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ORIGINAL ARTICLE

Genome-Wide Identification and Expression Profile Analysis of the *SnRK2* Gene Family in *Nicotiana tabacum*

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Abstract

SnRK2 protein kinase family plays an important role in plant response to abiotic stress and has been identified in various plants. This study aimed to identify *SnRK2* genes in tobacco and systematically analyze their expression under abscisic acid treatment and abiotic stress. We identified 22 *NtSnRK2* members, which were divided into three groups and located on 13 chromosomes, mainly at both ends of the chromosomes; additionally, 11 duplicated *NtSnRK2* gene pairs were observed. Phylogenetic analysis showed that these *SnRK2* members were divided into three groups in tobacco. The motifs of NtSnRK2 proteins in the same group were highly similar. Subcellular localization indicated that *NtSnRK2s* in Group3 were present in the nucleus, cytomembrane, and cytoplasm. Gene expression pattern analysis revealed that *NtSnRK2* genes played a role in the responses to several abiotic stresses (salt, drought, and low-temperature stress), indicating that they are widely involved in the adaptation of tobacco to adverse environmental conditions.

Keywords *Nicotiana tabacum* · *NtSnRK2s* · Genome-wide identification · ABA · Abiotic stresses

Introduction

The complex and changeable natural environment results in the generation of abiotic stressors that inevitably affect plants during their growth. Abnormal environmental factors, such as drought and flooding, hinder crop growth and decrease yield (Bailey-Serres and Voesenek 2008; Chaves et al. 2003; Wang

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et al. 2004). To adapt to environmental changes, plants have evolved a complex set of defense mechanisms to survive via natural selection (Blum 2017; Krasensky and Jonak 2012), and exploring these mechanisms is extremely important to improve crop yield and plant resistance.

SnRK2 is a plant protein kinase family, which is split into subgroups I to III (Umezawa et al. 2009, 2010) based on their sequence homology and respective responses to osmotic pressure and abscisic acid (ABA). The cDNA of *PKABA1*, a member of this gene family, was first identified from the cDNA library of *Triticum aestivum* L. endosperm treated with ABA (Anderberg and Walker-Simmons 1992); *PKABA1* can phosphorylate the substrate *Triticum aestivum* ABA response element-binding factor (TaABF) and be induced by ABA, low temperature, high salinity, and drought conditions (Johnson et al. 2008). Subsequently, AAPK (abscisic acid-activated protein kinase) of the SnRK2 family was identified in fava bean and found to play a role in Ca^{2+}-independent ABA signal transduction in guard cells (Li and Assmann 1996). In *Arabidopsis thaliana*, SnRK2 is composed of 10 family members, among which nine are induced by hypertonic stress, five by ABA, and none by low temperatures (Boudsocq et al. 2004). There are 11 SnRK2 family members in *Zea mays* L., among which five are induced by ABA, two are strongly induced by NaCl, two are induced by low temperatures, and three are inhibited by high temperatures. In addition, most members exhibit weak responses to salt stress (Huai et al. 2008). Ten *SnRK2* members have been identified in *Oryza sativa* L., all of which are induced by hypertonic stress; however, three of these could be induced by ABA (Kobayashi et al. 2004).

Arabidopsis overexpressing maize *SAPK8* and rice overexpressing *SAPK4* show higher salt tolerance than the wild-type plants (Diédhiou et al. 2008; Ying et al. 2011). After NaCl solution treatment, the *SAPK4* gene in rice decreases the accumulation of Na^+ and Cl^- in cells via reducing oxidative damage and regulating ion balance, thereby improving the salt tolerance of transgenic plants (Diédhiou et al. 2008). Overexpression of the *TaSnRK2.4* gene in wheat induces the expression of downstream stress-related genes. This leads to a series of physiological changes, such as decreased tissue osmotic potential, increased relative water content, and enhanced cell membrane stability, thus improving the salt tolerance and cold and drought resistance of transgenic plants (Mao et al. 2010).

On the whole, members of the SnRK2 family provide support for plants adapting to abiotic stress. Tobacco is not only a special industrial crop but also a model crop (Chen et al. 2017). Therefore, clarifying the molecular mechanism underlying the action of SnRK2 family in tobacco under adverse conditions can provide an important theoretical basis for improving tobacco stress resistance.

In this study, sequences of the SnRK2 protein in Arabidopsis were used to screen NtSnRK2 family members. Thereafter, we analyzed the physical properties, chromosome and subcellular localization, evolutionary relationship, and conserved motifs of NtSnRK2 family members. To clarify the function of *NtSnRK2* genes, we assessed the expression levels of *NtSnRK2* genes under abiotic stresses (salt, drought, and low temperature) and ABA treatment. Our study could provide theoretical guidance for the stress resistance breeding of tobacco.

