https://doi.org/10.17221/48/2022-PPS

Characterisation of the *HbSnRK2* gene family members and revealing specific *HbSnRK2.2* functions in the stress resistance of the rubber tree

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Citation: Zhang Y., Liu M.Y., Zhang D., Wang M., Wang L.F. (2023): Characterisation of the *HbSnRK2* gene family members and revealing specific *HbSnRK2.2* functions in the stress resistance of the rubber tree. Plant Protect. Sci., 59: 59–72.

Abstract: SNF1-related protein kinase (SnRK2) is a critical positive regulatory factor in the abscisic acid (ABA) signalling pathway. However, the roles of the *HbSnRK2* gene family members in the rubber tree, especially in response to stress, have not been thoroughly characterised. Here, we cloned six *HbSnRK2* genes from the rubber tree. Based on the phylogenetic analysis, the *HbSnRK2* family genes were divided into three groups. The motifs and intron numbers of HbSnRK2 were conserved. Analysis of *cis*-regulatory element sequences of all *HbSnRK2* genes identified ABRE and TC-rich elements in the prompter of all the *HbSnRK2* genes, illustrating that *HbSnRK2* could be adjusted by the ABA and stress responsiveness. The qRT-PCR analysis showed that the expression patterns of the six *HbSnRK2* genes differed in different tissues. The expression of these genes also differed under treatment with the plant hormone ABA, the *HbSnRK2.2* gene was especially significantly expressed under the ABA treatment. Moreover, the *HbSnRK2.2* gene responded to glyphosate, powdery mildew, heat stress and cold stress processes, which indicates that the *HbSnRK2.2* gene plays an important role in phytohormone signalling and stress response in rubber trees. Taken together, the study provides valuable information to further define the role of the *HbSnRK2* gene in rubber trees.

Keywords: SnRK2; *Hevea brasiliensis* Müll. Arg.; phytohormone; signal pathway

Supported by the Hainan Basic and Applied Basic Research Program (Natural Science) High-Level Talent Program (No. 2019RC164); the National Science Foundation of China (No. 31660187, No. 31660204 and No. 31860183); and Earmarked Fund for Modern Agro-industry Technology Research System (No. CARS-33-BC1).

https://doi.org/10.17221/48/2022-PPS

Abscisic acid (ABA), an essential phytohormone, is involved in seed germination, plant growth, development, biotic and abiotic stress responses (Lee et al. 2015). In the ABA signalling pathway, ABA binds to the pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA receptor (PYR/PYL/RCAR) proteins and represses the protein phosphatase 2C (PP2C) activity. The phosphorylation of SNF1-related protein kinase (SnRK2) protein triggers the expression of ABA-responsive element (ABRE)-binding protein/ABRE-binding factor (ABF) transcription factors (Fujii & Zhu 2009; Fujita et al. 2009).

SnRK2 is a plant-specific serine/threonine (Ser/Thr) protein kinase family that has a SNF1/AMP kinase domain. In *Arabidopsis*, there are 10 *SnRK2* genes (SnRK2.1-2.10) (Hrabak et al. 2003), and the *AtSnRK2* family can be divided into three groups (Mizoguchi et al. 2010). Studies have shown that the *SnRK2* genes in group III (*SnRK2.2*, *SnRK2.3*, *SnRK2.6*) are strongly activated in response to ABA, whereas those of group II (*SnRK2.7*, *SnRK2.8*) are weakly activated in response to ABA (Boudsocq et al. 2004; Furihata et al. 2006). Knocking out three *SnRK2* genes in group III nearly completely blocks the ABA response, showing that *AtSnRK2.2*, *AtSnRK2.3*, and *AtSnRK2.6* are essential components of ABA signalling. The *AtSnRK2* genes in group III can regulate the ABFs to response to drought stress in ABA signalling. The *snrk2.2/3/6* triple mutant exhibits significantly reduced tolerance to drought stress and is highly insensitive to ABA. Under drought stress, ABA and the drought stress-dependent gene expression is globally and drastically impaired, and both jasmonic acid (JA)-responsive and the expression of flowering-related genes are upregulated in the *snrk2.2/3/6* triple mutant, but not in the single and double mutant. These results indicate that the *SnRK2* genes in group III are the primary positive regulators of the ABA signalling in response to drought stress (Fujita et al. 2009).

Interestingly, all *AtSnRK2s* except *AtSnRK2.9* can be activated by osmotic stress in *Arabidopsis* protoplasts (Boudsocq et al. 2004). Analyses of the decuple (*SnRK2.1*/*2*/*3*/*4*/*5*/*6*/*7*/*8*/*9*/*10*) and septuple (*SnRK2.1*/*4*/*5*/*7*/*8*/*9*/*10*) *snrk2* mutants indicated that *SnRK2.2*/*3*/*6* are essential components of the osmotic stress responses as well as the ABA signalling (Fujita et al. 2011). *SnRK2.4* and *SnRK2.10* are involved in the maintenance of the root system architecture during salt stress (McLoughlin et al. 2012). Systemic immunity in *Arabidopsis* requires *SnRK2.8* to mediate the phosphorylation to activate NPR1 (Lee et al. 2015). Similarly, the inactivation of the functionally redundant members of the SnRK2 kinases leads to a reduction in the miRNA accumulation under stress conditions (Yan et al. 2017).

Commercial natural rubber (*cis*-1,4-polyisoprene) is almost exclusively extracted from rubber trees (*Hevea brasiliensis* Müll. Arg.). The cultivation of rubber trees often suffers various stress conditions, such as low temperatures, pesticides and diseases, all of which affect the rubber tree development and latex yield (Tungngoen et al. 2011; Fang et al. 2021). *SnRK2s* function as positive regulators in response to abiotic stress, via transgenic technology, serve as potential candidates to improve the rubber tree tolerance against adverse environmental conditions. COI1–JAZ3–MYC2 is the specific jasmonate signalling module to regulate the rubber biosynthesis in laticifer cells (Deng et al. 2018). Ethephon (ETH) is a routinely used stimulant that can increase the latex yield of rubber trees (Priya et al. 2007; Zou et al. 2015; Nie et al. 2016). ETH led to a significant increase in the latex yield at 8 h after being applied to ABA-treated rubber trees (Tungngoen et al. 2011). In the present study, the *SnRK2* gene family members in the rubber trees were isolated and characterised based on the genomic database of the rubber tree (Tang et al. 2016). The gene and protein structure, phylogeny, and expression pattern in the different tissues and defence responses were analysed. The results help provide an essential understanding of the *SnRK2* genes in rubber trees and facilitate the breeding of new rubber tree varieties to tolerant different stresses.

