al. 2015; Zhang et al. 2016; Yang et al. 2012; Coello et al. 2012).

In higher plants, the SnRK (sucrose nonfermenting-1-related protein kinase) family consists of three subfamilies (denoted SnRKl, SnRK2 and SnRK3) based on their sequence similarity and C-terminal domain structure characteristics (Hrabak et al. 2003; Kobayashi et al. 2004). Members of the SnRK1 subfamily are most closely related to *SNF1* from yeast and AMP-activated protein kinase (AMPK) from mammals (Hardy et al. 1994; Hardie 2007). The SnRK1 subfamily typically consists of relatively few members (only 3 members in *Arabidopsis*) that both encode relatively large proteins (55–60 kDa) and play a major role in global regulation of carbon metabolism and energy status (Hrabak et al. 2003;

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Coello et al. 2012). The SnRK2 and SnRK3 subgroups appear to be unique to plants and are more diverse (10 *SnRK2* genes and 25 *SnRK3* genes have been described in *Arabidopsis*), and members of these subfamilies are implicated in the response to various abiotic stresses, including heat, cold, drought, salinity, and osmotic stresses (Coello et al. 2012; McLoughlin et al. 2012; Yang et al. 2012; Fujita et al. 2013; Liang et al. 2015; Song et al. 2016; Zhang et al. 2016; Bai et al. 2017; Soma et al. 2017; Tan et al. 2018; Wang et al. 2018).

The first *SnRK2* gene, namely, *PKABA1*, was cloned from wheat and could be induced by dehydration, cold temperature and osmotic stress (Holappa and Walker-Simmons 1995; Gomez-Cadenas et al. 2001). To date, members of the SnRK2 family have been identified and comprehensively analyzed in many different plant species, including the dicotyledons *Arabidopsis* (Hrabak et al. 2003), apple (Shao et al. 2014), strawberry (Han et al. 2015), pak-choi (Huang et al. 2015), cotton (Liu et al. 2017b), and potato (Bai et al. 2017), and the monocotyledons rice (Kobayashi et al. 2004), maize (Huai et al. 2008), wheat (Zhang et al. 2016) and sugarcane (Li et al. 2017). SnRK2 N-termini contain conserved kinase domains, such as an ATP-binding domain (GXGXXGX) and a serine/threonine activation loop, while the C-terminus contains regulatory domains rich in acidic amino acids (AAs), such as aspartic acid (D) and glutamic acid (E). A phylogenetic analysis revealed that the SnRK2 family could be divided into three subgroups. Members of subgroups I and II are weak or unresponsive to abscisic acid (ABA) (Soma et al. 2017), while those of subgroup III are strongly induced by ABA (Wang et al. 2013).

Cumulative evidence has shown that SnRK2s are core components of the ABA-mediated signaling transduction network involved in the SnRK2-PP2C (a type 2C protein phosphatase) complex structure (Soon et al. 2012; Shen et al. 2017; Zhao et al. 2018). Among the ten *AtSnRK2*s (named *AtSnRK2.1*-*AtSnRK2.10*) identified in the model plant *Arabidopsis thaliana*, subgroup I members (including *AtSnRK2.1*, *AtSnRK2.4*, *AtSnRK2.5*, *AtSnRK2.9*, and *AtSnRK2.10*) are involved in the response to osmotic or salt stress and improve plant resistance to drought (Szymanska et al. 2019; Julkowska et al. 2015; McLoughlin et al. 2012; Kulik et al. 2012). Two members in subgroup II (*AtSnRK2.7* and *AtSnRK2.8*) are responsive to salt stress and could also strengthen plant tolerance to drought (Lee et al. 2015). Subgroup III contains three members (*AtSnRK2.2*, *AtSnRK2.3*, and *AtSnRK2.6*) that are involved in ABA signaling during seed germination/dormancy, root growth (Fujita et al. 2009; Nakashima et al. 2009; Fujii et al. 2011; Zheng et al. 2010), the development and ripening of some nonclimacteric fruit (Han et al. 2015) and the response to drought and chilling stress (Wang et al. 2018; Tan et al. 2018). In rice, all 10 *SAPK1* (stress/ABA-activated protein kinase) to *SAPK10* members are activated by hyperosmotic stress, and three of them (*SAPK8*, *SAPK9*, and *SAPK10*) are also activated by ABA (Kobayashi et al. 2004; Yu et al. 2012; Lou et al. 2018). The sensitivity of these SnRK2 genes to ABA and various stresses differ between *Arabidopsis* and rice, indicating that the functions of these core ABA signaling components are diverse. However, research on *SnRK2* genes in pepper is very limited.

Pepper (*Capsicum* spp.) is an important vegetable and spice plant. The *Capsicum* genus consists of more than 30 species, five of which are domesticated (*C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*) (Carrizo Garcia et al. 2016). Given the importance of the SnRK2 gene family in the stress resistance of plants and completion of whole-genome sequencing of the pepper (Kim et al. 2014; Qin et al. 2014), all *CaSnRK2*s predicted to belong to the SnRK2 family were identified and a comprehensive bioinformatics analysis was carried out in this study. The expression patterns of all *CaSnRK2* genes in different tissues, especially the fruit, and under different abiotic stresses were analyzed via transcriptome data and fluorescence quantitative PCR (qRT-PCR). The goal of this study is to fully understand the status of SnRK2s related to growth and abiotic stress in chili pepper (*Capsicum annuum* L.). This study provides a basis for further characterization of the physiological and biochemical functions of the *CaSnRK2*s in peppers.

Materials and methods

CaSnRK2 identification and sequence analysis

To identify *SnRK2* genes in the pepper genome, a BlastP search was performed against the Sol Genomics Network (https://solgenomics.net/) (Mueller et al. 2005) based on the *Capsicum annuum* cv. Zunla (v2.0), cv. CM334 (v1.55) and var. *glabriusculum* (v2.0) genomes using SnRK2 protein sequences from *Arabidopsis* as queries. The default parameters used a cut-off value of 1e–10. Additionally, the candidate sequences were analyzed via PROSITE (http://prosite.expasy.org/scanprosite) and the SMART program (http://smart.embl-heidelberg.de) to confirm the presence of related domains. The molecular weight (MW) and theoretical isoelectric point (pI)of the Ca*SnRK2* proteins were computed by ProtParam (https://web.expasy.org/protparam/).

Chromosomal location, gene structure analysis and conserved motif prediction

The chromosomal location information for *CaSnRK2*s was obtained by a BlastP query against the Pepper Genome Database (PGD, http://peppersequence.genomics.cn/page/species/index.jsp) (cultivar Zunla-1), which was constructed by our group and visualized using MapChart 2.2. The gene structures of all *CaSnRK2* genes were drawn by the online software GSDS (http://gsds.cbi.pku.edu.cn/index.php) (Hu

et al. 2015). The conserved motifs in the *CaSnRK2*s were analyzed using the online motif alignment and search tool MEME Suite 5.0.3 (http://meme-suite.org/tools/meme) (Bailey et al. 2015).

