Genome-Wide Identification and Characterization of Heat-Shock Transcription Factors in Rubber Tree

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Abstract: Heat-shock transcription factors (Hsfs) play a pivotal role in the response of plants to various stresses. The present study aimed to characterize the Hsf genes in the rubber tree, a primary global source of natural rubber. In this study, 30 Hsf genes were identified in the rubber tree using genome-wide analysis. They possessed a structurally conserved DNA-binding domain and an oligomerization domain. On the basis of the length of the insert region between HR-A and HR-B in the oligomerization domain, the 30 members were clustered into three classes, Classes A (18), B (10), and C (2). Members within the same class shared highly conserved gene structures and protein motifs. The background expression levels of 11 genes in cold-tolerant rubber-tree clone 93-14 were significantly higher than those in cold-sensitive rubber-tree clone Reken501, while four genes exhibited inverse expression patterns. Upon cold stress, 20 genes were significantly upregulated in 93-114. Of the upregulated genes, HbHsfA2b, HbHsfA3a, and HbHsfA7a were also significantly upregulated in three other cold-tolerant rubber-tree clones at one or more time intervals upon cold stress. Their nuclear localization was verified, and the protein–protein interaction network was predicted. This study provides a basis for dissecting Hsf function in the enhanced cold tolerance of the rubber tree.

Keywords: Hevea brasiliensis Muell. Arg.; heat-shock transcription factor; cold stress; cold-sensitive rubber-tree clones; cold-tolerant rubber-tree clones

1. Introduction

Heat-shock transcription factors (Hsfs) allow for abiotic-stress signal transduction via the transcriptional activation of stress-related genes [1]. The highly conserved DNA-binding domain (DBD), which is responsible for activating the expression of heat-shock protein (HSP) genes by binding to heat-shock elements (HSEs), is located in the N-terminal of all Hsfs. It is followed by an oligomerization domain (OD) that is composed of two hydrophobic heptad repeats (HR-A and HR-B) and connected to the DBD by a flexible linker of variable length (15–80 amino acid residues). On the basis of peculiarities of the HR-A/B region and the phylogenetic relationship, plant Hsfs are divided into three main classes: A, B, and C [1–3]. In addition, certain Hsfs possess a nuclear localization signal (NLS), nuclear export signal (NES), and C-terminal activation (AHA) domain [2,4].
By activating their target stress-related genes, Hsfs play an important role in regulating plant adaption to various abiotic stresses [1]. Genome-wide identification of Hsfs has been performed in plants such as Arabidopsis [5], rice [6,7], apple [8], soybean [9], wheat [10], Chinese cabbage [11], strawberry [12], banana [13], rape [14], and plum [15]. These works provide a basis for integrating Hsf function into the regulation of plant growth and development by adaption to avoidable abiotic stresses.

The rubber tree (Hevea brasiliensis Muell. Arg.) is a primary global source of natural rubber (NR). As a species indigenous to the Amazon Basin in South America, cold stress adversely affects its survival and growth. To meet the demand for NR, rubber-tree planting has been continuously expanded and is no longer limited to areas without cold stress. For example, rubber-tree plantations in China frequently suffer from sudden cold waves. Decades of selective breeding have resulted in a gradual improvement in the cold tolerance of rubber trees. Several cold-tolerant rubber-tree clones, such as 93-114, Zhanshi327-13, GT1, and Guiyan73-165, have been selected. Hsfs may be involved in enhanced cold tolerance. However, information regarding Hsfs is limited [16,17] despite four versions of the rubber-tree genome having been published [18–21].

In the present study, 30 HbHsf genes were identified from the rubber-tree genome published by Tang et al. [21] and isolated from rubber-tree clone 93-114. We then analyzed evolutionary relationships, gene structure, and protein domains, and performed expression analysis using real-time quantitative RT-PCR in cold-tolerant rubber-tree clone 93-114 and cold-sensitive rubber-tree clone Reken501. The expression of several Hsfs with differential expression between 93-114 and Reken501 was further analyzed in three other cold-tolerant and cold-sensitive rubber-tree clones. As a result, three HbHsfs were suggested to be involved in the enhanced cold tolerance of rubber-tree clones. Their nuclear localization was verified, and the protein–protein interaction network was predicted. The present work provides a basis for dissecting Hsf function in the enhanced cold tolerance of rubber tree.

2. Materials and Methods

2.1. Plant Materials and Growth Condition

Plantlets of 4 cold-tolerant rubber-tree clones (93-114, Zhanshi327-13, GT1, and Guiyan73-165) and 4 cold-sensitive rubber-tree clones (Reken501, Haiken1, Reken514, and Reken515) budded on rootstocks were grown on the Experimental Farm of the Chinese Academy of Tropical Agricultural Sciences (CATAS) on Hainan Island. When plantlets had developed 1 extension unit and leaves were completely matured in the field, they were transferred into a growth chamber (PGR15, CONVIRON, Canada) with light for 16 h at 125 µmol·m⁻²·s⁻¹, temperature of 28 °C, and relative humidity of 80% for 2 days. Thereafter, plantlets were transferred into another chamber under the same conditions at 4 °C instead of 28 °C.