MATERIAL AND METHODS

Identification of the *SnRK2* genes in the genome of the rubber tree

The amino acid sequences of *Arabidopsis* SnRK2.1-2.10 obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org) were used as queries for BLASTP searches against the rubber tree genome and Transcriptome Shotgun Assembly (TSA) database. Default BLAST settings were used, and low-complexity filtering and removal of redundant sequences were performed manually. All hits that were considered candidate

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sequences were submitted to the National Center for Biotechnology Information (NCBI) Conserved Domain Search database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to confirm the presence and completeness of each gene as a member of the *SnRK2* family. Based on the results that we searched in the rubber tree genome and TSA database, we also obtained information on the cDNA sequences and genomic sequences. Finally, all the identified protein sequences were aligned with ClustalW (http://www.clustal.org/) using known SnRK2 sequences to confirm that the sequences were candidate *HbSnRK2* genes. The molecular weight (MW) and isoelectric point (pI) of each HbSnRK2 protein were calculated using ExPASy (http://web.expasy.org/compute_pi/). The Protein Subcellular Localization Prediction Tool (PSORT, https://www.genscript.com/psort.html) was used to predict the subcellular localisation of HbSnRK2.

Phylogenetic analysis

To construct the phylogenetic tree, we downloaded the full-length amino acid sequences of the SnRK2s from *Arabidopsis*, rice, maize, and grapes from the NCBI protein database (http://www.ncbi.nlm.nih.gov/guide/). All the SnRK2s and HbSnRK2s obtained here were aligned with the integrated MUSCLE alignment program of Molecular Evolutionary Genetics Analysis (MEGA) 5, with the default parameters. The phylogenetic analysis was conducted by the maximum likelihood (ML) method using MEGA 5 software, and bootstrap tests were replicated 1 000 times. Additionally, all the HbSnRK2 protein sequences were aligned with MEGA 5 and used to construct the phylogenetic tree in the same way.

Gene structure, motif and *cis*-elements analysis

The exon-intron structure of the *HbSnRK2* genes were determined by aligning their coding sequences to their corresponding genomic sequences. A map of exon-intron structure was then obtained with the online Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.ch). The motifs in the six HbSnRK2 amino acid sequences were elucidated using the Multiple Expectation maximization for Motif Elicitation (MEME) tool (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). The optimum motif widths were set between 16 and 100 residues; the maximum number of motifs was nine. The *cis*-elements of the promoters were identified by PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalup.html).

Sequence alignment and tertiary structure prediction

Multiple alignments of the HbSnRK2 proteins were obtained using DNAMAN software (version 7.0). The conserved protein kinase domains were detected by the ScanProsite (http://prosite.expasy.org/scanprosite/) program. The tertiary structure of the HbSnRK2 proteins was predicted in SWISS-MODEL (https://swissmodel.expasy.org/).

Plant materials and treatments

Clones of rubber tree CATAS73397 were planted at the experimental farm of the Chinese Academy of Tropical Agricultural Sciences, Danzhou city, Hainan Province, China. We used different developmental stages as materials. Mature leaves, roots, stems, bark, laticifer tissue and flowers were collected from three 18-year-old trees (CATAS73397). At least five leaf, root, stem, bark, laticifer and flower samples were harvested from each tree. The leaf samples were collected from the budding rootstocks of rubber trees treated with 200 μmol/L of glyphosate, 200 μmol/L of jasmonic acid (JA), 200 μmol/L of ABA, 2% (v/v) ethephon (ETH), heat, cold, glyphosate and powdery mildew (caused by *Oidium heveae* B.A. Steinm.). The control plants were sprayed with distilled water consisting of 0.05% (v/v) ethanol and 0.1% (v/v) Tween-20. The treatments were applied according to the methods of our recent study (Yu et al. 2017). All the harvested samples were immediately frozen in liquid nitrogen and subsequently stored at −80 °C. Each sample included three independent biological replicates.

Molecular cloning of *SnRK2* genes in the rubber trees

The cDNA from a mixed population of leaves was used as a template for amplifying the rubber tree *SnRK2s* genes. The specific primers (Table 1) for gene cloning were designed based on the revised putative sequences. Polymerase chain reaction (PCR) was performed with PrimeSTAR® HS DNA Polymerase (TaKaRa, China), and the amplification conditions were empirically optimised. The PCR products were recovered and ligated to the pMD18-T vector (TaKaRa, China).

qRT-PCR analysis

The expression profiles of *HbSnRK2*s were analysed via quantitative real-time- PCR (qRT-PCR) in conjunction with the specific primer pairs.

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Table 1. Primers used for the *HbSnRK* gene clone and qRT-PCR

Primers	Function	Sequences 5'–3'
HbSnRK2.6-F	gene clone	TGTGGGAAAAGATGGAGGAG
HbSnRK2.6-R		TTTCGCCACATGGTGGTAT
HbSnRK2.2-F		CCGTGCCTTTGTTTCTTGTT
HbSnRK2.2-R		AGAACCACCCCCTCTCTTGT
HbSnRK2.5-F		GAAACGTTTGCTGCCTCTTC
HbSnRK2.5-R		GCTGTTTTTCTATGCTAACCTACCA
HbSnRK2.3-F		CGGATTGATTAGCGGGTTTA
HbSnRK2.3-R		ACTCTTTCTGCCCAATACGC
HbSnRK2.1-F		TTGTTCTAGCTCTTGTGCTACATTG
HbSnRK2.1-R		AGAAATCTGATGCTCTTAATCATGG
HbSnRK2.4-F		TCACTGAATCTACCAGCACTG
HbSnRK2.4-R		GAAACTCTCAACATCACCCTG
HbACTIN-F	qRT-PCR	GATGTGGATATCAGGAAGGA
HbACTIN-R		CATACTGCTTGGAGCAAGA
HbSnRK2.6-F		AGCGTTCAATACTCCATACC
HbSnRK2.6-R		TCAGATGTACCAGCAATAGC
HbSnRK2.2-F		CTTAATGTCCAGTATTCCATCC
HbSnRK2.2-R		TTGTCTGCCATATACCGATT
HbSnRK2.5-F		AGTCTTCAGTGCTTCATTCA
HbSnRK2.5-R		GCCTATTGTCTTCTCATCCA
HbSnRK2.3-F		AGTTCCTCCTTCGGTATCT
HbSnRK2.3-R		CATCTTCCTCTTCTTCTTCTTC
HbSnRK2.1-F		GTCCGCTACACAATTCCA
HbSnRK2.1-R		GAACTTCTTCAATGCTCTGT
HbSnRK2.4-F		GATGCTGGTTGGTGCTTA
HbSnRK2.4-R		ATAGTCTGGAATGGAGTAGTG

The PCR was performed using a CFX96^{TM} Real-Time System (Bio-Rad, Hercules, CA, USA) in a 20 µL (total volume) reaction system consisting of 1× SYBR Premix Ex Taq (TaKaRa, China), 0.4 µL of each primer, and 1 µL of cDNA template (approximately 100 ng/µL) per sample; ddH_2O was added to bring the final volume to 20 µL. The reaction programme is the same as that used by pervious study (Qin et al. 2015).