Multiple sequence alignment and phylogenetic analysis

The full-length SnRK2 AA sequences of *Arabidopsis* (*Arabidopsis thaliana*) (Hrabak et al. 2003), potato (*Solanum tuberosum*) (Bai et al. 2017), tomato (*Solanum lycopersicum*), apple (*Malus domestica*), corn (*Zea mays*) (Huai et al. 2008), rice (*Oryza sativa* Japonica) (Kobayashi et al. 2004) and pepper were aligned using ClustalW software. A phylogenetic tree based on the alignment was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software (Tamura et al. 2013) with the NJ method and 1000 bootstrap replicates.

Analysis of *cis*-elements within the promoters

To analyze the *cis*-elements within the *CaSnRK2* promoters, the 1500 bp (bp) sequence upstream of the initiation codons (ATG) of each *CaSnRK2* gene was retrieved from the PGD. The *cis*-elements were predicted by the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002), including those for phytohormones and the stress responses. The data were processed using the Microsoft Office Excel software, and then the tables and figures were constructed.

Expression pattern analysis based on transcriptome data

To evaluate the expression patterns of the *CaSnRK2* genes, previously generated Illumina transcriptome sequencing data were utilized (Liu et al. 2017a; Qin et al. 2014). The transcriptome data included different tissues, such as root, stem, leaf, floral bud and fully blossomed flower tissues as well as tissues from seeds and fruits at different developmental stages. For stress treatments, the 40-day old seedlings were subjected to 200 mM NaCl (salt stress), 400 mM D-mannitol (osmotic stress), 30 mM H_2O_2 (oxidative stress), 42 ℃ or 10 ℃ (heat or cold stress). Leaf and root tissues from five plants were collected from treated and control plants at 0, 1.5, 3, 6, 12 and 24 h post treatment and mixed as one biological replicate. Only genes with fragments per kilobase million (FPKM) > 1 were collected for further analysis. The expression levels of the *CaSnRK2* genes were calculated using the $\log_2$ (FPKM). A heatmap was subsequently created using MeV 4.9 (Saeed et al. 2003).

Plant materials, total RNA extraction and qRT-PCR analysis

To validate the transcriptome data downloaded from public databases, we designed qRT-PCR primers using Zunla-1 (*Capsicum annuum* L.) seedlings. The seedlings at the six-leaf stage were subjected to heat (38 ℃) and NaCl (300 mM) treatments for 3, 6, and 12 h. Leaves harvested at 0 h were regarded as the controls (Wu et al. 2016). Eight plants were included in each group, and their leaves were collected from the same position on the seedlings with three biological replicates.

Total RNA was extracted from developing fruits using the EZNA™ Total RNA Kit I (Omega Biotek, Norcross, GA, USA) and subsequently treated with RNase-free DNase I (Takara, Dalian, China) to remove any genomic DNA contamination. First-strand cDNA was synthesized from the total RNA with the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Dalian, China) using random primers in accordance with the product manual. The TB Green™ *Premix Ex Taq*™ II kit (Takara Bio Inc., Dalian, China) was used for qRT-PCR analysis on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) following the manufacturers' instructions. The PCR program was initiated for 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 55 °C for 30 s, and the program was completed with a melting curve analysis. *CaActin* (AY572427 in GenBank) was used as an internal control. The transcription level of each *CaSnRK2* gene was calculated using the $2^{-\Delta\Delta Cq}$ method as previously described (Livak and Schmittgen 2001). qRT-PCR was conducted for three technical replicates, and SPSS 18.0 (SPSS Inc., USA) was used for the statistical analysis. The primers used for the qRT-PCR analysis of the *CaSnRK2* genes were designed using the Primer Premier 5.0 software.

Results

Pepper genome contains nine SnRK2 genes

The first Blast search identified 22, 21 and 21 *SnRK2* candidates in the Zunla-1, CM334 and chiltepin genomes, respectively. After genes with a percent identity < 50.00 were removed, 9, 8 and 9 suspected *SnRK2* genes remained in the Zunla-1, CM334 and chiltepin genomes, respectively. All these protein sequences were downloaded for further study. A total of 9 full-length *CaSnRK2* genes were ultimately confirmed in the cultivated pepper Zunla-1 (*Capsicum annuum* L.) and its wild progenitor chiltepin (*Capsicum annuum* var. *glabriusculum*). These genes were named *CaSnRK2.1-CaSnRK2.9* according to their genomic positions. The lengths of the CaSnRK2 proteins ranged from 236 AA (CaSnRK2.4)

to 331 AA (CaSnRK2.6), and the corresponding open reading frames (ORFs) ranged from 711 to 996 bp (sequences supplied in Supplemental File S1). The MWs ranged from 26.67 to 38.15 kDa, and the theoretical isoelectric point (pI) ranged from 4.46 (CaSnRK2.2) to 7.63 (CaSnRK2.5) (Table 1). All CaSnRK2s have a highly conserved N-terminal catalytic domain that contains an ATP-binding site and a protein kinase activation signature. The C-terminal regions of the CaSnRK2s are quite divergent and contain regions rich in the D/E AA (Fig. 1).

CaSnRK2 positions and gene and protein structures

Based on the pepper genome sequence, nine *CaSnRK2* genes were mapped onto different chromosomes (Supplemental Fig. 1). *CaSnRK2.6* and *CaSnRK2.7* were mapped onto chromosome 8, and *CaSnRK2.1*, *CaSnRK2.2*, *CaSnRK2.3*, *CaSnRK2.4*, *CaSnRK2.5* and *CaSnRK2.8* were mapped onto chromosomes 1, 2, 4, 5, 6 and 12, respectively. *CaSnRK2.9* could not be mapped on any chromosome but on a pseudochromosome designated as Chr00.

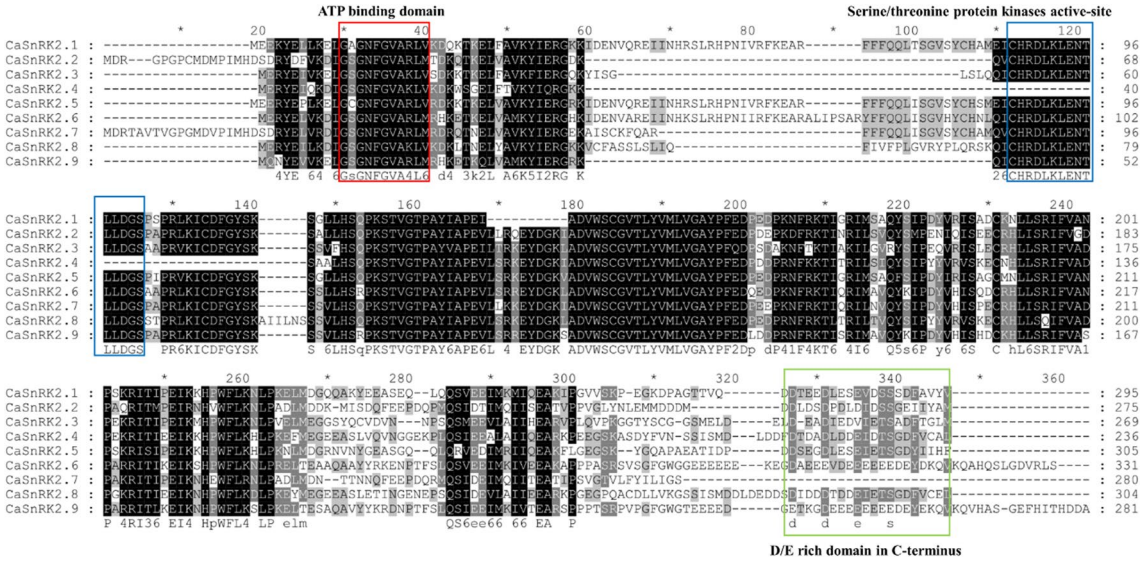

Fig. 1 Multiple sequence alignment of the CaSnRK2 amino acid sequences. The CaSnRK2 proteins were aligned using the ClustalX2 program with the default settings. Identical amino acid residues are covered in black, similar residues are indicated in gray, and gaps in the sequences are indicated by dashes. The predicted functional domains are boxed. The ATP-binding domain, serine/threonine protein kinases active-site and the D/E rich domain in C-terminus are marked by red, blue and green box, respectively