2.2. Stress Treatments

To evaluate stress intensity, plantlets of cold-tolerant rubber-tree clone 93-114 and cold-sensitive rubber-tree clone Reken501 were treated for 2 days at 4 °C, and then recultured for 5 days at 28 °C. The length of withered stems was surveyed once a day. The extent of cold tolerance was represented by the ratio of withered length to the total length of the stem (%). Each variety included 5 plantlets.

The same stress intensity as above was performed for gene-expression analysis in 3 cold-tolerant rubber-tree clones and 3 cold-sensitive rubber-tree clones, in addition to 93-114 and Reken501. Each clone included 120 plantlets and remained at 28 °C for 2 days. Thereafter, plantlets were divided into 2 groups. Each group included 60 plantlets. One group was treated at 4 °C, while the other remained at 28 °C as a control. Bark samples were collected at 0, 4, 8, and 24 h. At each time interval, bark-tissue samples were collected from the stem of 15 plantlets. Bark tissue from every 5 plantlets was mixed as 1 sample. This was done in triplicate.
2.3. Real-Time Quantitative PCR Analysis

Total RNA was extracted using a plant RNA extraction kit (TIANGEN, Beijing, China). Approximately 1 µg of RNA was used for reverse transcription, and cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific Inc., Waltham, MA, USA). Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was used to design gene-specific primers for cloning the full-length cDNA of each HbHsf gene (Supplementary Table S1). qRT-PCR experiments were carried out in triplicate with the CFX384 real-time PCR system (Bio-Rad Laboratories, Inc., v. Bio-Oxford, USA) using an SYBR Prime Script RT-PCR Kit (TaKaRa, Dalian). Relative gene expression was determined using Actin7a as an endogenous control gene [22]. Relative expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method. Primers for qRT-PCR are listed in Supplementary Table S2.

2.4. Bioinformatics Analysis

Hsf genes were predicted from the whole genome sequence of *Hevea brasiliensis* ([https://www.ncbi.nlm.nih.gov/genome/?term=rubber%20tree%20genome](https://www.ncbi.nlm.nih.gov/genome/?term=rubber%20tree%20genome)) [21]. All the nucleotide and amino acid sequences of predicted HbHsf genes were used as queries to perform BLAST searches in the public NCBI database ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). If 2 or more protein sequences at the same gene locus overlapped, only the longest sequence was used. Multiple sequence alignment of Hsf proteins from the rubber tree was carried out using the DNAMAN program (Lynnon Biosoft, Vaudreuil, QC, Canada). Full-length amino acid sequences of Hsf proteins from the rubber tree (HbHsf), *Arabidopsis* (AtHsf), and *Populus trichocarpa* (PtHsf) were used to generate a phylogenetic tree through MEGA5.10 [12]. Firstly, the alignment was built and aligned by ClustalW, then analyzed using the unrooted neighbor-joining method with pairwise deletion. Bootstrap value analysis was performed with 1000 replications to assess tree reliability. Deduced amino acid sequences were analyzed using the Compute pI/MW Tool ([http://www.expasy.org/tools/pi_tool.html](http://www.expasy.org/tools/pi_tool.html)) for computation of the theoretical isoelectric point and protein molecular weight. Exon–intron structures of HbHsf genes were visualized using online program GSDS 2.0 ([http://gsds.cbi.pku.edu.cn/](http://gsds.cbi.pku.edu.cn/)), which aligned the respective coding sequences with corresponding full-length sequences. The protein domains of the HbHsf proteins were identified using MEME online tools ([http://meme.nbcr.net/meme/](http://meme.nbcr.net/meme/)).

2.5. Subcellular-Localization Analysis

For subcellular-localization assays, the entire coding sequences of HbHsfA2b, HbHsfA3a, and HbHsfA7a with no stop codons were amplified with specific primers (Supplementary Table S3). HbHsfA2b, HbHsfA3a, and HbHsfA7a were fused with GFP under the control of the 35S promoter in the pCAMBIA1302 vector. HbHsfA2b::GFP, HbHsfA3a::GFP, and HbHsfA7a::GFP fusion constructs, and empty GFP vectors were introduced into living onion epidermal cells by *Agrobacterium* (GV3101)-mediated transformation [23]. The expression of fusion constructs was measured after 24 h incubation in the dark by a confocal laser scanning microscope (Zeiss LSM 800, Jena Germany). DAPI was used to stain the nucleus.