Materials and Methods

Identification of *NtSnRK2* Genes

We obtained *SnRK2* gene sequences of *Oryza sativa*, *Zea mays*, *Nicotiana tabacum*, and *Arabidopsis thaliana* from the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/), Maize Genetics and Genomics Database (https://www.maizegenetics.net/), National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/mapview/index.html), and the Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/), respectively. Based on the identified protein sequences of the *AtSnRK2* gene, the candidate sequences of tobacco *SnRK2* were obtained using the Basic Local Alignment Search Tool (BLAST; *E*-value = 1e − 10). The hidden Markov model (HMM) obtained from the Pfam database (http://pfam.xfam.org, PF00069) (Finn et al. 2016) was used to predict the NtSnRK2 protein, and the HMMER software (HMM) was used to search for SnRK2 proteins from tobacco sequences. The predicted SnRK2 domains were detected using the SMART online tool (https://smart.embl-heidelberg.de/) to confirm the final protein sequences of *NtSnRK2s*. The ExPASy ProtParam online software (https://www.expasy.org/) was used to analyze the biophysical properties of the encoded *NtSnRK2s*. The subcellular localization and trans-membrane spanning region of NtSnRK2*s* were separately predicted using the WoLF PSORT online tool (https://www.genscript.com/wolf-psort.html) and TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/), respectively.

Chromosome Localization and Collinearity Analyses of *NtSnRK2* Genes

We retrieved the chromosomal distribution information regarding *NtSnRK2* genes from the Sol Genomics Network (https://solgenomics.net/). The MCScanX program was used to identify the Synteny blocks of tobacco genome containing the *SnRK2* genes (Wang et al. 2012). For gene duplication analysis, the chromosomal distribution of *NtSnRK2s* and the collinearity relationships between *NtSnRK2* homologs were verified and visualized using Circos (http://circos.ca/).

Phylogenetic Analysis of *SnRK2* Proteins

The Clustal X program with default parameters was used to perform the multiple sequence alignment of *SnRK2* genes in four species (*Z. mays*, *A. thaliana*, O. sativarice, and tobacco). MEGA 7.0 (Molecular Evolutionary Genetics Analysis) software with the neighbor-joining method was used to construct the unrooted phylogenetic tree, and set the Bootstrap value to 1000.

Gene Structure and Conserved Motifs Analysis of *NtSnRK2s*

We employed the Gene Structure Display Server 2.0 (GSDS 2.0) (http://gsds.cbi.pku.edu.cn/) to predict *NtSnRK2s* exon–intron structures using cDNAs and their

corresponding genome sequence alignment. The conserved motifs in the NtSnRK2 protein sequences were discovered using MEME online software (https://meme-suite.org/meme/tools/meme), and the number of motif searches was set at 15.

Subcellular Localization of NtSnRK2 Proteins in Group 3

The open reading frame (ORF) of *NtSnRK2* without a termination codon was fused upstream of green fluorescent protein (GFP) in the pJIT163-GFP vector under the control of the CaMV 35S promoter. The fusion construct was transformed into tobacco protoplasts by PEG-mediated transformation (Abel and Theologis 1994). Tobacco protoplasts were enzymically isolated from tobacco leaves of 3-week-old plants (Mahdieh and Mostajeran 2009). Incubated at 25 °C for 15 h after transformation, fluorescence signals were observed in the range of 500–570 nm wavelengths with a laser scanning confocal microscope (Leica TCSNT, Germany).

Gene Expression Pattern Analysis

K326 (*Nicotiana tabacum* L., cv. Kentucky 326), a widely cultivated flue-cured tobacco variety, was used to analyze the expression of *NtSnRK2* genes. The tobacco seeds were planted in mixed soil (humus: vermiculite = 1:1) with water and germinated in a sieve plate (25 ℃, 16 h light and 8 h dark). Seedlings cultured for 4 weeks in treatment groups were exposed to drought (PEG 6000 solution, − 0.5 MPa water potential), salt (300 mM NaCl solution), cold (4 °C), or ABA (50 μM, spray), respectively. Leaves were collected at 0, 1, 3, 6, 12, 24, 48, and 72 h post-treatment, frozen using liquid nitrogen, and stored at − 80 °C for RNA isolation. Total RNA was isolated using TRIzol reagent (Applygen, Beijing, China), then treated with RNase-free DNase to remove residual DNA. Complementary DNA (cDNA) was then synthesized using a reverse transcription (RT) kit (Takara, Dalian, China).

The gene expression patterns were detected using qRT-PCR, which was performed in an ABI PRISM 7000 system (Applied Biosystems, Waltham, MA, USA) using SYBR Green RT-PCR kits (Takara, Dalian, China) according to the manufacturer's instructions; all reactions were performed in triplicate. The *L25* gene was used as an internal control to quantify the relative transcript levels (Gregor and Delaney 2010). Results were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen 2001). The specific gene primers are listed in Table S1.

Results

Identification and Chromosomal Localization of the *SnRK2* Gene in Tobacco

Arabidopsis SnRK2 protein sequences were used as the query sequences, and a local BLASTP search was used to identify the SnRK2 members in the tobacco genome. The candidate genes with default *E*-values were searched via the SnRK2 domain (PF00069) using the HMMER3.0 program to verify the screening results.