Statistical analysis

IBM-SPSS (version 24.0) statistical software was used for the statistical analysis. Duncan's multiple range test was applied to compare the significant differences among the expression levels in the different tissues, and Tukey's honestly significant difference (HSD) test was used to compare the significant differences of all treatments against their controls. The significant level was $\alpha = 0.05$.

RESULTS

Identification of the *SnRK2* gene family members in the rubber trees

To identify the *SnRK2* gene family members in the rubber trees, *AtSnRK2* genes were used as a query to search in the rubber tree genome. After removing the redundant sequences, a total of six *HbSnRK2* genes were identified within the rubber tree genome and were then cloned; we named them *HbSnRK2.1*, *HbSnRK2.2*, *HbSnRK2.3*, *HbSnRK2.4*, *HbSnRK2.5* and *HbSnRK2.6* according to the order of their NCBI accession number. The characteristics of the *HbSnRK* gene family, including the gene length, open reading frame (ORF) length, pI, MW and subcellular localisation are listed in Table 2. The ORF length ranged from 336 bp (*HbSnRK2.4*) to 364 bp (*HbSnRK2.2*), the MW ranged from 96.98 kDa (Hb-

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Table 2. Basic information of the *HbSnRK* gene family members

Gene name	NCBI accession number	Coding sequence (bp)	Amino acid (bp)	Isoelectric point	Molecular weight (kDa)	Subcellular localization
HbSnRK2.1	MF785116	1 017	338	4.99	103 760.04	cytoplasm
HbSnRK2.2	MF785117	1 095	364	5.06	102 756.3	cytoplasm
HbSnRK2.3	MF785118	1 065	354	5.07	105 291.98	cytoskeleton
HbSnRK2.4	MF785119	1 011	336	4.99	110 419.4	cytoplasm
HbSnRK2.5	MF785120	1 089	362	5.02	122 659.54	cytoplasmic matrix
HbSnRK2.6	MF785121	1 017	338	4.99	96 985.77	cytoskeleton

SnRK2.6) to 122.65 kDa (HbSnRK2.5), and the pI ranged 4.99 (HbSnRK2.6) to 5.07 (HbSnRK2.3). All the HbSnRK2 proteins had a low pI (pI < 6.0). The subcellular localisation prediction revealed that HbSnRK2.1, HbSnRK2.2 and HbSnRK2.4 were present in the cytoplasm of the plant cells, HbSnRK2.3 and HbSnRK2.6 were present in the cytoskeleton, and HbSnRK2.5 was present in the cytoplasmic matrix.

Phylogenetic analysis of the *HbSnRK2* family

To determine the phylogenetic relationship of the SnRK family between the rubber trees and the other species, an ML phylogenetic tree was constructed using the full-length amino acid sequences of HbSnRK2. Consistent with the previous classification (Fujii et al. 2007), the *HbSnRK2* family clustered into three groups: group I, II, and III (Figure 1A). *HbSnRK2.3* belonged to group I with *AtSnRK2.1*, *AtSnRK2.4*, *AtSnRK2.5*, *AtSnRK2.9* and *AtSnRK2.10*. *HbSnRK2.1*, *HbSnRK2.4*, *HbSnRK2.6* with *AtSnRK2.7*, *AtSnRK2.8* clustered in group II. *HbSnRK2.2*, *HbSnRK2.5* together with *AtSnRK2.2*, *AtSnRK2.3*, *AtSnRK2.6* in group III.

We summarised the distribution of the *SnRK2* genes from several plant species (Figure 1B). The results showed that all higher plants have three groups of *SnRK2*s, and the *SnRK2* genes in mosses and ferns all belonged to group III. According to the data and increasing amounts of evidence, the *HbSnRK2* genes in group III were the most ancient from an evolutionary standpoint, and the *HbSnRK2* genes in group I was the most recent during the genetic evolutionary process.

Exon-intron organisation, conserved motifs and domains of the *HbSnRK2* family

HbSnRK2 amino acid sequences were used to construct a phylogenetic tree. The tree showed that the homologous gene pairs clustered into the same clade in Figure 1A. To obtain a deep understanding of the structure of the *HbSnRK2* genes, we analysed the polymorphisms in the exon-intron structure of the *HbSnRK2* family members to explore the structural evolution of the *SnRK2* genes in the rubber trees. In addition, the *HbSnRK2.6*, *HbSnRK2* family members in same group had a similar length and exon-intron structure. All the *HbSnRK2* genes had eight introns, except for *HbSnRK2.6*, which had nine introns (Figure 2A). By using MEME to detect the conserved motifs in the six HbSnRK2 proteins, we found that HbSnRK2 in the same group shared the same motif structures. HbSnRK2.1, HbSnRK2.4 and HbSnRK2.6 in group II had eight motifs; HbSnRK2.3 had seven motifs; HbSnRK2.2 and HbSnRK2.5 had nine motifs (Figure 2B). Motifs 1–5 and 8 were shared among all the HbSnRK2s. Motif 6 was present in all the proteins except HbSnRK2.3. Motif 7 and motif 9 were present in the HbSnRK2 proteins of group III, suggesting that motif 7 and motif 9 were associated with the ABA response.

Prior studies had shown that SnRK2s had a highly conserved N-terminal domain which was similar to SNF1/AMP kinases and a short C-terminal domain which was less conserved (Halford & Grahame Hardie 1998). The analysis of HbSnRK2 amino acid sequences confirmed the theories mentioned above. Furthermore, there are two domains (domain I and domain II) at the C-terminus which are not conservative and the Ser/Thr kinase domain is conserved (Figures 3A and 3B). Moreover, we used SWISS-MODEL to predict the tertiary structure of the HbSnRK2 protein which found that these protein kinases consisted of α-helix, random coil and β-turn, which is basically consistent with the prediction results of the secondary structure of HbSnRK2 (Figure 3C).