2.6. Predicted Protein–Protein Interaction-Network Construction

The predicted protein–protein interaction network of HbHsfA2b, HbHsfA3a, and HbHsfA7a was generated by STRINGV10.0 (University of Zurich, Zurich, Switzerland) online software, which visualizes known and predicted protein–protein interactions. Parameters were set as follows: Meaning of network edges—confidence (line thickness indicates strength of data support); active interaction sources—check all; minimum required interaction score—high confidence (0.700); and max number of interactors to show—no more than 10 interactors [24].
2.7. Statistical Analysis

Data were shown as the mean ± standard deviation and tested for statistical significance with a paired Student’s t-test. \( p < 0.05 \) was selected as the point of minimal statistical significance in all analysis.

3. Results

3.1. Isolation and Identification of Hsf Genes in Rubber Tree

A total of 30 Hsf genes were predicted in the sequenced genome of rubber-tree clone CATAS 7-33-97 through the BLAST search in NCBI. They contained apparently complete Hsf-type DNA-binding domains and oligomerization domains. Using predicted HbHsfs coding sequences, 30 homologous genes were isolated from rubber-tree clone 93-114. Identified HbHsfs were designated on the basis of names of their presumptive Arabidopsis and P. trichocarpa orthologs (Figures 1 and 2A). ORF sequences of isolated HbHsfs shared identity with the corresponding HbHsfs from rubber-tree clone CATAS 7-33-97. HbHsf genes contained one or two introns. Introns ranged from 66 (HbHsfB4d) to 5347 bp (HbHsfA1b) in length. HbHsfs ranged from 216 (HbHsfB2a) to 566 (HbHsfA3a) amino acid residues in length, and predicted isoelectric points (pI) varied from 4.73 (HbHsfA3a) to 9.36 (HbHsfB2a), and molecular weights (MW) from 24.83 (HbHsfB2a) to 63.73 kDa (HbHsfA3b) (Supplementary Table S4).

![Figure 1.](image)

Figure 1. (A) Gene organization of HbHsfs and (B) motifs identified by MEME tool. Ten motifs (1–10) were indicated by different colors.
which were characterized by the insertion length between HR-A and HR-B regions. In comparison with
while two genes (HbHsfA9b and HbHsfA9b) had two introns. Introns were inserted into the highly
conserved DNA-binding domain-coding regions, and most of the introns were phase-zero introns,
except for phase-one introns HbHsfA9b and HbHsfA2b (Figure 1A).

Ten motifs were detected in HbHsf proteins using MEME software (Figure 1B). Prediction by the
InterProscan database annotated Motifs 1, 2, and 5 as DBDs, Motif 3 as an HR-A/B domain, Motif 4 as
an HR-A/B motif, Motif 7 as an AHA motif, Motif 8 as an NES motif, and Motif 9 as an NES motif.
Motifs 6 and 10 were not annotated in the database. Motifs 1 and 2 were present in all of the HbHsfs
members. Motif 5 was found in most of the HbHsf members except for HbHsfC1a, HbHsfC1b, and
HbHsfB4b. Motif 4 was only present in HbHsfBs, while Motif 3 was absent from HbHsfBs except for
HbHsfB3a. Motif 7 was only found in eight HbHsfAs. Motif 8 was present in seven HbHsfAs, and
Motif 9 was absent from HbHsfBs except for HbHsfB4b.

Thirty HbHsfs, 21 Arabidopsis Hsfs (AtHsfs), and 28 P. trichocarpa Hsfs (PtHsf) were used to
construct a phylogenetic tree (Figure 2A). Thirty HbHsf proteins were grouped into different subgroups
of Arabidopsis Hsfs and P. trichocarpa Hsfs. All HbHsfs were divided into three classes (A, B, and C),
which were characterized by the insertion length between HR-A and HR-B regions. In comparison with
C, insertions in B and A contained seven and 21 amino acid residues, respectively (Figure 2B). Class
A included 18 HbHsf members, Class B had 10 members, and Class C only contained two members
(Figure 2).

3.2. Expression Patterns of HbHsfs in Cold-Tolerant and Cold-Sensitive Rubber-Tree Clones in Response to
Cold Stress

The difference in the cold-tolerance phenotype between cold-tolerant rubber-tree clone 93-114 and
cold-sensitive rubber-tree clone Reken501 was remarkable when plantlets were treated at 4 °C for two
days, and then recultured at 28 °C for five days. The leaves and stems of Reken501 began to shrivel after
being recultured for one day. Stems of most Reken501 plantlets had withered after being recultured
for five days, while stems of a few 93-114 plantlets exhibited withering (Figure 3A). The stem–wither
ratio was 82.77% for Reken501 and 6.06% for 93-114 (Figure 3B). By using this experimental system,
expression patterns of 30 HbHsfs in the two clones in response to cold stress were analyzed (Figure 4).
Background expression levels of 11 genes in the bark tissue of 93-114 were significantly higher than