Finally, 22 NtSnRK2 proteins were identified. Table 1 lists the characteristics of *NtSnRK2*, including the chromosomal localization, gene length, instability index, molecular mass, theoretical isoelectric point, number of amino acids, and subcellular localization. The results indicated a slight discrepancy among these *NtSnRK2* genes: the lengths of *NtSnRK2*s were 2488–6043 bp, the protein sequences of *NtSnRK2*s comprised 282–363 amino acids, the molecular weights of *NtSnRK2s* were 32.77–41.57 kDa, and the isoelectric points of *NtSnRK2* proteins ranged from 4.59 to 6.36, suggesting that all members of the NtSnRK2 gene family encode acidic proteins. The hydrophobicity index of proteins encoded by *NtSnRK2s* was less than zero, which indicated that *NtSnRK2* proteins were hydrophobic. Subcellular localization was predicted using the WoLF PSORT online tool and revealed that members of the *NtSnRK2* family were distributed in the cytoplasm, cytoskeleton, and mitochondria, respectively. Among the 22 *NtSnRK2* members, 13 members exhibited an instability index greater than 40, which indicates that these members are unstable.

According to the chromosomal distribution information regarding 22 *NtSnRK2* genes (Fig. 1), 16 *NtSnRK2* genes were located on 13 chromosomes, and the other six were located on the scaffolds. Two *NtSnRK2* genes were observed on chromosomes 6, 10, and 13, while one was observed on 10 other chromosomes (1, 3, 4, 5, 9, 14, 15, 17, 22, and 24). In addition, the location information and gene sequence homology analysis results showed that the 16 *NtSnRK2* genes successfully located on the ends of the chromosome, and there were 11 duplicated gene pairs in 22 *NtSnRK2* genes.

Phylogenetic Analysis of *SnRK2* Proteins

We used MEGA 7.0 with the neighbor-joining method to construct an unrooted phylogenetic tree and examine the evolutionary relationship among *SnRK2* members. The phylogenetic tree contained 52 *SnRK2* members from four species: 10 *SnRK2*s from *Arabidopsis*, 10 from rice, 10 from maize, and 22 from tobacco. The 52 *SnRK2*s were divided into three subgroups (Group 1–3, Fig. 2): LOC107766520, LOC107810314, LOC107817827, LOC107791496, LOC107798612, and LOC107826957 were clustered in Group 1; LOC107766134, LOC107765115, LOC107807198, LOC107819672, LOC107817951, and LOC107829367 belonged to Group 2; and the other tobacco SnRK2 proteins were assigned to the same branch (Group 3). It was found that each group contained SnRK2 members from tobacco, maize, rice, and Arabidopsis. Compared to maize or rice, SnRK2 proteins from tobacco and Arabidopsis were highly homologous. Furthermore, homologous SnRK2 proteins from maize and rice showed a close homology.

It is worth considering that in Group 1, LOC107798612 and LOC107826957 showed high similarity with *AtSnRK2.1* and *AtSnRK2.7*, while *AtSnRK2.7* could respond to osmotic stress and had a weak induction response to ABA. Similarly, LOC107766520, LOC107810314, LOC107817827, and LOC107791496 were closely related to *AtSnRK2.4* and *AtSnRK2.10*, whereas *AtSnRK2.4* and *AtSnRK2.10* were induced by osmotic stress but not by ABA. In Group 2, LOC107777197, LOC107780983, LOC107802713, and LOC107770300 had high homology with

Table 1 *SnRK2* gene family characteristics in *Nicotiana tabacum*

Gene ID	Chromosome	Gene length	Number of amino acids	Molecular weight (kDa)	Theoretical iso-electric point	Instability index	Protein hydro-phobicity	WoLF PSORT[a]
LOC107792070	chr13	4922	354	40.34	4.59	42.46	− 0.33	Cyto
LOC107766520	chr13	3551	353	40.79	5.97	51.89	− 0.51	Cysk
LOC107810314	scaffold	3719	336	38.72	5.92	52.67	− 0.49	Cysk
LOC107829367	chr1	3186	288	32.78	5.24	40.61	− 0.21	Cyto
LOC107817951	chr6	2926	299	34.18	5.37	41.83	− 0.23	Cyto
LOC107819672	chr3	3387	282	32.37	5.10	35.25	− 0.31	Cyto
LOC107807198	scaffold	4415	313	35.58	5.02	39.22	− 0.33	Cysk
LOC107807681	scaffold	5624	362	41.06	4.76	65.75	− 0.32	Cyto
LOC107790910	chr10	4300	344	39.33	4.84	41.14	− 0.32	Cysk
LOC107769218	chr4	4391	363	41.30	4.85	41.17	− 0.34	Cyto
LOC107824451	scaffold	3426	362	40.96	4.76	35.03	− 0.30	Cyto
LOC107791496	chr9	3212	356	41.08	5.78	56.12	− 0.60	Cysk
LOC107780983	chr24	3815	339	38.23	5.59	34.83	− 0.39	Cysk
LOC107766134	chr15	2943	348	39.55	5.43	32.56	− 0.31	Cyto
LOC107827494	chr17	6043	354	40.34	4.59	42.46	− 0.33	Cyto
LOC107817827	chr6	3250	355	40.84	5.98	52.39	− 0.58	Cysk
LOC107798612	chr14	4295	362	41.57	6.08	53.06	− 0.59	Cysk
LOC107826957	scaffold	4347	361	41.45	6.12	54.51	− 0.58	Cysk
LOC107777197	chr22	2397	339	38.39	5.38	37.95	− 0.40	Cyto
LOC107770300	chr10	4003	339	38.29	6.19	31.86	− 0.41	Cysk
LOC107802713	chr5	4836	339	38.17	6.36	30.43	− 0.41	Cysk
LOC107765115	scaffold	2488	295	33.33	4.98	29.08	− 0.67	Mito