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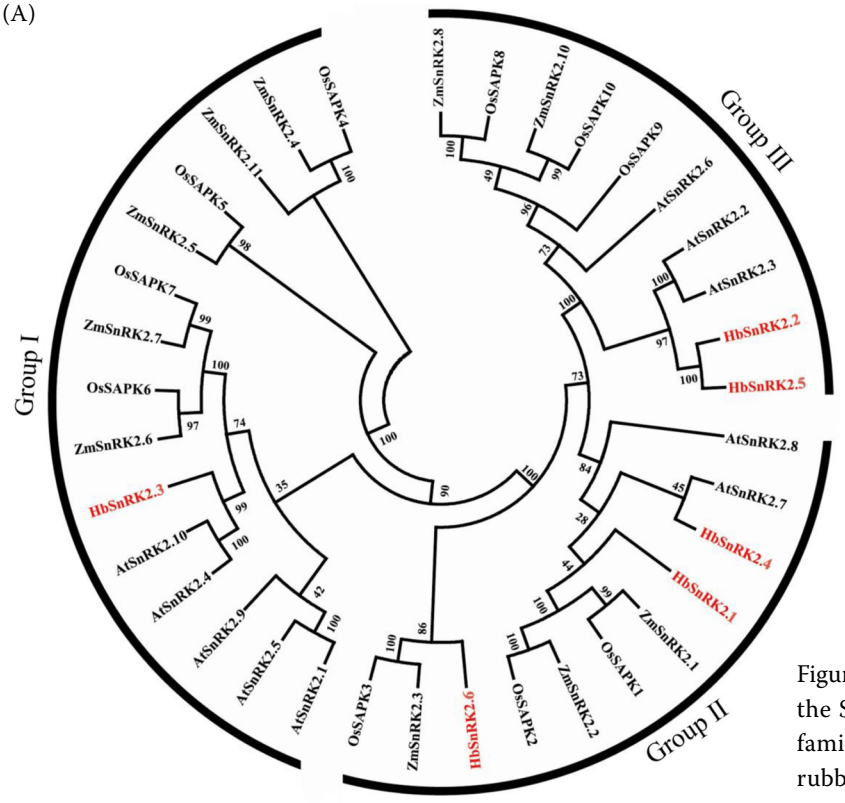
(A)



(B)

		I	II	III	Sum
arbor	Hevea brasiliensis Muell.Arg.	1	3	2	6
dicotyledon	Ricinus communis L.	1	3	2	6
herb	*Gossypium hirsutum* L.	2	8	10	20
	Solanum tuberosun L.	5	2	1	8
	Arabidopsis thaliana	5	2	3	10
monocotyledon	Zea mays L.	5	2	3	10
	Oryza sativa L.	5	2	3	10
pteridophyte	Moellendorf"sSpikemossHerb	0	0	6	6
non-seed bryophyte	Physcomitrella patens	0	0	4	4

Figure 1. Phylogenetic relationships between the SNF1-related protein kinase (SnRK2) families from *Arabidopsis*, maize, rice and rubber trees

(A) Molecular phylogenetic analysis using the Maximum Likelihood method in MEGA5 for 39 SnRK2 amino acid sequences from *Arabidopsis*, maize, rice and rubber trees. (B) Distribution of the *SnRK2* genes in the different plant species

The members of the HbSnRK2s family are marked in red. Three groups are separated by black lines. Bootstrapping (1 000 replicates) was used in the phylogenetic tree

Search for *cis*-elements involved in the osmotic stress regulation of *HbSnRK2* genes

Cis-elements are the binding site of the transcription factors involved in the gene regulation. In our study, a 1 kb upstream genome sequence region of six *HbSnRK2* genes were identified. According to information within the PLACE database (Figure 4), 13 *cis*-acting regulatory elements related to abiotic stress and phytohormone responsiveness were identified. All the *HbSnRK2* genes had different *cis*-elements at different quantities and positions. *HbSnRK2.4* had the largest number of *cis*-elements, and only *HbSnRK2.6* had a MYB (drought-inducible) element. Five genes (excluding *HbSnRK2.5*) had an ABA-responsive element, and *HbSnRK2.3* had the maximum *cis*-elements to response to ABA. In addition to *HbSnRK2.6*, the *HbSnRK2* members had a heat stress responsiveness element. All *HbSnRK2* except for *HbSnRK2.1* had ARE to the response anaerobic induction. *HbSnRK2.3* and *HbSnRK2.5* had an ERF element to response to ethylene. *HbSnRK2.4* and *HbSnRK2.6* had no *cis*-element to response to auxin but had it to the TCA-element.

Expression pattern of the *HbSnRK2* genes in the different tissues

To explore the steady-state expression patterns of the *HbSnRK2* genes in different tissues, a qRT-PCR analysis was used to determine the transcript levels in six different tissues of the rubber tree (root, stem, bark, leaf, latex, and flower). This

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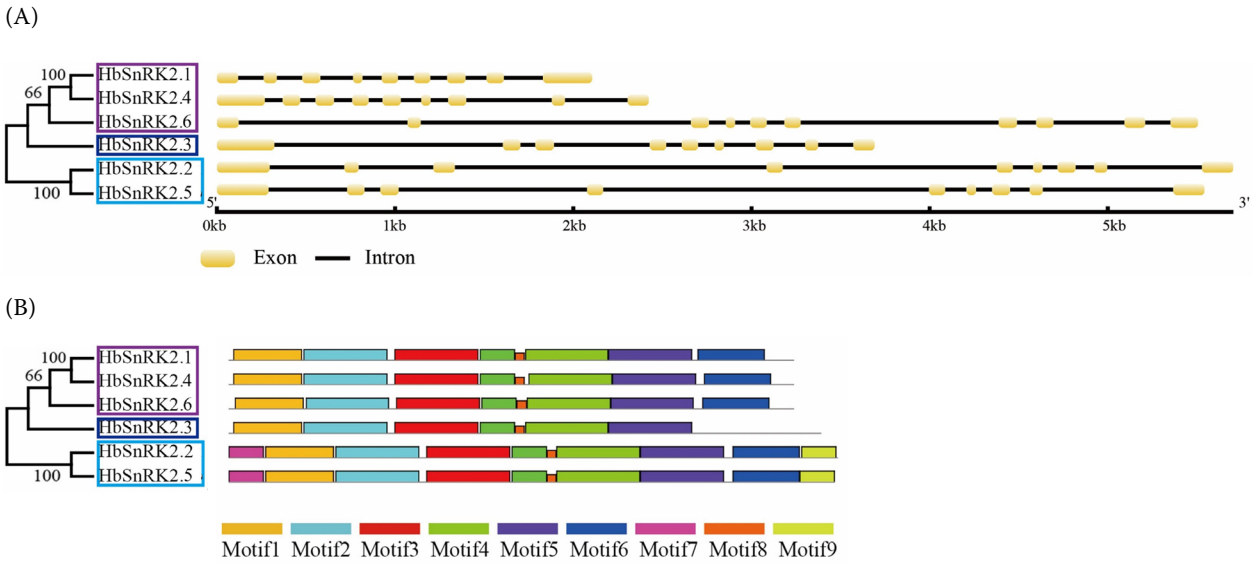