![Figure 2. Unrooted phylogenetic tree of rubber tree, Arabidopsis, and Populus trichocarpa heat-shock transcription-factor (Hsf) family (A) and multiple sequence alignment of HR-A/B regions (oligomerization domain (OD)) of HbHsf proteins (B). Amino acid sequences of Hsf proteins were analyzed using the neighbor-joining method, with genetic distance calculated by MEGA5.10.](image-url)
those of Reken501, while the reverse was true for \textit{HbHsfA9a}, \textit{HbHsfA9b}, and \textit{HbHsfB2c}. Upon cold stress, 20 of 30 \textit{HbHsfs} were upregulated at 4 h and/or 8 h in 93-114. By contrast, only four genes were upregulated at corresponding time intervals in Reken501. Three genes—\textit{HbHsfA2b}, \textit{HbHsfA4a}, and \textit{HbHsfB4a}—were significantly upregulated in 93-114, while \textit{HbHsfA9b} was upregulated in Reken501 during cold stress. Expression patterns of \textit{HbHsfC1a} and \textit{HbHsfC1b} were similar between 93-114 and Reken501. The expression of all four \textit{HbHsfB4} members was higher in 93-114 than in Reken501.

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**Figure 3.** Evaluation of cold-stress intensity of rubber trees. (A) Phenotype and (B) statistical analysis of cold tolerance of rubber-tree clones Reken501 and 93-114.
**Figure 4.** Expression profiles of 30 HbHsf genes in stem bark of Reken501 and 93-114 in response to cold stress. **and *:** very significant difference (\( p < 0.01 \)) and significant difference (\( p < 0.05 \)), respectively, in expression levels between corresponding time intervals in Reken501 and 93-114.

HbHsfs that were upregulated in both 93-114 and Reken501, and those that were significantly upregulated at two or more time intervals upon cold stress in 93-114, were selected for further analysis of their expression patterns in three other cold-tolerant and cold-sensitive rubber-tree clones. The same experimental system was used. They included HbHsfA1b, HbHsfA2b, HbHsfA3a, HbHsfA4a, HbHsfA7a, and HbHsfB4a (Figure 5). Of the six genes, HbHsfA2b, HbHsfA3a, and HbHsfA7a were significantly
upregulated at one or more time intervals in all three cold-tolerant rubber-tree clones in comparison with the three cold-sensitive rubber-tree clones. Background expression of HbHsfA2b was significantly upregulated in the three cold-tolerant rubber-tree clones, but only in the most cold-tolerant rubber-tree clone (GT1) during cold stress (Figure 5B). Background expression of HbHsfA3a was significantly upregulated and then further upregulated during cold stress in cold-tolerant rubber-tree clones Zhanshi327-13 and GT1. It was significantly upregulated in Guiyan73-165 at 24 h during cold stress in comparison with the other cold-sensitive rubber-tree clones (Figure 5C). Background expression of HbHsfA7a was significantly upregulated in the three cold-tolerant rubber-tree clones. It was also upregulated during cold stress, except for at 4 h, where there was no difference between cold-tolerant rubber-tree clone Guiyan73-165 and the three cold-sensitive rubber-tree clones (Figure 5E).

Figure 5. Expression profiles of HbHsfA1b (A), HbHsfA2b (B), HbHsfA3a (C), HbHsfA4a (D), HbHsfA7a (E) and HbHsfB4a (F) genes in three cold-sensitive rubber-tree clones (Haiken1, Reken514, and Reken515), and three cold-tolerant rubber-tree clones (GT1, Zhanshi327-13, and Guiyan73-165) under cold treatment. ** and *: very significant difference (p < 0.01) and significant difference (p < 0.05), respectively.

3.3. Subcellular Localization and Predicted Protein–Protein Interactions of HbHsfA2b, HbHsfA3a, and HbHsfA7a

Subcellular localization of HbHsfA2b, HbHsfA3a, and HbHsfA7a was performed by transiently expressing vectors 35S::GFP, 35S::HbHsfA2b::GFP, 35S::HbHsfA3a::GFP, and 35S::HbHsfA7a::GFP in living onion epidermal cells. Confocal imaging showed that cells transformed with vector 35S::GFP displayed fluorescence throughout the cells, whereas the green fluorescence signal was exclusively detected in the nucleus of cells that were transformed with 35S::HbHsfA2b::GFP, 35S::HbHsfA3a::GFP, and 35S::HbHsfA7a::GFP (Figure 6A). The protein–protein interaction network of HbHsfA2b, HbHsfA3a, and HbHsfA7a was predicted. Ten proteins were involved in the interaction with the three HbHsfAs. They were DREB2A, ROF1, HSP101, HSP90.1, HSF1, MBF1C, HSP70, HSF3, HSP70b, and HSPB (Figure 6B).
4. Discussion