Cyto cytoplasm, *Cysk* cytoskeletal, *Mito* mitochondria

[a]Best possible subcellular localization of *Nicotiana tabacum* SnRK2 based on WoLF PSORT

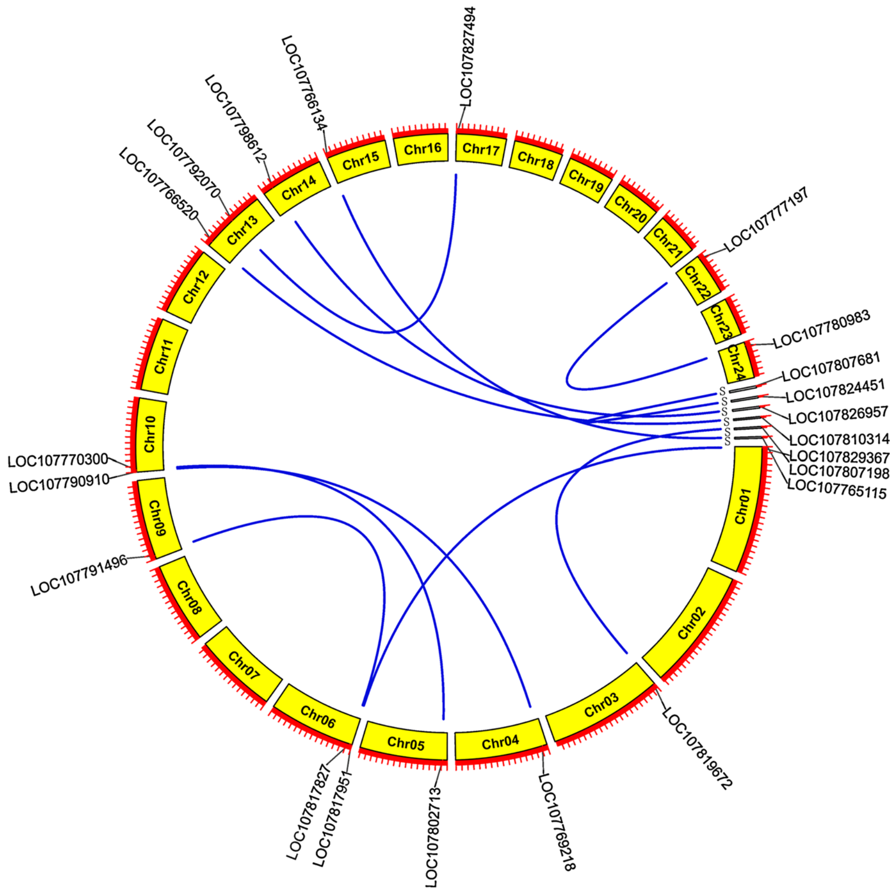

Fig. 1 Chromosome location and duplications of *SnRK2* genes in the *Nicotiana tabacum* genome. The chromosomal distributions of *NtSnRK2* genes and synteny relationships were displayed using Circos software. The blue lines indicate segmental duplication (Color figure online)

ZmSnRK2.3 and *OsSAPK3*, while *OsSAPK3* was induced by ABA, and *ZmSnRK2.3* was induced by low temperature and salt stress. In Group 3, LOC107792070 and LOC107827494 were similar to *AtSnRK2.2* and *AtSnRK2.3*; LOC107769218, LOC107790910, LOC107807681, and LOC107824451 were highly homologous with *AtSnRK2.6*; and *AtSnRK2.2*, *AtSnRK2.3*, and *AtSnRK2.6* were strongly induced by ABA (Huai et al. 2008; Kobayashi et al. 2004).