Figure 2. Intron/exon structures and motifs of the *SnRK* family in the rubber trees

(A) The phylogenetic tree was constructed from a complete alignment of six HbSnRK amino acid sequences by the maximum likelihood method. Gene Structure Display Server was used to output the gene structure. The yellow box and black line represent the coding sequence and intron, respectively. Gene models are drawn to scale as indicated at the bottom. (B) Motif analysis of SnRK in the rubber trees. Conserved motifs in the HbSnRK family were identified by MEME, the colour box represents the different conserved motifs

result suggested that the expression of six *HbSnRK2* genes varied among the different tissues (Figure 5). All the *HbSnRK2* genes were weakly expressed in the latex, and *HbSnRK2.3* and *HbSnRK2.6* did not detect the expression as well. The relative expression of *HbSnRK2.2* was highest in the *HbSnRK2* gene family that might be related to the formation and secretion of latex. Only *HbSnRK2.3* and *HbSnRK2.5* expressed it in the stem. The expression level of *HbSnRK2.3* and *HbSnRK2.5* was very low in the leaf. *HbSnRK2.1* and *HbSnRK2.3* were not expressed or weakly expressed in the bark. The *HbSnRK2* gene was relatively high in both the root and leaf tissues. While *HbSnRK2.3* was primarily expressed in the flowers. *HbSnRK2.1* was expressed in the root and flower; *HbSnRK2.2* was expressed in the root, leaf, bark, latex and flower; *HbSnRK2.3* was expressed in five organs, just not in the latex; *HbSnRK2.4* was expressed in root, bark and flower; *HbSnRK2.5* was expressed in the root, stem, leaf and flower; *HbSnRK2.6* was expressed in the leaf, bark and flower. According to this result, we can know that the expression pattern of all the *HbSnRK2* genes was very low in the latex. Individual *HbSnRK2s* exhibited different and overlapping patterns of expression.

Expression profiles of the *HbSnRK2* genes response to the phytohormone treatments

HbSnRK2 plays an essential role in the ABA, ETH and JA hormone signalling pathways. To explore the relationship between the *HbSnRK2* genes and the phytohormone stimulation, a qRT-PCR analysis was performed on the leaves of the rubber tree seedlings grown in histoculture under different phytohormone treatments. The *HbSnRK2* genes showed different expression levels under the ABA treatment, especially the expression level of the *HbSnRK2.2* gene belonging to the third group was significantly increased at 6 h, indicating that the *HbSnRK2.2* gene was sensitive to the ABA treatment (Figure 6A). So, we selected the *HbSnRK2.2* gene for the next step in our study. The expression of the *HbSnRK2.2* gene showed a rising trend under the ETH treatment, followed by a decreasing trend, with the highest expression at 6 h (Figure 6B). The expression of the *HbSnRK2.2* gene under the JA treatment reached its highest expression at 10 h (Figure 6C). We speculate that, at 6 h, the *HbSnRK2.2* gene is regulated by the synergistic action of the ABA and ETH signalling pathways, whereas it is influenced by the interaction of ABA and JA signalling pathways at 10 hours. This indicates that the

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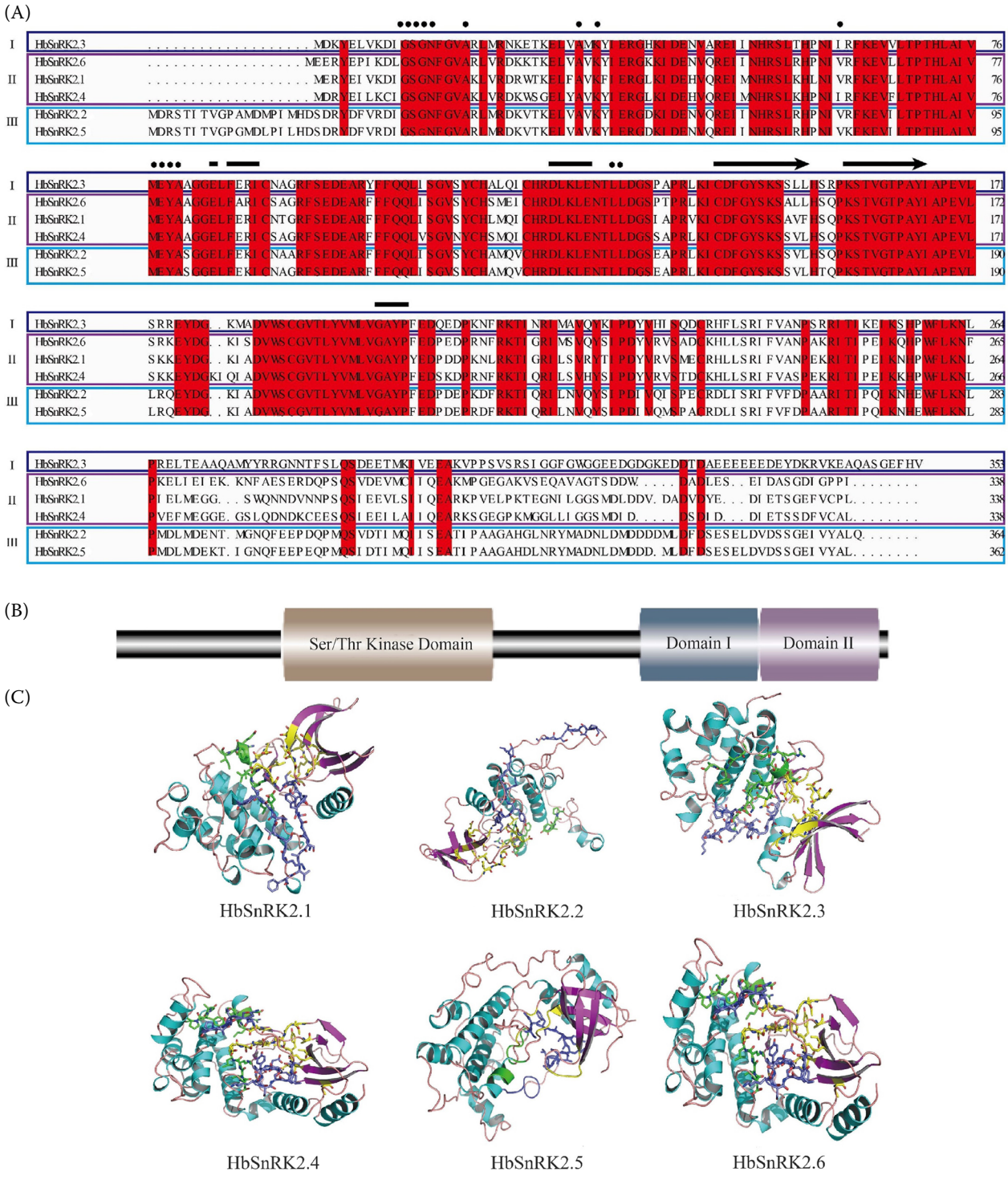