Plant Hsfs are terminal components of a signal-transduction chain mediating the expression of abiotic-stress-responsive genes [1]. The number of Hsf members is 18 in plums [15], 21 in Arabidopsis [5], 25 in rice [6], 25 in apples [8], and 32 in cassavas [25]; these plants are diplontic. The number of Hsf members increases in allopolyploids—there are 43 in triploid banana plants [13], 59 in tetraploid soybean plants [9], and 56 in sextaploid wheat plants [10]. On the basis of the version of the rubber-tree genome [21] and verification by RT-PCR, 30 HbHsf genes were isolated in the present study. The number of HbHsf genes falls in the range of diploid plants. Expansion of the Hsf gene family seems to be the result of the allopolyploid process. HbHsfs are structurally similar to Hsfs from other plants,
in that DBD contains an intron insertion, HR-A/B domains are highly homologous to those of the corresponding Class A Hsf members from *Arabidopsis* and *P. trichocarpa*, and the number of inserted amino acids between the HR-A and HR-B domains is the same as that in *Arabidopsis* [5], rape [14], and cotton [26]. This structural conservation suggests that the functions of Hsf in different plants are conserved.

During evolution, gene-family expansion is implemented through tandem duplication and segmental duplication. Segmental-duplication genes with different functions are on different chromosomes or scaffolds. Tandem-duplication genes that have similar functions are located on the same chromosome, and the distance between the two genes is relatively close [27,28]. In *P. trichocarpa*, only gene pair *PtHsfA6a/A6b* is from tandem duplication, while other clusters are from segmental duplications [29]. In peanuts, cluster *AiHsf7/8* is located on chromosome five, and the distance between the two genes is about 2 Kb. Gene pair *AiHsf7/8* is from tandem duplication [27]. In the rubber tree, due to the lack of a high-density genetic map, identification of paralogs that are derived from Whole Genome Duplication (WGD) or segmental duplication has been largely restricted. On the basis of identification of tandem-duplication events in the rubber tree by Zou et al. [28], only gene pair *HbHsfC1a/C1b* was located on same scaffold 0795, and the distance between the two genes was about 2 Kb. Other clusters, such as *HbHsfA1a/A1c*, *HbHsfA2a/A2b*, *HbHsfA3a/A3b*, *HbHsfA4a/A4b*, *HbHsfA5a/A5b*, *HbHsfA9a/A9b*, *HbHsfB3a/B3b*, and *HbHsfB4a/B4c* were located on different scaffolds (Table S4). Therefore, gene pair *HbHsfC1a/C1b* was from tandem duplication, whereas other gene pairs were from segmental duplications. Compared to tandem-duplicated gene pair *HbHsfC1a/C1b*, some segmentally duplicated gene pairs had differential expression patterns in response to cold and/or heat stress (Figure 4 and Figure S1), suggesting these duplicated genes display functional diversity.

Although the rubber tree is tropical, the cold tolerance of cultivars has been gradually improved by selective breeding. Among 30 *HbHsfs*, *HbHsfA2b*, *HbHsfA3a*, and *HbHsfA7a* were significantly upregulated at one or more time intervals in four cold-tolerant rubber-tree clones in comparison with four cold-sensitive rubber-tree clones (Figures 4 and 5). Their corresponding homologs *OsHsfA2* [6], *MeHsfA3a* [30], and *OsHsfA7* [7] are upregulated upon cold stress. *OsHsfA2* and *OsHsfA7* genes are also induced by high temperature. In *P. trichocarpa*, transcription levels of *PtHsfA2* and *PtHsfA7ab* are increased under high-temperature treatment [29]. In rubber-tree clone 93-114, 20 of 30 *HbHsfs* were upregulated under cold stress (Figure 4). Of the 20 genes, the expression levels of 14 *HbHsf* genes, including *HbHsfA2b*, *HbHsfA3a*, and *HbHsfA7a*, were also upregulated upon heat stress (42 °C; Figure S1).