Gene Structure and Motif Analyses of *NtSnRK2s*

To further understand the function of the *NtSnRK2* gene family, a neighbor-joining phylogenetic tree, including 22 NtSnRK2 protein sequences, was constructed, and motifs were analyzed using the MEME online software. The neighbor-joining phylogenetic tree showed that the 22 NtSnRK2s were split into 11

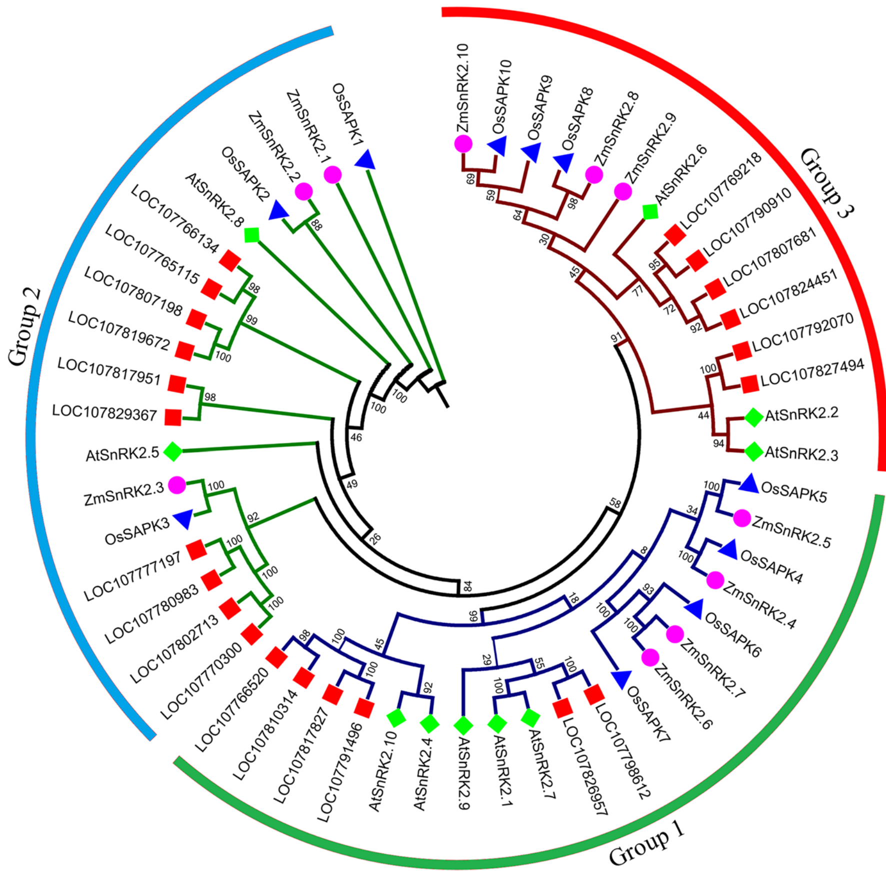

Fig. 2 Phylogenetic analysis of *SnRK2* proteins from *Oryza sativa, Zea mays*, *Arabidopsis thaliana*, and *Nicotiana tabacum*. The MEGA7.0 program was used to generate the unrooted phylogenetic tree using the neighbor-joining method with 1000 bootstrap replicates. The areas covered by the green, blue, and red arcs represent Group 1, Group 2, and Group 3, respectively. The red square represents *Nicotiana tabacum*, the green diamond represents *Arabidopsis thaliana*, the purple circle represents *Zea mays*, and the blue triangle represents *Oryza sativa* (Color figure online)

varieties (NtSnRK2.1–NtSnRK2-11) based on differences in their genetic sequences (Fig. 3a). The results of gene structure analysis (Fig. 3b) showed that there was a little discrepancy (6–9 exons) in the number of introns among different members of the NtSnRK2 family. Group 1 had the most exons, ranging from eight to nine, while 15 of the *NtSnRK2* gene family members had nine, four had eight, two had seven, and one had six.

As revealed by the motif analysis (Fig. 3c), NtSnRK2 members contained 15 conserved motifs. Furthermore, the protein motifs of Group 1 and Group 3 were relatively conserved. Motifs 1, 3, 7, 8, 9, and 11, which can be found in each NtSnRK2 protein, may be vital for their common functions, and more than 86.4% of the NtSnRK2 members contained motifs 2, 4, 5, and 6. It is worth