Table 1 *SnRK2* genes identified in pepper (*Capsicum annuum*) genome

Gene name	Locus ID	Chromosomal location	CDS (bp)	Putative Proteins			Corresponding Locus ID	
				Length (AA)	MW (KDa)	pI	In CM334	In Chiltepin
CaSnRK2.1	*Capana01g000732*	Chr01:14,773,000..14,777,100 (– strand)	888	295	33.51	5.96	*CA01g04580*	*Capang01g000665*
CaSnRK2.2	*Capana02g003135*	Chr02:156,268,373..156,273,353 (+ strand)	828	275	31.19	4.46	*CA02g23140*	*Capang02g002844*
CaSnRK2.3	*Capana04g000996*	Chr04:24,411,026..24,414,600 (+ strand)	810	269	30.00	5.51	*CA00g36560*	*Capang04g000948*
CaSnRK2.4	*Capana05g000287*	Chr05:5,497,009..5,500,969 (– strand)	711	236	26.67	5.01	*CA05g02310*	*Capang05g000242*
CaSnRK2.5	*Capana06g001594*	Chr06:40,308,643..40,318,991 (+ strand)	918	305	34.77	7.63	–	*Capang06g001507*
CaSnRK2.6	*Capana08g001898*	Chr08:138,059,533..138,062,849 (+ strand)	996	331	38.15	6.10	*CA08g11790*	*Capang01g004459*
CaSnRK2.7	*Capana08g002441*	Chr08:146,898,342..146,901,416 (+ strand)	843	280	31.84	6.00	*CA08g12560*	*Capang01g004977*
CaSnRK2.8	*Capana12g002655*	Chr12:224,954,961..224,959,202 (– strand)	915	304	34.23	4.80	*CA12g03040*	*Capang12g002297*
CaSnRK2.9	*Capana00g002391*	Chr00:473,248,789..473,250,826 (+ strand)	846	281	32.20	5.97	*CA00g67200*	*Capang07g000015*

To understand the structure of the *CaSnRK2* genes, we compared the genomic DNA sequences to analyze the exon/intron organization using the online tool Gene Structure Display Server (GSDS) 2.0. The results revealed that pepper *SnRK2* genes contained 4 to 8 introns, with four out of nine *CaSnRK2* genes containing five introns. The detailed gene structure is presented in Fig. 2a.

To further interpret the structural diversity of the SnRK2 proteins, 10 conserved motifs were predicted using MEME (Fig. 2b). The length of these motifs varied from 7 to 41 AAs; the specific AA sequences of each motif are also provided (Supplementary Table 1). Motifs 1, 2, 4, 6 and 10 were detected in all SnRK2 members. Furthermore, some conserved motifs in specific proteins were identified; for instance, motif 8 was detected only in *CaSnRK2.1*, *CaSnRK2.5* and *CaSnRK2.6*.

Phylogenetic analysis

To further investigate the evolutionary relationships among the CaSnRK2 proteins and proteins in other plant species, a phylogenetic tree was constructed based on a multiple alignment of 64 selected full-length SnRK2 protein sequences (Supplemental File S2). These sequences included 10 AtSnRK2s from *Arabidopsis* (*Arabidopsis thaliana*), 10 OsSAPKs from rice (*Oryza sativa* Japonica), 10 ZmSnRK2s from corn (*Zea mays*), 9 MdSnRK2s from apple (*Malus domestica*), 8 StSnRK2s from potato (*Solanum tuberosum*), 8 SlSnRK2s from tomato (*Solanum lycopersicum*) and 9 CaSnRK2s from pepper (*Capsicum annuum*) (Supplementary Table 2). The phylogenetic tree showed that all SnRK2 proteins could be clustered into three subgroups (I, II and III) corresponding to the *Arabidopsis SnRK2* gene classification (Hrabak et al. 2003). Subgroup I consisted of 4 members (*CaSnRK2.1*, *CaSnRK2.5*, *CaSnRK2.6* and *CaSnRK2.9*). *CaSnRK2.3*, *CaSnRK2.4* and *CaSnRK2.8* belonged to subgroup II, and *CaSnRK2.2* and *CaSnRK2.7* were included in subgroup III (Fig. 3).

Analysis of *cis*-elements in the promoter region of all CaSnRK2s

To better understand the possible functions of the *CaSnRK2* genes, the likely *cis*-elements within the promoter region were identified using PlantCARE online tools (Supplementary Table 3). The *cis*-elements that responsive to the plant hormones and stresses were focused in this study. In total, 81 *cis*-acting elements were detected in the promoters of the *CaSnRK2* genes, including 9 types of hormone-related elements and 7 stress-related elements (Fig. 4). With respect to the hormone responsive *cis*-elements, the abscisic acid-responsive elements (ABREs) were found to