Figure 3. Protein structures of the HbSnRK2 family members

(A) Structure alignment of the HbSnRK2 proteins. Active sites (including the ATP-binding sites and substrate-binding sites) are shown by dots, polypeptide-binding sites are shown by lines, and activation loops are displayed as lines with arrows. (B) Model of the SnRK2 protein structure, including the Ser/Thr kinase domain. Domain I, which is required for osmotic stress responses, and domain II, which is required for ABA responses. (C) Predicted tertiary structure of the HbSnRK2 proteins. The α-helices are blue, and the β-sheets are rose red

The yellow colour represents the ATP-binding site, the green colour represents the polypeptide-binding site, and the dark blue colour represents Ser/Thr sites (A-loop)

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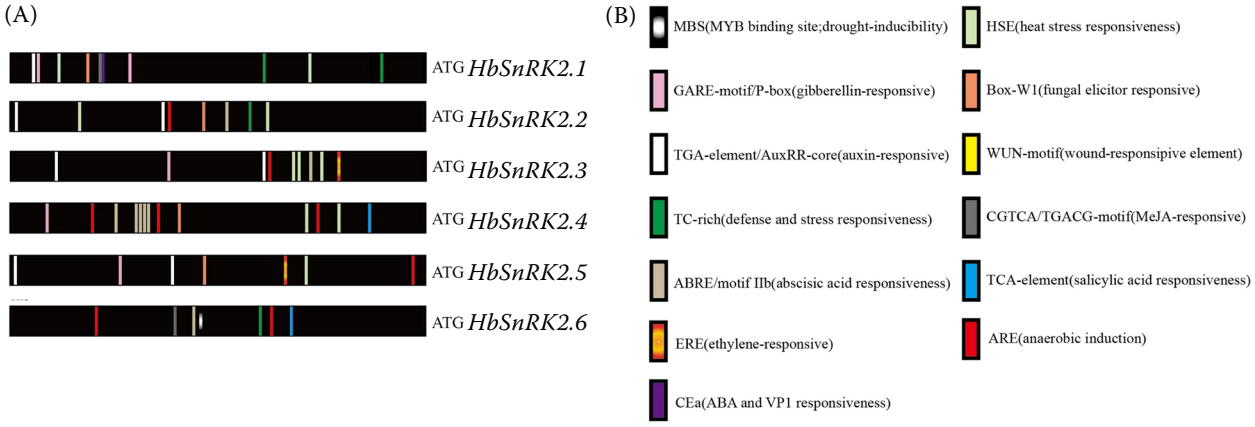

Figure 4. *Cis*-acting regulatory elements within the *HbSnRK2* promoter
(A) Distribution of the *cis*-elements in the promoter sequences of *HbSnRKs*. (B) the boxes with different colours represent the different *cis*-elements

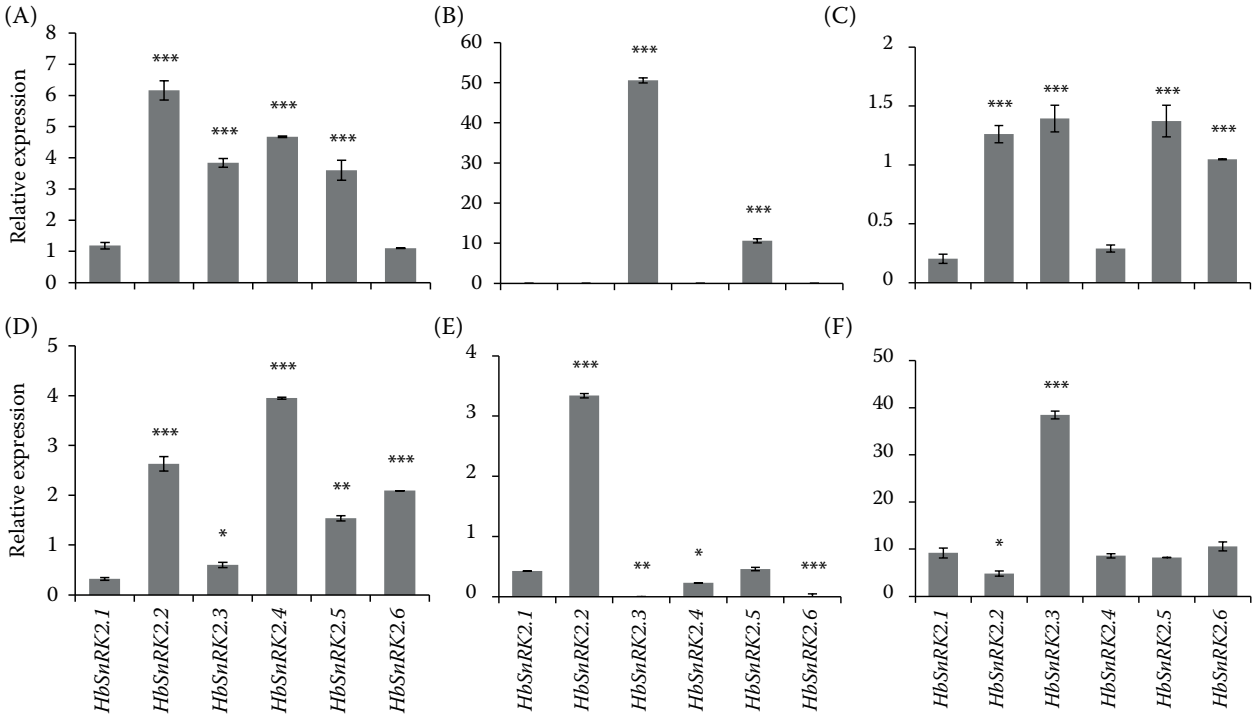

Figure 5. qRT-PCR analysis of the tissue specific expression patterns of the *SnRK* genes in the rubber trees
The total RNA was isolated from the roots (A), stems (B), leave (C), barks (D), latices (E) and flowers (F)
The *x*-axes indicate the different *HbSnRKs*, the *y*-axes indicate the expression fold change. Tissue-specific expression analyses were performed using *HbActin* as an internal control. The error bars represent the means ± standard errors of three replicates
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

HbSnRK2.2 gene is closely related to the hormone signalling.