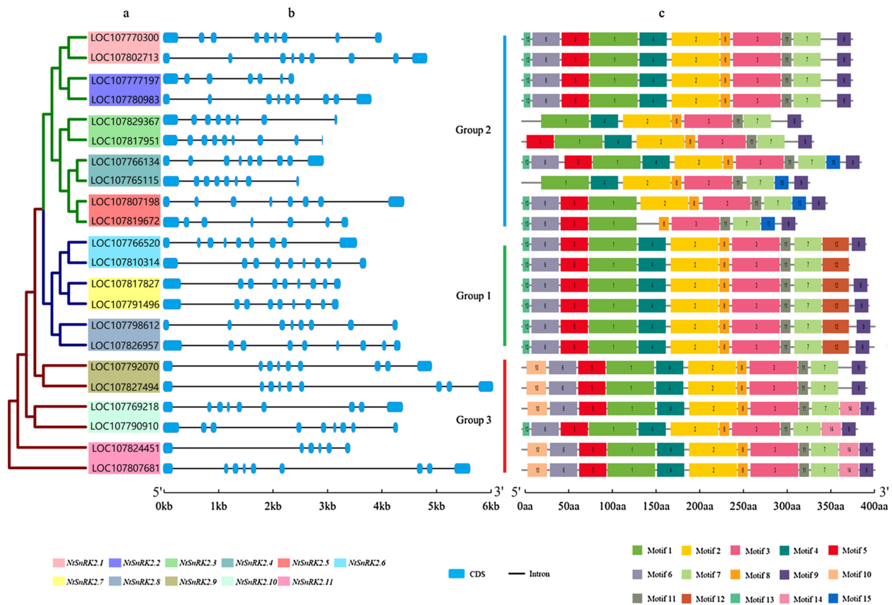

Fig. 3 Phylogenetic tree, exon–intron structure, and motif analysis of *NtSnRK2s*. **a** Phylogenetic analysis of *NtSnRK2s* using the neighbor-joining method. The boxes of different colors represent *NtSnRK2.1* to *NtSnRK2.11* from top to bottom. **b** Gene structure analysis of *NtSnRK2* genes. Exons and introns are represented by blue rectangles and black lines, respectively. **c** *NtSnRK2* protein motifs were analyzed using MEME tools. The order of the motifs corresponds to their position in the protein sequence. Conserved motifs are shown in different colored boxes (Color figure online)

mentioning that motifs 12 and 15 were specific to Groups 1 and 2, respectively, while motifs 10 and 14 were specific to Group 3. These motifs may explain the functional differences among the three groups.

Subcellular Localization of *NtSnRK2* Proteins in Group 3

The NtSnRK2.9-11 proteins in Group 3 contained a trans-membrane spanning region (DVWSCGVTLYVMLVGAYPF) and a potential N-myristoylation site (SGVSY/FCH), indicating that NtSnRK2.9-11 might mediate the association between the nuclear and cell membrane systems (Fig. S1). To determine the subcellular localization of the three NtSnRK2 proteins, their coding regions were separately fused in-frame with the *GFP* gene, and the fusion constructs were then individually transfected into tobacco protoplasts. The GFP-labeled NtSnRK2 protein in tobacco protoplasts was used to assess the cellular distribution of green fluorescence. As shown in Fig. 4, the NtSnRK2.9-11 proteins in Group 3 were separately targeted to the cell outlines, likely the nucleus, cytomembrane, and cytoplasm.

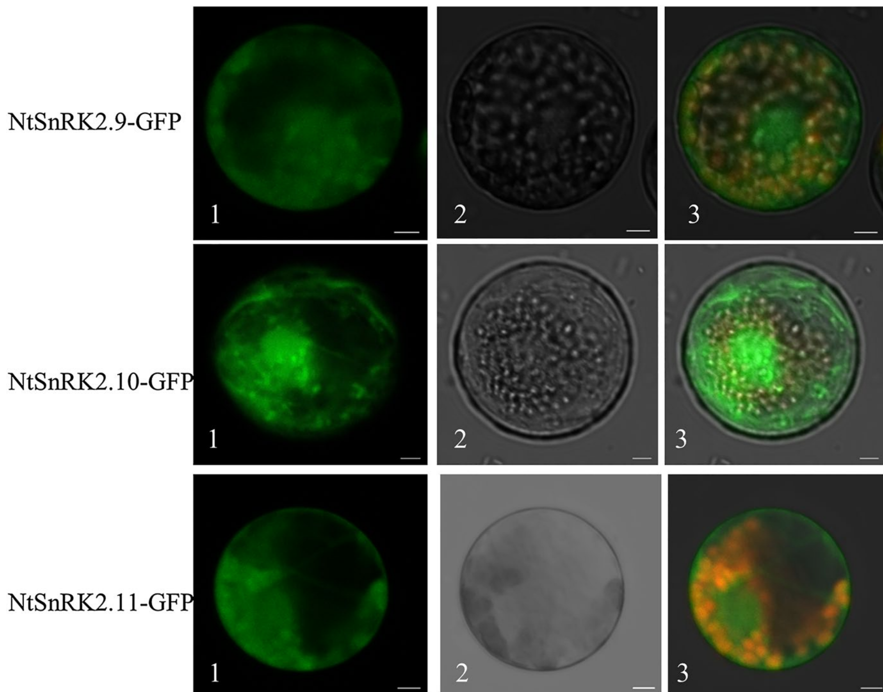

Fig. 4 Subcellular localization of NtSnRK2 proteins in Group 3. The ORF of *NtSnRK2* without a termination codon was fused upstream of GFP in the pJIT163-GFP vector under the control of 35S promoter. NtSnRK2–GFP fusion proteins were transiently expressed in tobacco protoplasts by PEG-mediated transformation. Fluorescence signals were observed in the range of 500–570 nm wavelengths with a laser scanning confocal microscope. 1, GFP fluorescence; 2, bright field images; 3, Chl autofluorescence; 4, merged images. Bar = 100 μm