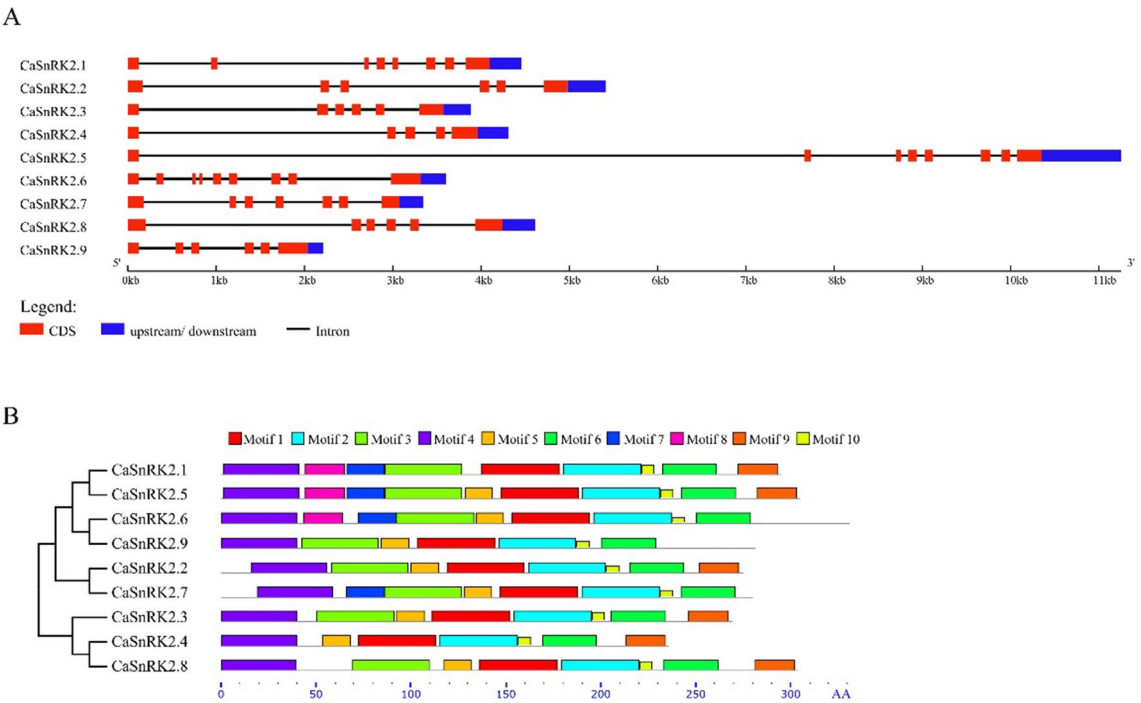

Fig. 2 The *CaSnRK2* gene structures. **a** The intron/exon distribution in all *CaSnRK2* genes. The black lines and red boxes indicate introns and exons, respectively. The blue boxes indicate upstream and downstream. **b** MEME-based analysis to investigate 10 conserved motifs in the CaSnRK2 proteins. Each colored box represents a conserved region. Details are provided in Supplementary Table 1

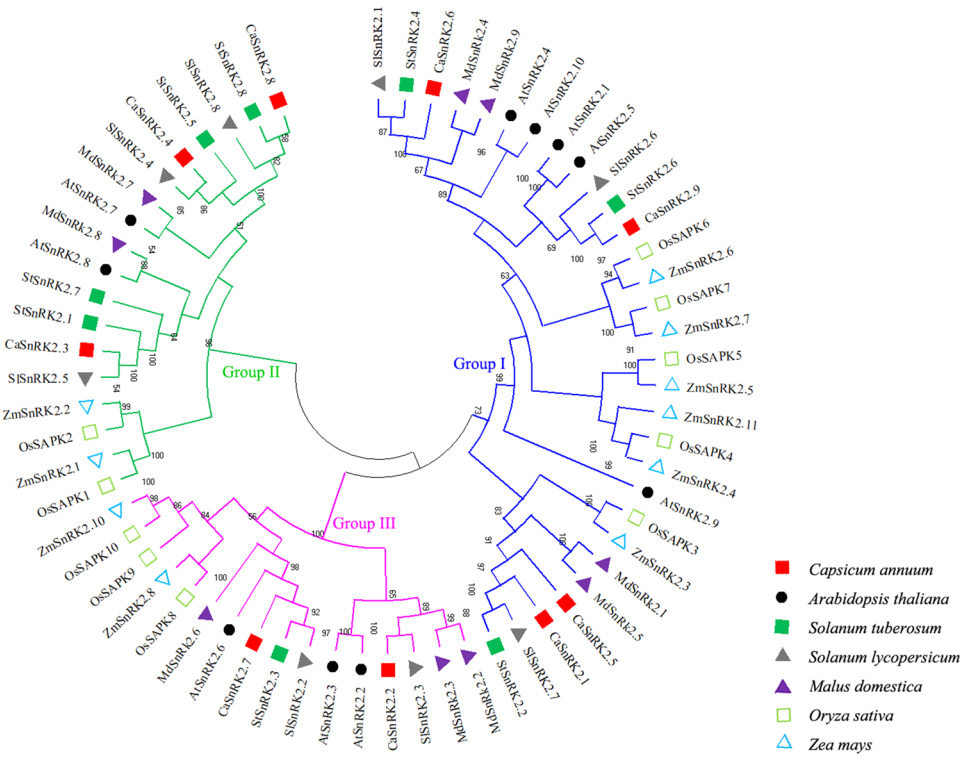

Fig. 3 Phylogenetic analysis of SnRK2 members from *Arabidopsis* (*Arabidopsis thaliana*), potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), apple (*Malus domestica*), corn (*Zea mays*), rice (*Oryza sativa* Japonica) and pepper (*Capsicum annuum*). The phylogenetic tree was constructed by MEGA 6.0 with the neighbor-joining (NJ) method and 1000 bootstrap replications. Values less than 50 were removed. Members in the same clade with a unique color belong to the same subgroup

Genes	Plant hormones responsive elements									Stress responsive elements							Total
	ABA	*Auxin*	*GA*			*Eth*	*SA*	*MeJA*		*Heat*	*Drought*	*Wound*	*Anaerobic*	*Anoxic*	*Fungal elicitor*	*Defense and stress*	
	ABRE	**TGA-element**	**GARE-motif**	**P-box**	**TATC-box**	**ERE**	**TCA-element**	**CGTCA-motif**	**TGACG-motif**	**HSE**	**MBS**	**WUN-motif**	**ARE**	**GC-motif**	**W-Box**	**TC-rich repeats**	
CaSnRK2.1	2	0	0	0	0	0	0	1	1	0	0	1	1	0	2	1	9
CaSnRK2.2	2	2	1	1	0	2	1	2	2	1	2	1	1	1	2	0	21
CaSnRK2.3	1	0	0	0	0	1	0	1	1	0	1	1	0	0	0	2	8
CaSnRK2.4	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	3
CaSnRK2.5	3	0	1	0	0	1	1	0	0	0	1	1	2	0	0	1	11
CaSnRK2.6	1	0	0	0	0	0	0	1	1	0	0	1	2	0	0	0	6
CaSnRK2.7	2	1	0	0	0	0	0	2	2	0	0	1	0	0	1	0	9
CaSnRK2.8	0	1	1	0	0	3	1	1	1	0	0	0	0	0	0	0	8
CaSnRK2.9	2	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0	6
	13	4	3	3	2	9	3	8	8	1	5	6	6	1	5	4	**81**

Fig. 4 The cis elements that respond to plant hormones and stress signals within the *CaSnRK2* gene promoters

be the most abundant elements in 7 of the nine *CaSnRK2*s. Subgroup I *CaSnRK2*s (including *CaSnRK2.1*, *CaSnRK2.5*, *CaSnRK2.6* and *CaSnRK2.9*), which were not induced by ABA, contained several ABREs in their promoter regions. Ethylene-responsive elements (EREs) and methyl jasmonate (MeJA)-responsive elements (the CGTCA and TGACG motifs, respectively) were also found in 5 and 6 *CaSnRK2* genes, respectively. Other hormone-related *cis*-elements, such as salicylic acid-responsive elements (TCA elements), gibberellin-responsive elements (GARE motifs, P-boxes and the TATC-box), and auxin-responsive elements (the TGA-box and TGA elements), were also found in some *CaSnRK2* genes.

With respect to stress-responsive *cis*-elements, heat stress elements (HSEs) and anoxic stress (GC motif)-related elements were detected only in *CaSnRK2.2*. The wound stress (WUN motif)-responsive element was the most frequent element within the 6 *CaSnRK2* promoters. Moreover, one WUN motif was detected in *CaSnRK2.1*, *CaSnRK2.2 CaSnRK2.3*, *CaSnRK2.5*, *CaSnRK2.6* and *CaSnRK2.7*. The *CaSnRK2* promoter also contained other stress-responsive elements, such as an MBS (a MYB binding site involved in drought inducibility), ARE (a *cis*-acting regulatory element essential for anaerobic induction), W-box (a fungal elicitor-responsive element) and TC-rich repeat region (a defense-related and stress-responsive element). These results indicated that *CaSnRK2* genes might be involved in transcriptional control of both the hormone and stress responses.