Expression characteristics of the *HbSnRK2.2* genes in response to the abiotic and biotic stress

We explored the expression of *HbSnRK2.2* after a powdery mildew infection and the glyphosate, heat (40 °C) as well as cold (10 °C) treatments (Figure 7). We found that the *HbSnRK2.2* genes were significantly differentially expressed in response to the different treatments. The *HbSnRK2.2* genes had a response to all the treatments. *HbSnRK2.2* was significantly expressed under the glyphosate treatment, reaching the highest expression at 6 h (Figure 7A).

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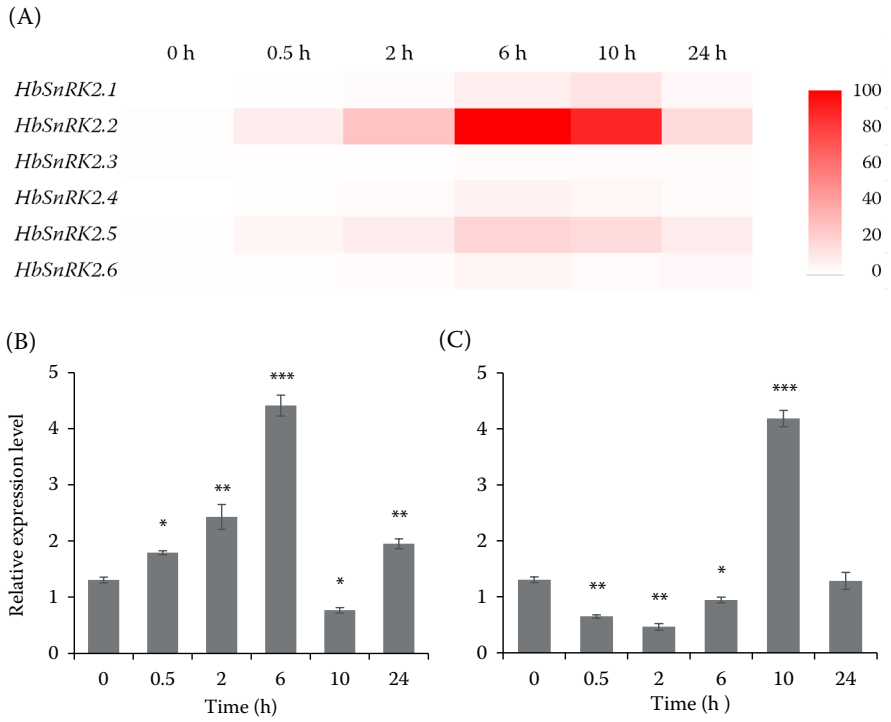

Figure 6. Relative expression levels of *HbSnRK2s* from the leaf under the phytohormone treatments

(A, B and C) The expression patterns in the leaf under the abscisic acid, ethephon and jasmonic acid treatment, respectively. The gene expression analysis was executed by qRT-PCR 24 h for each treatment and 0 h for the control sample. The values represent the experiment among three independent biological repetitions. The bars represent the SD

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

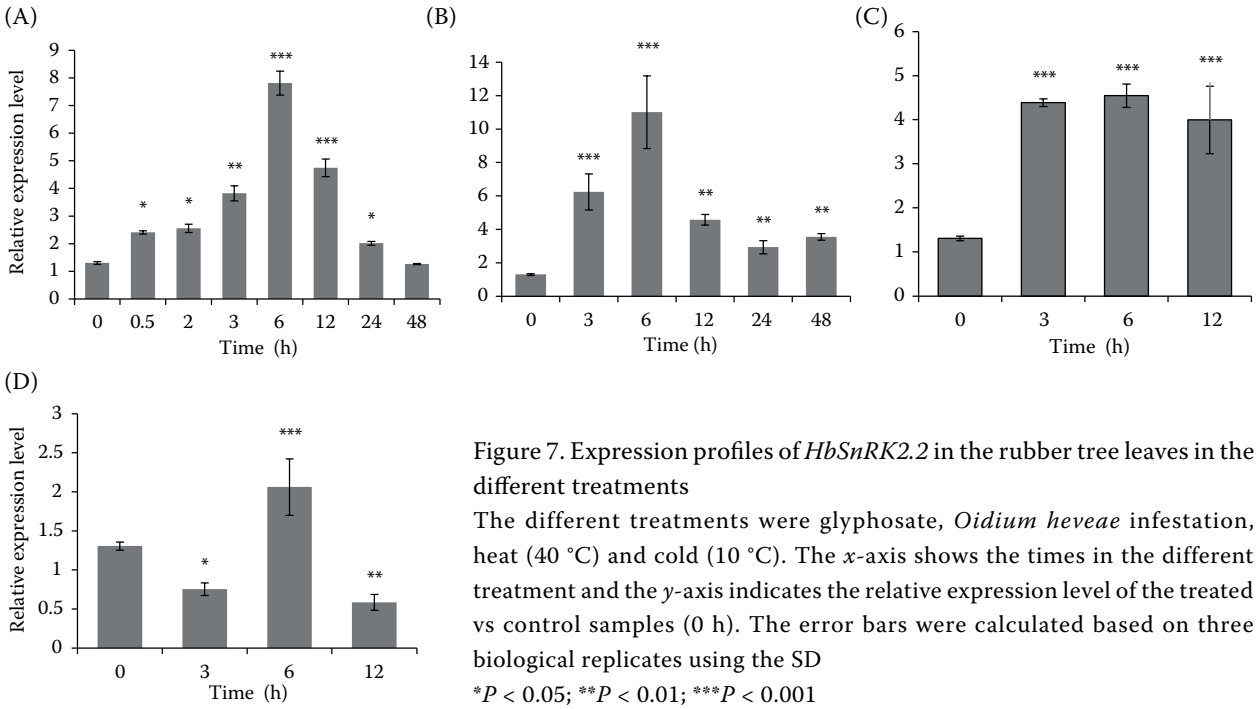

Figure 7. Expression profiles of *HbSnRK2.2* in the rubber tree leaves in the different treatments

The different treatments were glyphosate, *Oidium heveae* infestation, heat (40 °C) and cold (10 °C). The *x*-axis shows the times in the different treatment and the *y*-axis indicates the relative expression level of the treated vs control samples (0 h). The error bars were calculated based on three biological replicates using the SD

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

After the powdery mildew infection, the *HbSnRK2.2* gene expression increased and was higher after the early infection than the late infection (Figure 7B). The *HbSnRK2.2* gene expression was higher under the high temperature treatment than under the low temperature treatment (Figures 7C and 7D). Except for the low temperature treatments, the *HbSnRK2.2* gene expression increased under all the other treatments. The powdery mildew, glyphosate and high temperature treatments all showed an increase and then a decrease in the *HbSnRK2.2* gene expression at 6 h of treatment. Therefore, we thought 6 h was the threshold for the internal self-regulation of plants under adverse stress.