Expression Patterns of *NtSnRK2s* Under ABA Treatment and Abiotic Stress

To assess the potential functions of *NtSnRK2s* during abiotic stress, qRT-PCR was performed to determine the expression pattern of *NtSnRK2s* after ABA, drought, high salinity, and cold treatments. As shown in Fig. 5, although *NtSnRK2* transcription was induced with a different expression profile, the expression patterns of *NtSnRK2s* in the same group were similar across the treatment groups. Distinct patterns were observed under ABA treatment among the three *SnRK2* groups: three Group 1 *NtSnRK2* members (*NtSnRK2.6-8*) showed no response to ABA; Group 2 *NtSnRK2s* (*NtSnRK2.1-5*) were weakly induced; and Group 3 *NtSnRK2*s (*NtSnRK2.9-11*) were strongly activated by ABA. We observed the rapid transcription induction of all *NtSnRK2s* under NaCl stress. The transcription of *NtSnRK2s* in Group 1 gradually increased and peaked at 48 h. Group 2 and 3 *NtSnRK2s* responded rapidly, reaching a peak at 3 or 6 h, and then their expression decreased. Under cold stress, the transcription of *NtSnRK2*s in Group 2 peaked at 1 h, declined gradually, and reached a maximum at 24 h. The expression of Group

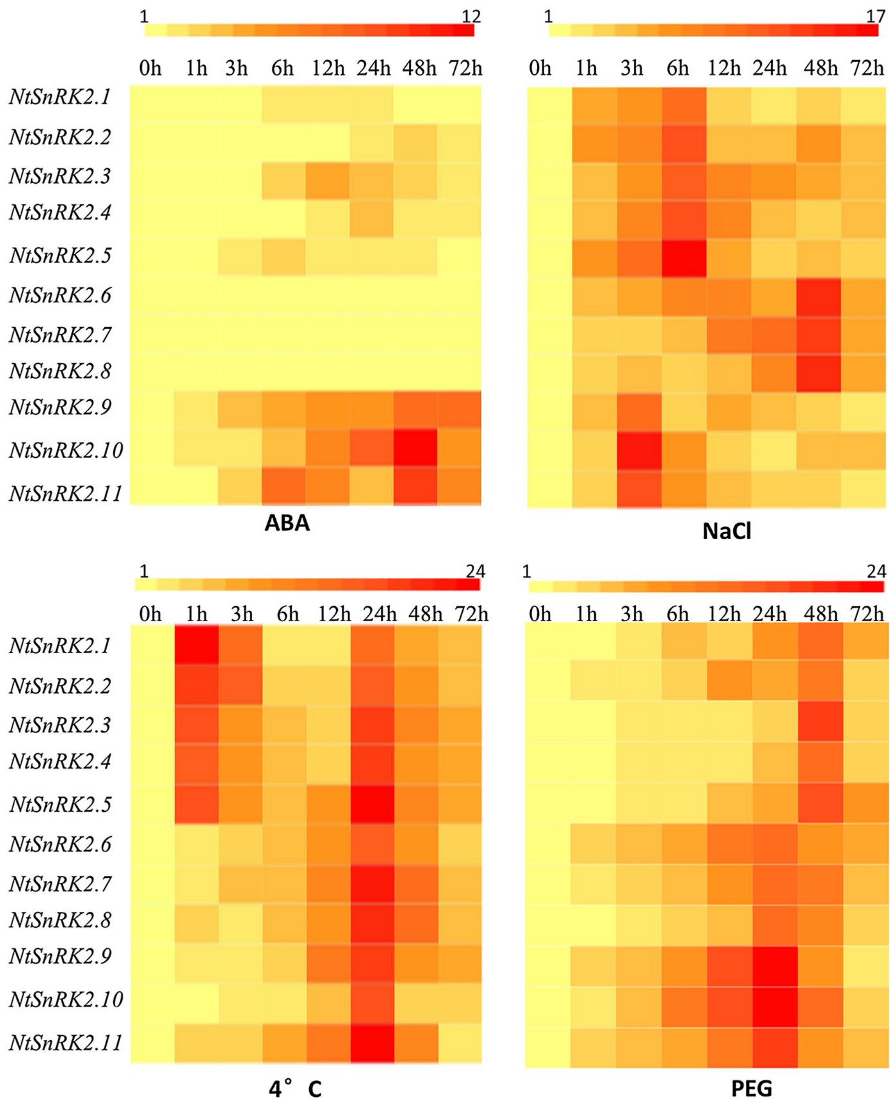

Fig. 5 Heat map of *NtSnRK2s* gene expression under ABA, NaCl, low temperature, and PEG treatments. The data from quantitative RT-PCR was used for hierarchical cluster analysis with MeV 4.9.0. The expression values of each *NtSnRK2* were log2 transformed. Yellow indicates low level expression and red indicates high level expression (Color figure online)

1 and 3 *NtSnRK2s* increased gradually, reached a maximum at 24 h, and then decreased. All *NtSnRK2*s showed a similar expression pattern in response to PEG treatment. Transcription was induced gradually, peaked at 24 (Group 1 and 3) or 48 h (Group 2), and then decreased. These results suggest that *NtSnRK2s* may participate in abiotic stress responses in different manners.