CaSnRK2 expression profiles in different tissues/organs and developing fruits

To understand the specific expression of *CaSnRK2* genes in pepper, we analyzed the expression profiles of these genes in the roots, stems, leaves, buds, and flowers (including whole flowers, petals, ovaries and anthers) and developing fruits (which also included the pericarp, placenta and seeds) based on published transcriptome data (Fig. 5 and Supplemental Fig. 2), and their FPKM values are shown in Supplementary Table 4. Some genes, including *CaSnRK2.2*, *CaSnRK2.3*, *CaSnRK2.4* and *CaSnRK2.6*, presented high transcript accumulation in all tissues. *CaSnRK2.5* and *CaSnRK2.8* were expressed at low levels in nearly all tissues, especially in the roots, stems and leaves. Some genes were expressed at relatively low levels in most tissues but at relatively high levels in specific tissues (e.g., *CaSnRK2.1* in the roots and *CaSnRK2.9* in the roots and buds). The expression patterns of the *CaSnRK2* genes varied in the pepper tissues, implying multiple roles in pepper tissue development.

To further explore the functions of the *CaSnRK2*s during fruit development and ripening, we investigated their expression characteristics. *CaSnRK2.2* and *CaSnRK2.6* were both

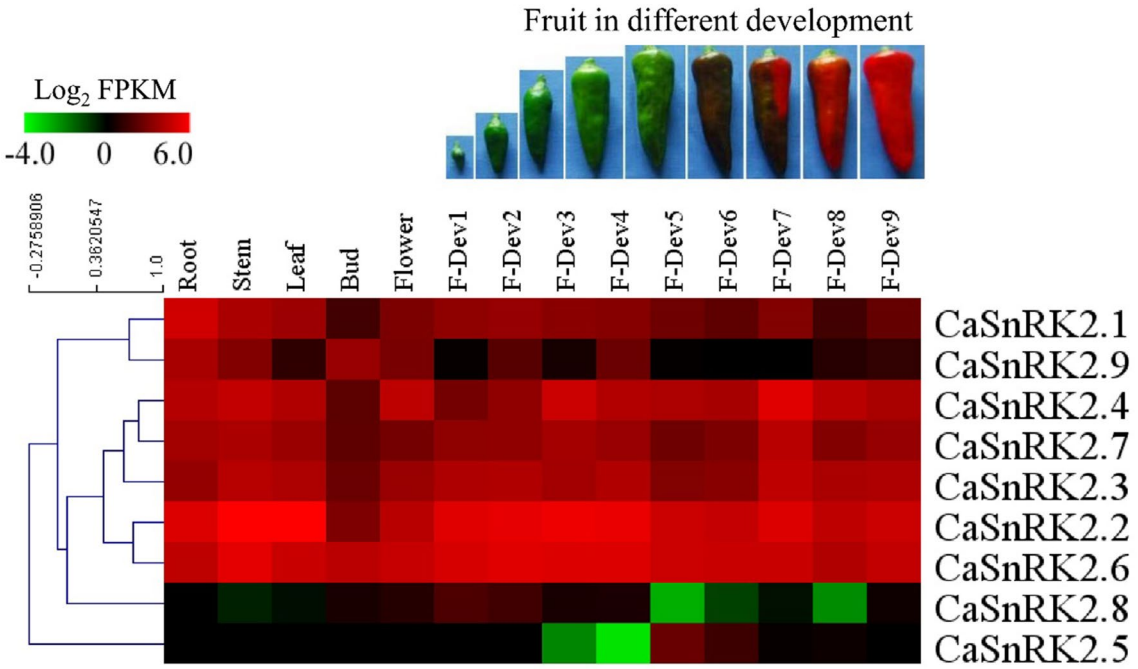

Fig. 5 Expression abundance of *CaSnRK2*sin different tissues and at different developmental stages. The tissues include roots, stems, leaves, buds and flowers as well as whole fruits at different developmental stages. Dev1–5 indicate 0–1 cm, 1–3 cm, 3–4 cm, 4–5 cm, and mature green fruits, respectively. Dev6 indicates the breaker stage fruit. Dev7–9 indicate 3, 5, and 7 days after breaking. The bar in the upper left corner indicates the $\log_2$-corrected FPKM values, and the different colors indicate different expression levels. Red indicates relatively high expression, and green indicates relatively low expression. ZL1 indicates pepper (*Capsicum annuum*) cultivar Zunla-1

strongly expressed throughout the fruit development and ripening process. *CaSnRK*2.1, *CaSnRK*2.3 and *CaSnRK*2.7 were expressed at moderate levels, while *CaSnRK*2.5, *CaSnRK*2.8 and *CaSnRK*2.9 were expressed at low levels at all stages. During the fruit development and expansion stage (from Dev1 to Dev5), *CaSnRK*2.2, *CaSnRK*2.4, *CaSnRK*2.6 and *CaSnRK*2.7 expression continued to increase and peaked at the Dev3 stage, followed by a decreasing expression pattern. During the fruit color change process until ripening (from Dev5 to Dev9, the fruit size remained unchanged), the expression of most of the *CaSnRK2*s except *CaSnRK*2.5, *CaSnRK*2.8 and *CaSnRK*2.9 was strongly induced and peaked at the Dev7 stage, followed by a sharp decrease.

Expression patterns of CaSnRK2s in response to abiotic stress

Research has increasingly shown that *SnRK2* genes are highly important for tolerance to various abiotic stresses. To further explore the biological functions of the *CaSnRK2*s under abiotic stresses (cold, oxidative, heat, drought, and salinity), available transcriptome data (Liu et al. 2017a) were gathered to construct heatmaps (Fig. 6), and the FPKM values of these genes are shown in Supplementary Table 5.

In the leaf tissue, two genes (*CaSnRK2.5* and *CaSnRK2.9*) with nonexistent or extremely low expression (FPKM < 1) were removed from the subsequent analysis. Overall, the other seven members in the leaf tissue responded to at least one treatment (Fig. 6a). Among these members, *CaSnRK2.1*, *CaSnRK2.3* and *CaSnRK2.8* were significantly induced by four treatments. *CaSnRK2.8* showed immediate transcript accumulation that peaked at 3 h in response to those treatments. *CaSnRK2.1* responded after 6 or 12 h. *CaSnRK2.7* appeared to be upregulated by H_2O_2, heat and mannitol. *CaSnRK2.4* and *CaSnRK2.6* expression was neither upregulated nor downregulated significantly under any of these stresses despite stress exposure varying from 0 to 24 h. This finding implied that these genes might not have any function in leaf tissue. Moreover, *CaSnRK2.2* expression was downregulated at the early stage (from 0 to 3 h) in response to these stresses but not to a very high degree (fold change (FC), treatment FPKM/control FPKM < 2).