Hsfs act as key components in regulating the enhanced production of reactive-oxygen-species (ROS) scavengers and HSPs that facilitate plant stress tolerance [1]. *OsHsfA2a/cf, AtHsfA2a*, and *OsHsfA7* are induced by oxidative stress [6,7,31]. *APX2*, a stress-related gene in *Arabidopsis*, is the target gene of *HsfA2* [2,32]. Ectopic expression of CHsfA2b in *Arabidopsis* enhances the transcriptional activity of *Atapx2* by directly binding to the HSE on the promoter of *Atapx2* [33]. *AtHsfA2* knockout lines and *HsfA2* overexpression lines indicate that *HsfA2* is one of the key regulators in protecting organelles against oxidative damage [32,34]. *HbHsfA2b, HbHsfA3a*, and *HbHsfA7a* may be involved in regulating the activity of ROS scavengers, because the activity of peroxidase, catalase, and superoxide dismutase in the leaves of cold-tolerant rubber-tree clone 93-114 was significantly higher than that in cold-sensitive rubber-tree clone Reken501 during cold stress [35]. In addition, available data showed that heat-shock proteins are associated with chilling tolerance in many plants and are transcriptionally regulated by Hsfs [36]. The interaction of Hsfs with other proteins is essential for activating their target stress-related genes, including HSPs and ROS scavengers [37]. Interactions of *HbHsfA2b, HbHsfA3a*, and *HbHsfA7a* with several HSPs, Hsfs, and DREB2A were predicted (Figure 6B). In *Arabidopsis*, activation of sHSP by HsfA2 depends on the formation of an ROF1–HSP90.1–HsfA2 complex by interactions [38]. The predicted protein–protein interaction network showed that *HbHsfA2b* could interact with ROF1 and HSP90.1 (Figure 6B). Moreover, some of the FvHsf proteins of *Fragaria vesca* were reported to localize to both the nucleus and cytosol, such as FvHsfA2a, FvHsfA3a, FvHsfA4a, FvHsfA5a,
FvHsfB2b, and FvHsfC1a [12]. While HbHsfA2b was only detected in the nucleus (Figure 6A), the expression of a number of sHSPs was significantly activated upon cold stress in cold-tolerant rubber-tree clone 93-114 in comparison with cold-sensitive rubber-tree clone Reken501 [16]. These data suggest that HbHsfA2b may be involved in activating some sHSPs in the rubber tree in a similar manner to HsfA2 in Arabidopsis. It would be worthwhile to elucidate the interaction between HbHsfA2b, HbHsfA3a, and HbHsfA7a, their interaction with proteins such as HSPs and DREBs, and the identity of their target genes.

5. Conclusions

A total of 30 HbHsf genes were identified in the rubber tree. They were structurally conserved and may function in a similar manner to corresponding members in other plants. Most of the HbHsfs were significantly upregulated in cold-tolerant rubber-tree clones upon cold stress. Specifically, the upregulation of HbHsfA2b, HbHsfA3a, and HbHsfA7a may contribute to the enhanced cold tolerance of the rubber tree. The present work provides a basis for dissecting HbHsf function in the enhanced cold tolerance of the rubber tree.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/12/1157/s1.
Table S1: Primer sequences for cloning full-length cDNA of HbHsf genes. Table S2: Primer sequences for quantitative real-time PCR analysis. Table S3: Primer sequences for the subcellular localization of HbHsfA2b, HbHsfA3a, and HbHsfA7a. Table S4: Summary information of HbHsf family genes in the rubber tree. Figure S1: Expression profiles of 30 rubber tree HbHsfs in the leaves of rubber tree clone 93-114 plantlets upon heat (42 °C) treatments. p < 0.05; * p < 0.01.

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References

1. Guo, M.; Liu, J.H.; Ma, X.; Luo, D.X.; Gong, Z.H.; Lu, M.H. The plant heat stress transcription factors (HSFs): Structure, regulation, and function in response to abiotic stresses. Front. Plant Sci. 2016, 7, 114. [CrossRef] [PubMed]
2. Scharf, K.D.; Berberich, T.; Ebersberger, I.; Nover, L. The plant heat stress transcription factor (Hsf) family: Structure, function and evolution. BBA Gene Regul. Mech. 2012, 1819, 104–119. [CrossRef] [PubMed]
3. Von Koskull-Döring, P.; Scharf, K.D.; Nover, L. The diversity of plant heat stress transcription factors. Trends Plant Sci. 2007, 12, 452–457. [CrossRef] [PubMed]
4. Wiederrecht, G.; Seto, D.; Parker, C.S. Isolation of the gene encoding the S. cerevisiae heat shock transcription factor. Cell 1988, 54, 841–853. [CrossRef]
5. Nover, L.; Bharti, K.; Döring, P.; Mishra, S.K.; Ganguli, A.; Scharf, K.D. Arabidopsis and the heat stress transcription factor world: How many heat stress transcription factors do we need? Cell Stress Chaperon 2001, 6, 177–189. [CrossRef]
6. Chauhan, H.; Khurana, N.; Agarwal, P.; Khurana, P. Heat shock factors in rice (Oryza sativa L.): Genome-wide expression analysis during reproductive development and abiotic stress. Mol. Genet. Genom. 2011, 286, 171–187. [CrossRef]
7. Mittal, D.; Chakrabarti, S.; Sarkar, A.; Singh, A.; Grover, A. Heat shock factor gene family in rice: Genomic organization and transcript expression profiling in response to high temperature, low temperature and oxidative stresses. Plant Physiol. Biochem. 2009, 47, 785–795. [CrossRef]
8. Giorno, F.; Guerriero, G.; Baric, S.; Mariani, C. Heat shock transcriptional factors in Malus domestica: Identification, classification and expression analysis. BMC Genom. 2012, 13, 639. [CrossRef] [PubMed]
9. Chung, E.; Kim, K.M.; Lee, J.H. Genome-wide analysis and molecular characterization of heat shock transcription factor family in Glycine max. J. Genet. Genom. 2013, 40, 127–135. [CrossRef]
10. Xue, G.P.; Sadat, S.; Drenth, J.; McIntyre, C.L. The heat shock factor family from *Triticum aestivum* in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. *J. Exp. Bot.* 2014, 65, 539–557. [CrossRef]