Discussion

*SnRK2*s are a class of Ser/Thr protein kinases unique to plants, which play significant roles in abiotic stress and various signaling pathways. Numerous studies have demonstrated that each *SnRK2* gene is differently involved in the response to multi-environmental stress. In the present study, we identified 11 *NtSnRK2* gene members from the *Nicotiana tabacum* genome database using a homology search of the protein sequences of *SnRK2* in *Arabidopsis*. A total of 15 motifs were present in the NtSnRK2 proteins, and the motif distribution patterns were similar within subgroups (Fig. 3c). This finding indicated that tobacco *SnRK2* genes were relatively conserved during evolution.

Gene replication is an important mechanism in gene family evolution. As a general biological phenomenon, gene repetition can provide the most primitive genetic material basis for biological evolution, produce new genes or subfunctional genes, and promote species differentiation and diversity (Hu et al. 2009; Leitch et al. 2008; Yang et al. 2008). Thus, more gene members are commonly present in polyploid species. Compared with other plants (10 *SnRK2* genes have been identified in maize, rice, and *Arabidopsis*), the *Nicotiana tabacum SnRK2* family displays more members (Table 1). This may be because *Nicotiana tabacum* is a tetraploid plant, and replication of new genes occurred in the process of tetraploidization.

To better analyze the evolutionary relationship among SnRK2s in different species, we constructed a SnRK2 phylogenetic tree, which comprised four species (tobacco, maize, rice, and Arabidopsis). Similar to that in previous studies, NtSnRK2 proteins were divided into three groups (Umezawa et al. 2010). Homologous SnRK2 proteins from maize and rice showed high homology, and tobacco SnRK2 proteins showed the highest similarity to their counterparts from *Arabidopsis* (Fig. 2). These data implied that the differentiation of SnRK2 family genes occurred after the separation of monocots and dicots.

Protein kinases localize to certain cell compartments to perform their proper function, and scanning sequences often specify their intracellular locations. It has been reported that the NtSnRK2 proteins in Group 1 and 2 are present in the cell membrane, cytoplasm, and nucleus (Zhang et al. 2014a, b). All *NtSnRK2* proteins in Group 3 also contained a conserved transmembrane-spanning motif (DVWSCGVTLYVMLVGAYPF) and a potential N-myristoylation site (SGVSY/FCH). The N-terminal myristoylation and transmembrane-spanning motifs are crucial for proteins to function in mediating membrane targeting and signal transduction in plant responses to environmental stresses (Ishitani et al. 2000; Podell and Gribskov 2004). To study the cellular localization of the NtSnRK2s proteins in Group 3, the *NtSnRK2s*::GFP construct driven by the CaMV 35S promoter was transiently expressed in tobacco protoplasts. As predicted, the NtSnRK2s proteins in Group 3 were all localized to the cell membrane, cytoplasm, and nucleus (Fig. 4).

Gene expression patterns can be a direct indication of a gene's function in plant growth and development. A study regarding stress resistance in maize indicated

that the expression levels of *ZmSnRK2.2*, *2.4*, *2.5*, *2.7*, and *2.10* were enhanced after ABA treatment; the expression levels of *ZmSnRK2.5* and *ZmSnRK2.6* were obviously induced by NaCl treatment; *ZmSnRK2.3* and *ZmSnRK2.7* expression was significantly increased under low temperatures, while the expression levels of *ZmSnRK2.5*, *2.6*, and *ZmSnRK2.9* decreased under high-temperature conditions (Huai et al. 2008). In Arabidopsis, *SnRK2* subfamily II and III members participate in ABA-dependent signal transduction (Boudsocq et al. 2004; Zheng et al. 2010). In our study, the expression of Group 3 *NtSnRK*s was strongly induced by ABA treatment; the expression levels of Group 2 *NtSnRK*s were also responsive to ABA, but the response was weak; and the expression of Group 1 *NtSnRK*s was not activated by ABA treatment (Fig. 5). Similar results were observed in rice, maize, and wheat in previous studies (Huai et al. 2008; Kobayashi et al. 2004; Zhang et al. 2014a).

All *NtSnRK2* members were activated by NaCl treatment (Kobayashi et al. 2004), similar to the results of *OsSAPK* in rice (Diédhiou et al. 2008), but genes of Groups 2 and 3 were more sensitive to salt stress and strongly induced transcription 3 and 6 h after exposure (Fig. 5), respectively. All *NtSnRK2* members were activated by low temperature or drought; among them, Group 2 genes were highly sensitive to low-temperature stress and expressed strongly after exposure to low temperature for 1 h (Fig. 5)—this result differs from that of other similar studies (Zhang et al. 2014b).

The present study focused on the identification of evolved and conserved motifs of *SnRK2* gene family members from *Nicotiana tabacum*, and the expression of each *NtSnRK2* family member was analyzed under a variety of abiotic stresses. Together, our results can provide an important theoretical basis for improving the stress resistance of tobacco.

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Author Contributions FH and JHL conceived and designed the experiments. JYS, JTM, JWL, and CJL performed the experiments and participated in data analysis. XWZ and JCL performed the qRT-PCR experiments. YC revised the manuscript.

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Data Availability The data sets supporting the results of this article are included within the article and its additional file.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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