In the root tissue (Fig. 6b), the expression of *CaSnRK*s was time-dependent under different stresses, demonstrating that the responses of the plants to stress involved a complex process. Most *CaSnRK*s, including *CaSnRK2.1*, *CaSnRK2.2*, *CaSnRK2.3*, *CaSnRK2.4*, *CaSnRK2.5* and *CaSnRK2.7*, were inducible at 1.5 h in the early stage, while

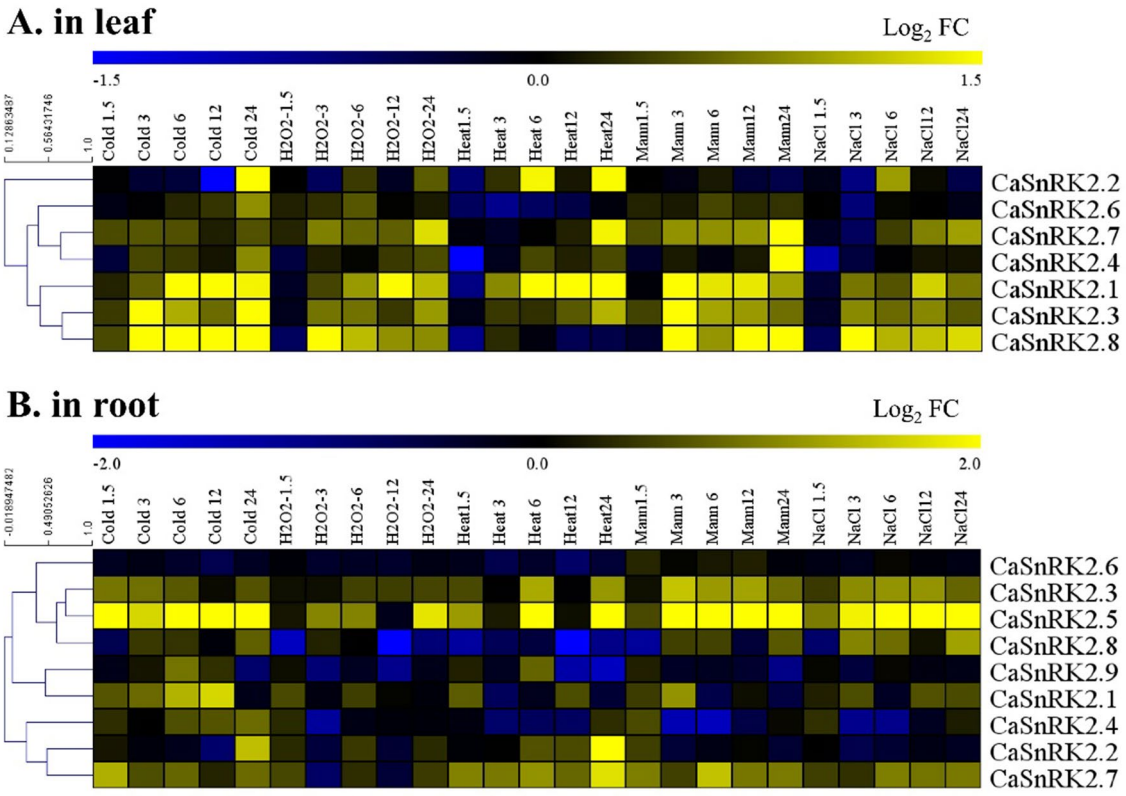

Fig. 6 Expression abundance of *CaSnRK2*s under cold, H_2O_2 (oxidative), heat, drought (osmotic), and salinity stresses in the pepper. **a** leaf tissue. **b** Root tissue. Genes with similar profiles across arrays are grouped on the left by the hierarchical clustering method

CaSnRK2.8 expression was downregulated. *CaSnRK2.5* exhibited a significantly high FC (> 10-fold) of upregulated expression under cold, mannitol and NaCl stresses, especially during the 6–24 h time period. With respect to different stresses, *CaSnRK2.1* was significantly induced under cold stress, *CaSnRK2.7* was induced under heat stress, and *CaSnRK2.3* was induced under mannitol and NaCl stress. Some genes demonstrated interesting and unique expression profiles. For example, *CaSnRK2.2* was induced only at 24 h under cold/heat stress ($\log_2$ FC > 1), while *CaSnRK2.4* was repressed only at 3 or 6 h under mannitol/NaCl stress ($\log_2$ FC < -1), suggesting their potential functions in response to these stresses. In addition, the expression of one gene, *CaSnRK2.6*, did not vary in response to any of the tested stresses (|$\log_2$ FC| < 1).

To further validate the transcriptome data, qRT-PCR was employed to assess the expression patterns of these genes under heat and salt stress (Fig. 7). The primers used are listed in Supplementary Table 6. Apart from two genes (*CaSnRK*2.1 and *CaSnRK*2.9), the other members were all inducible by the two stresses. The expression profiles of most genes were basically consistent with the published data. *CaSnRK*2.2, *CaSnRK*2.3 and *CaSnRK*2.8 were all significantly upregulated in response to heat stress ($p < 0.01$),