11. Huang, X.Y.; Tao, P.; Li, B.Y.; Wang, W.H.; Yue, Z.C.; Lei, J.L.; Zhong, X.M. Genome-wide identification, classification, and analysis of heat shock transcription factor family in Chinese cabbage (*Brassica rapa* *pekinensis*). *Genet. Mol. Res.* 2015, 14, 2189–2204. [CrossRef] [PubMed]

12. Hu, Y.; Han, Y.T.; Wei, W.; Li, Y.J.; Zhang, K.; Gao, Y.R.; Zhao, F.L.; Feng, J.Y. Identification, isolation, and expression analysis of heat shock transcription factors in the diploid woodland strawberry *Fragaria vesca*. *Front. Plant Sci.* 2015, 6, 736. [CrossRef] [PubMed]

13. Wei, Y.; Hu, W.; Xia, F.; Zeng, H.; Li, X.; Yan, Y.; He, C.; Shi, H. Heat shock transcription factors in banana: Genome-wide characterization and expression profile analysis during development and stress response. *Sci. Rep.* 2016, 6, 36864. [CrossRef] [PubMed]

14. Zhu, X.; Huang, C.; Zhang, L.; Liu, H.; Yu, J.; Hu, Z.; Hua, W. Systematic analysis of *Hsf* family genes in the *Brassica napus* genome reveals novel responses to heat, drought and high CO₂ stresses. *Front. Plant Sci.* 2017, 8, 1174. [CrossRef] [PubMed]

15. Wan, X.; Yang, J.; Guo, C.; Bao, M.; Zhang, J. Genome-wide identification and classification of the *Hsf* and *sHsp* gene families in *Prunus mume*, and transcriptional analysis under heat stress. *Peer J.* 2019, 7, e7312. [CrossRef] [PubMed]

16. Deng, D.; Wang, J.; Li, Y.; Wu, S.; Yang, S.; Chao, J.; Chen, Y.; Zhang, S.; Shi, M.; Tian, W. Comparative transcriptome analysis reveals phytohormone signalings, heat shock module and ROS scavenger mediate the cold-tolerance of rubber tree. *Sci. Rep.* 2018, 8, 4931. [CrossRef]

17. Deng, D.; Wang, J.X.; Wang, J.; Tian, W.M. Two *HbHsfA1* and *HbHsfB1* genes from the tropical woody plant rubber tree confer cold stress tolerance in *Saccharomyces cerevisiae*. *Braz. J. Bot.* 2018, 41, 711–724. [CrossRef]

18. Lau, N.S.; Makita, Y.; Kawashima, M.; Taylor, T.D.; Kondo, S.; Othman, A.S.; Shu-Chien, A.C.; Matsui, M. The rubber tree genome shows expansion of gene family associated with rubber biosynthesis. *Sci. Rep.* 2016, 6, 28594. [CrossRef] [PubMed]

19. Pootakham, W.; Sonthirod, C.; Naktang, C.; Ruang-Areerate, P.; Yoocha, T.; Sangsrakru, D.; Theerawattanasuk, K.; Rattanawong, R.; Lekawipat, N.; Theerawattanasuk, K.; Rattanawong, R.; Lekawipat, N.; Tangphatsornruang, S. De novo hybrid assembly of the rubber tree genome reveals evidence of paleotetraploidy in *Hevea* species. *Sci. Rep.* 2017, 7, 41457. [CrossRef]

20. Rahman, A.Y.; Usharraj, A.O.; Misra, B.B.; Forsslund, K.; Helling, D.; Huerta-Cepas, J.; Simonovic, M.; Roth, A.; Santos, A.; Tsafou, K.P.; et al. Draft genome sequence of the rubber tree *Hevea brasiliensis*. *BMC Genom.* 2013, 14, 75. [CrossRef]

21. Tang, C.; Yang, M.; Fang, Y.; Luo, Y.; Gao, S.; Xiao, X.; An, Z.; Zhou, B.; Zhang, B.; Tan, X.; et al. The rubber tree genome reveals new insights into rubber production and species adaptation. *Nat. Plants* 2016, 2, 16073. [CrossRef] [PubMed]

22. Li, H.; Qin, Y.; Xiao, X.; Tang, C. Screening of valid reference genes for real-time RT-PCR data normalization in *Hevea brasiliensis* and expression validation of a sucrose transporter gene *HbSUT3*. *Plant Sci.* 2011, 181, 132–139. [CrossRef] [PubMed]