https://doi.org/10.17221/48/2022-PPS

DISCUSSION

SnRK2s plays a prominent role in the plant signalling system and in response to numerous biotic and abiotic stresses. SnRK2 originates from the duplication of SnRK1, then evolves rapidly and adapts to stressful environments throughout evolution (Halford & Hey 2009). SnRK2s existed before the divergence of angiosperms (Zhang et al. 2014), and most of the *SnRK2* genes maintain their original gene structure (the same as that in mosses) after expansion. Here, we cloned six *SnRK2* genes in rubber trees. Consistent with a previous classification (Halford & Grahame Hardie 1998), the *HbSnRK2* family could be divided into three groups (Figure 1A). A previous study inferred that the *SnRK2* genes of group I and group II originated from group III (Umezawa et al. 2009; Huang et al. 2015). Based on these theories, we proposed that the expansion of group III played an important role in the evolution of the *SnRK2* gene family. The differences in the expression patterns could be caused by the differences in the gene structure, activation or binding substrate specificity. Compared with the *SnRK2* genes in the other plant species, the *HbSnRK2* genes had many common structural characteristics. Similar with the present study, most *AtSnRK2*s have eight introns, except for *AtSnRK2.6* (nine introns) and *AtSnRK2.8* (five introns). The *HbSnRK2* genes in the same group had the same structure, five *HbSnRK2* genes had eight introns except for *HbSnRK2.6,* which had nine introns (Figure 2A).

Motifs play an essential role in the protein structure; transcription factors bind to motifs to regulate the gene expression. Motifs 1–4 were present in the N-terminal regions of all the HbSnRK2 proteins, which suggested that they might be part of the protein kinase domain. Unlike those at the N-terminus, the motifs in the C-terminus of HbSnRK2 presented intergroup diversity and intragroup similarity.

We evaluated the effects of the *cis*-elements on their gene expression levels (Figure 4). The expression of the *HbSnRK* genes changes by the different inductions via the *cis*-elements. For instance, ABRE binding factors were ABA-response elements. However, according to our study, not all the *HbSnRK* members were induced by stress even it contained *cis*-elements to respond to the corresponding stress. The amino acid sequence determines the functional activity and tertiary structure. The protein structure is closely related to the kinase function. HbSnRK2s in the same group had a similar protein structure which might have a similar potential function.

The environment restricts the growth of the rubber tree and decreases the latex yield, and adverse conditions cause considerable losses for the rubber economy. Phytohormones mobilise a series of genes that protect plants from the ensuing oxidative damage caused by prolonged stress (Wasilewska et al. 2008). As key regulators of the ABA responses, SnRK2s is involved in many processes that resist environmental pressures in plants (Wang et al. 2015). A recent study conclude that ABA does not regulate all stress-induced *SnRK2s*, indicating that the activation of *SnRK2s* in response to stresses and phytohormones is controlled by different mechanisms (Coello et al. 2012). In our study, the expression of *HbSnRK2.2*, which belong to group II, was markedly upregulated from 0.5 h to 24 h in response to the ABA treatment, especially at 6 h (Figure 6A). We suggest that the overexpression of *HbSnRK2* persisted after the regulation of other decisive genes in response to ABA, in combination with the results of another study (Zhang et al. 2011). Moreover, ETH and JA induced the expression of several *HbSnRK2.2* genes (Figures 6B and 6C) suggesting that the *HbSnRK2.2* gene may be involved in the crosstalk of the ABA, JA and ET signalling pathways. This contributes to the biosynthesis and regulation of natural rubber (Peng et al. 2009; Zhu & Zhang 2009; Pirrello et al. 2014). The interaction of the ABA, JA and ETH signalling pathways might be beneficial to plant responses to abiotic and biotic stresses (Lackman et al. 2011; Ahmad et al. 2016; Aleman et al. 2016).

SnRK2s plays an essential role in regulating the gene expression in response to abiotic and biotic stresses (Wu et al. 2017). It has been shown that *SnRK2s* is a major regulator of the response to cold, NaCl and other abiotic stresses (Shao et al. 2014; Liu et al. 2017). The gene expression profile could provide vital information to explore the function of genes. Our research showed that the *HbSnRK2*.2 gene is activated in response to plant stresses to varying degrees. Our study showed that the *HbSnRK2.2* gene was significantly induced by glyphosate at 6 h (Figure 7A). Glyphosate destroys plants by inhibiting the shikimic acid pathway. Furthermore, the shikimic acid pathway and the ABA synthesis

https://doi.org/10.17221/48/2022-PPS

pathway are important in the production of latex. Hence, we speculated that there is competition or that there are other relationships between these two pathways. Our results show that the *HbSnRK2.2* gene expression rises and then decreases with the heat (Figure 7C), while under cold conditions (Figure 7D), the *HbSnRK2.2* gene expression shows a down-regulation trend. Contrary to our results, the expression of *ZmSnRK2.3*, *ZmSnRK2.7* and *ZmSnRK2.11* was strongly induced under cold treatment, and the expression of most *ZmSnRK2*s was shown to be inhibited under heat treatment (Huai et al. 2008). Powdery mildew is an important leaf disease of rubber trees. The expression of *HbSnRK2*.2 significantly changed after the powdery mildew infection (Figure 7B). This result indicated that *HbSnRK2*.2 may play a role in the resistance of powdery mildew. Taken together, the results suggest that *HbSnRK2.2* may have an important role in the regulation of the rubber tree biosynthesis and plant disease resistance. Therefore, to clarify the role of *HbSnRK2.2* in the rubber synthesis and the relationship between various signalling pathways, this will be the focus of the next research.

CONCLUSION

Six *HbSnRK2* genes were cloned and identified in rubber trees. Based to the structural features of the proteins determined through the phylogenetic analysis, the *HbSnRK2* genes were divided into three groups: group I (*HbSnRK2.3*), group II (*HbSnRK2.1*, *HbSnRK2.4* and *HbSnRK2.6*) and group III (*HbSnRK2.2* and *HbSnRK2.5*). The gene structures and motifs of the six *HbSnRK2* genes were similar to those in other plant species. The qRT-PCR analysis revealed different expression profiles under different treatments (stresses and phytohormones). This study provides essential information for the future functional characterisation of *HbSnRK2* and affords the basis for the further characterisation of the resistant functions of *HbSnRK2s*.

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Received: April 18, 2022
Accepted: October 5, 2022
Published online: January 25, 2023