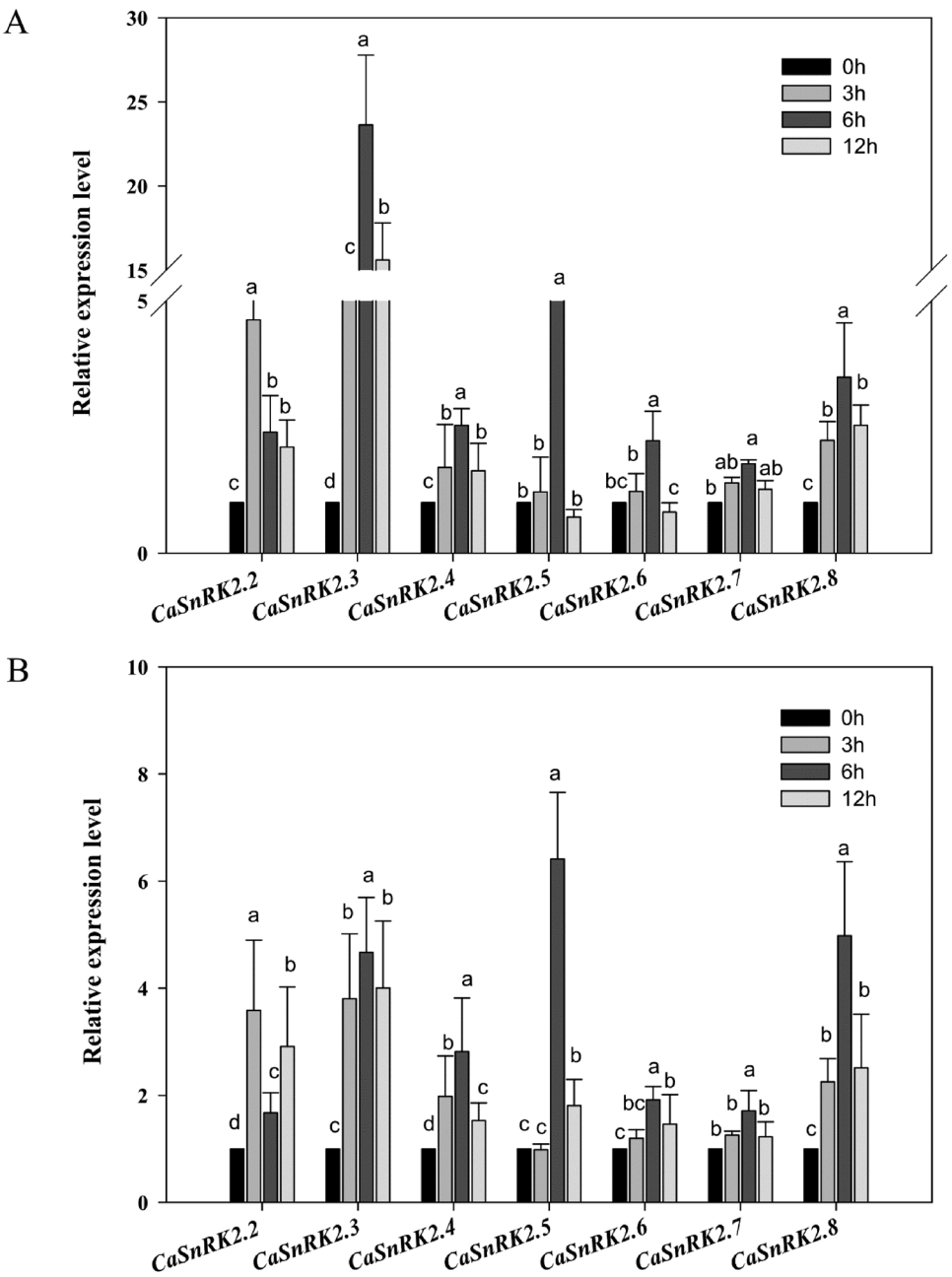

Fig. 7 The expression profiles of *CaSnRK2*s under heat (**a**) and salt (**b**) stress viaqRT-PCR. Total RNA was extracted from leaves of six-leaf stage pepper seedlings. For heat and salt stress, seedlings were suffered to 38 ℃ and 300 mM NaCl for 0, 3, 6, and 12 h. Eight plants were included in each group, and their leaves were collected from the same position on the seedlings with three biological replicates. The characters on top of the error bars represent the significant difference ($\alpha < 0.05$) compared to the control

while the expression levels of *CaSnRK*2.6 and *CaSnRK*2.7 did not change significantly under the two stresses.

Discussion

Plant perception of adversity and growth regulation are achieved through the signal transduction mechanism in vivo, and the key step of signal transduction is the phosphorylation and dephosphorylation by protein kinase and protein phosphatase. SnRK2 is a serine/threonine protein kinase unique to plants that has been cloned in *Arabidopsis*, rice, maize, tobacco and many other plants. SnRK2 responds to environmental signals, regulates the expression of related genes and protein activity, participates in a variety of signal transduction pathways in plants, and plays an important role in plant growth, development and stress resistance (Kulik et al. 2011; Rosenberger and Chen 2018). However, this gene family is poorly understood in pepper (*Capsicum annuum*). Fortunately, whole-genome sequencing data are available for pepper (Qin et al. 2014; Kim et al. 2014) and provide a great opportunity to explore this gene family at the genome level comprehensively.

Identification and characterization of *SnRK2* genes in pepper

Thus far, comprehensive studies have been conducted on genome-wide identification of *SnRK2* genes, and researchers have identified these genes in a variety of plants, such as 10 members in *Arabidopsis thaliana* (Hrabak et al. 2003), *Oryza sativa* (Kobayashi et al. 2004) and *Zea mays* (Huai et al. 2008), nine members in *Malus domestica* (Shao et al. 2014) and *Fragaria ananassa* (Han et al. 2015), 15 members in *Brassica rapa* ssp. *chinensis* (Huang et al. 2015), 20 members in *Gossypium hirsutum* (Liu et al. 2017b), etc. In the current study, a genome-wide identification revealed nine *CaSnRK2* genes from the 'CM334' and 'Zunla-1' databases of pepper genome named *CaSnRK2.1-CaSnRK2.9*. Multiple sequence alignment results demonstrated that the N-terminus of this family contained a relatively conservative protein kinase catalytic domain while the C-terminus is relatively specific and consists of two parts, an activated domain responding to abiotic stress and an ABA AA regulatory domain (D/E). The result is similar to the discoveries for *Malus prunifolia* (Shao et al. 2014) and *Solanum tuberosum* (Bai et al. 2017). The SnRK2 gene number in pepper is very similar to those in the *Arabidopsis*, rice, apple and strawberry genomes but less similar to those in *Brassica rapa* and cotton. *Brassica rapa* has more *SnRK2s* than pepper due to the salicoid duplication and *Brassica*-specific whole genome duplication (WGD) events (Huang et al. 2015). Thus, gene duplication likely contributed more to pepper SnRK2 expansion than to genome size (pepper genome is 3.48 Gb).

Through the phylogenetic analysis of 64 *SnRK2* genes from pepper, tomato, potato, apple, *Arabidopsis*, rice and maize, we conducted a comprehensive phylogenetic tree. Consistent with previous reports, the *SnRK2* genes were divided into three groups (Hrabak et al. 2003; Bai et al. 2017), with each group including two to four pepper *SnRK2* genes. The gene structure analysis revealed that *CaSnRK2s* contained four to eight introns. Similar characteristics have been reported in potato (Bai et al. 2017), *Arabidopsis* (Hrabak et al. 2003) and maize (Huai et al. 2008), suggesting their evolutionary conservation. The conserved motif identification showed an irregular distribution of 10 motifs in CaSnRK2s. Among them, motifs 1, 2, 4, 6 and 10 were in all SnRK2 members, motif 8 was only present in subgroup I, and motif 7 was unique to groups II and III. The results implied that they might contribute to the functional specificity of different groups, which requires deeper research.

Potential functions of *CaSnRK2* genes in growth and development

The tissue-specific expression of *SnRK2* genes provides insights into their potential function and has been investigated in many plants. In *Arabidopsis*, *SnRK2.10* was predominantly expressed in the vascular tissue while *SnRK2.4* was expressed throughout the roots. Both genes are involved in root growth and architecture (McLoughlin et al. 2012). *SnRK2.8/SRK2C* was expressed mainly in the roots, whereas *SnRK2.7/SRK2F* was expressed primarily in guard cells and vascular tissues of the roots, leaves and apical meristems (Mizoguchi et al. 2010). *TaSnRK2.3* in wheat was strongly expressed in booting spindles but less so in the seedling roots, leaves and emerging spikes (Tian et al. 2013). The rice *SAPK9* gene was most highly expressed in the leaves, and its expression was upregulated in response to drought stress and ABA treatment (Dey et al. 2016). In potato, *StSnRK2.1*, *StSnRK2.2*, *StSnRK2.5* and *StSnRK2.6* expression was highest in the roots while *StSnRK2.3*, *StSnRK 2.7* and *StSnRK 2.8* expression was significantly higher in the leaves and stems (Bai et al. 2017). In tomato, *SlSnRK2.1* was expressed only in the roots while all the other *SlSnRK2*s were expressed in all vegetative organs (Sun et al. 2011). Here, we assessed expression data in different tissues and organs obtained from public databases. Most *CaSnRKs* exhibited moderate expression in different tissues and organs, although some genes showed very high or very low expression in certain tissues. For example, *CaSnRK2.1* was expressed at relatively low levels in most tissues but at an especially high level in the roots, and *CaSnRK2.9* was also expressed in the roots and buds, implying vital roles for these genes in the regulation of root and flower growth.