23. Xu, K.; Huang, X.; Wu, M.; Wang, Y.; Chang, Y.; Liu, K.; Zhang, J.; Zhang, Y.; Zhang, F.; Yi, L.; et al. A rapid, highly efficient and economical method of *Agrobacterium*-mediated in planta transient transformation in living onion epidermis. *PLoS ONE* 2014, 9, e83556. [CrossRef] [PubMed]

24. Szklarczyk, D.; Franceschini, A.; Wyder, S.; Forslund, K.; Helle, D.; Huerta-Cepas, J.; Simonovic, M.; Roth, A.; Santos, A.; Tsafou, K.P.; et al. STRING v10: Protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 2015, 43, D447–D452. [CrossRef] [PubMed]

25. Wei, Y.; Liu, G.; Chang, Y.; He, C.; Shi, H. Heat shock transcription factor 3 regulates plant immune response through modulation of salicylic acid accumulation and signaling in cassava. *Mol. Plant Pathol.* 2018, 19, 2209–2220. [CrossRef] [PubMed]

26. Wang, J.; Sun, N.; Deng, T.; Zhang, L.; Zuo, K. Genome-wide cloning, identification, classification and functional analysis of cotton heat shock transcription factors in cotton (*Gossypium hirsutum*). *BMC Genom.* 2014, 15, 961. [CrossRef]

27. Wang, P.; Song, H.; Li, C.; Li, P.; Li, A.; Guan, H.; Hou, L.; Wang, X. Genome-wide dissection of the heat shock transcription factor family genes in *Arachis*. *Front. Plant Sci.* 2017, 8, 106. [CrossRef]
28. Zou, Z.; Yang, L.; Gong, J.; Mo, Y.; Wang, J.; Cao, J.; An, F.; Xie, G. Genome-wide identification of *Jatropha curcas* aquaporin genes and the comparative analysis provides insights into the gene family expansion and evolution in *Hevea brasiliensis*. *Front. Plant Sci.* 2016, 7, 395. [CrossRef]

29. Zhang, J.; Liu, B.; Li, J.; Zhang, L.; Wang, Y.; Zheng, H.; Lu, M.; Chen, J. Hsf and Hsp gene families in *Populus*. Genome-wide identification, organization and correlated expression during development and in stress responses. *BMC Genom.* 2015, 16, 181. [CrossRef]

30. Yu, X.Y.; Yao, Y.; Hong, Y.H.; Hou, P.Y.; Li, C.X.; Xia, Z.Q.; Geng, M.T.; Chen, Y.H. Differential expression of the Hsf family in cassava under biotic and abiotic stresses. *Genome* 2019, 62, 563–569. [CrossRef]

31. Schramm, F.; Larkindale, J.; Kiehlmann, E.; Ganguli, A.; Englisch, G.; Vierling, E.; Von Koskull-Döring, P. A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of *Arabidopsis*. *Plant J.* 2008, 53, 264–274. [CrossRef] [PubMed]

32. Miller, G.; Mittler, R. Could heat shock transcription factors function as hydrogen peroxide sensors in plants? *Ann. Bot.* 2006, 98, 279–288. [CrossRef] [PubMed]

33. Wang, X.; Huang, W.; Yang, Z.; Liu, J.; Huang, B. Transcriptional regulation of heat shock proteins and ascorbate peroxidase by CtHsfA2b from African bermudagrass conferring heat tolerance in *Arabidopsis*. *Sci. Rep.* 2016, 6, 28021. [CrossRef] [PubMed]

34. Zhang, L.; Li, Y.; Xing, D.; Gao, C. Characterization of mitochondrial dynamics and subcellular localization of ROS reveal that HsfA2 alleviates oxidative damage caused by heat stress in *Arabidopsis*. *J. Exp. Bot.* 2009, 60, 2073–2091. [CrossRef]

35. Wang, J.X.; Li, Y.; Tian, W.M. Physiological responses of two rubber tree clones with differential cold-tolerant potential to cold stress. *J. Rubber Res.* 2016, 20, 117–129. [CrossRef]

36. Usman, M.G.; Rafii, M.Y.; Martini, M.Y.; Yusuff, O.A.; Ismail, M.R.; Miah, G. Molecular analysis of Hsp70 mechanisms in plants and their function in response to stress. *Biotechnol. Genet. Eng. Rev.* 2017, 33, 26–39. [CrossRef]

37. Jacob, P.; Hirt, H.; Bendahmane, A. The heat-shock protein/chaperone network and multiple stress resistance. *Plant Biotech.* J. 2017, 15, 405–414. [CrossRef]

38. Meiri, D.; Breiman, A. *Arabidopsis ROF1(FKBP62) modulates thermotolerance by interaction with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs.* *Plant J.* 2009, 59, 387–399. [CrossRef]

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