Fruit development and ripening is a complex process that involves a series of complex physiological and biochemical reactions, such as cell division, expansion, and aging. The phytohormone ABA is an important regulator of fruit ripening, and it is likely involved in the regulation of fleshy fruit development and ripening in both climacteric and nonclimacteric fruits (Hou et al. 2018; Liao et al. 2018; Sun et al. 2017; Forlani et al. 2019). SnRK2s act as the core components of the ABA-mediated signaling network, and their roles in fruit development and ripening have attracted considerable attention from researchers. In the nonclimacteric fruit strawberry, *FaSnRK2.6*, *FaSnRK2.3* and *FaSnRK2.5* expression declined throughout fruit growth and development. FaSnRK2.6 was proven to be a negative regulator of strawberry fruit development and ripening (Han et al. 2015). In tomato fruits, which are climacteric, *SlSnRK2.2*, *SlSnRK2.3*, *SlSnRK2.4*, and *SlSnRK2C* were expressed at high levels at all fruit maturity stages while *SlSnRK2.1* and *SlSnRK2.5* were expressed at low levels. The researchers speculated that *SlSnRk2.3* and *SlSnRK2.4* might play key roles in the regulation of fruit development and ripening at all maturity stages and that *SlSnRK2.3* might play a more important role during the breaker stage (Sun et al. 2011). In cucumber fruits, which are also nonclimacteric, the absolute *CsSnRK2.1* expression level was much greater than that of *CsSnRK2.2* during all fruit developmental stages (Wang et al. 2012). To understand the function of *CaSnRK* in pepper fruit development, we analyzed their expression levels in nine developmental stages from fruit development to ripening. In our study, *CaSnRK2.2* and *CaSnRK2.6* were both strongly expressed throughout the fruit development and ripening process. Especially during the ripening process when the fruit turns color, the expression of most of the *CaSnRK2*s except *SnRK*2.5, *SnRK*2.8 and *SnRK*2.9 was strongly induced and peaked at the breaker stage (Dev7). These results demonstrated that variations in *CaSnRK2* expression were associated with the pepper fruit development and ripening process. We can also presume that ABA signal transduction may be involved in regulation of fruit development and ripening at the transcriptional level.

Possible functions of *CaSnRK2* members under abiotic stress

When plants are subjected to abiotic stresses, such as drought, high temperature, low temperature or salinity, the signals related to these stresses will be transformed into resistance factors through a series of signal transduction pathways. These resistance factors will activate the transcription factors related to adversity and combine with the *cis*-acting regulatory elements of corresponding genes to start the expression of corresponding genes and respond to adversity stress. Zhang et al. (2016) found a large number of *cis*-acting elements, such as ABRE, LTREs, ACGTAT ERD1 and DRE, in response to different hormones and abiotic stresses in the study of the SnRK2 gene family of wheat (Zhang et al. 2016). Similar results were also verified in potato (Bai et al. 2017) and cotton (Liu et al. 2017b). In this study, the results showed that almost all genes in the SnRK2 gene family of pepper contain ABRE *cis*-acting elements except *CaSnRK2.4* and *CaSnRK2.8*. *CaSnRK2.2* contains TGA, GARE, ERE, TCA, P-boxes, TATC-box, etc. and a total of 21 *cis*-acting elements, while other genes contain 3 to 11 elements. All genes in the SnRK2 gene family may be involved in the drought resistance stress response mechanism and wound or fungal elicitor-responses in plants, and *CaSnRK2.2* is more comprehensively involved in stress and hormone responses.

Previous investigations have shown that *SnRK2* genes are involved in various types of abiotic stresses and play an important role in regulation via ABA-dependent or ABA-independent signaling pathways (Yoshida et al. 2002; Tan et al. 2018; McLoughlin et al. 2012; Kobayashi et al. 2004; Ding et al. 2015; Soma et al. 2017). *AtSnRK2.4* and *AtSnRK2.10* are rapidly and transiently activated in *Arabidopsis* roots after exposure to salt (McLoughlin et al. 2012; Szymanska et al. 2019). In rice, all 10 *SnRK2* members could be induced and activated by NaCl and the gene expression levels were significantly increased (Kobayashi et al. 2004). The expression profiling revealed that *SAPK1* and *SAPK2* expression was strongly induced by drought, NaCl, and polyethylene glycol (PEG) treatment but not by ABA treatment (Lou et al. 2018). Overexpression of *SAPK4* or *SAPK6* (*OSRK1*) could increase salt tolerance in rice (Diedhiou et al. 2008; Nam et al. 2012), while *SAPK9* positively regulated ABA-mediated drought stress signaling pathways (Dey et al. 2016). In wheat (*Triticum aestivum*), *TaSnRK2* expression was induced by water deficit and salt and cold stress, and in *Arabidopsis*, *TaSnRK2.4* overexpression enhanced salt tolerance (Zhang et al. 2016). In potato (*Solanum tuberosum*), *StSnRK2.4* expression was greater in the shoot tissues than root tissues and could be induced by NaCl, ABA and PEG, suggesting that *StSnRK2.4* might be sensitive to stress signals and function in osmotic stress responses in potato shoot tissues (Bai et al. 2017). In cotton (*Gossypium hirsutum*), five *GhSnRK2* genes (*GhSnRK2.3/2.7/2.8/2.9/2.10*) were notably upregulated under salt and PEG treatment (Liu et al. 2017b). In our study, seven SnRK2 members responded to at least one treatment in the leaves, with *CaSnRK2.1*, *CaSnRK2.3* and *CaSnRK2.8* significantly induced by four treatments: cold, H_2O_2, mannitol and NaCl. Roots seem to be more sensitive to stress because the level of gene-induced expression is significantly greater in those tissues than in other tissues. Most *CaSnRK*s, including *CaSnRK2.1*, *CaSnRK2.2*, *CaSnRK2.3*, *CaSnRK2.4*, *CaSnRK2.5* and *CaSnRK2.7*, were inducible at

the early stage. *CaSnRK2.5* exhibited the greatest increase in expression (FC > 10-fold) under cold, mannitol and NaCl stresses. According to the phylogenetic analysis, *CaSnRK2.7* is a very close homologue of *Arabidopsis OST1/SnRK2.6* kinase, which is the main regulator of stomatal movements in response to stress (Liang et al. 2015). Therefore, *CaSnRK2.7* should play a role similar to that of *SnRK2.6* in leaves. Subsets of *CaSnRK2* genes that exhibit different expression profiles in response to abiotic stress have been identified, and they indicate that *CaSnRK2* plays a role in abiotic stress signaling in hot peppers.

In summary, pepper *SnRK2* genes were analyzed at the genome-wide level in this study and nine *CaSnRK2* genes were identified. Analyses of the gene structure, conserved motifs, *cis*-elements and expression patterns in different tissues and in response to different abiotic stresses revealed conserved and diverse characteristics of the members of the SnRK2 gene family. Among them, *CaSnRK2.2* and *CaSnRK2.6* may be involved in pepper fruit development and ripening process, while *CaSnRK2.1*, *CaSnRK2.3*, *CaSnRK2.5* and *CaSnRK2.7* may play important roles in abiotic stresses. This study lays a foundation for an improved understanding of the function of *CaSnRK2*s during pepper growth and development and response to stress.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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