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Genome-Wide Analysis and Expression Profiling of the Heat Shock Factor Gene Family in *Phyllostachys edulis* during Development and in Response to Abiotic Stresses

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Abstract: Heat shock transcription factors (Hsfs) play crucial roles in regulating plant responses to heat and other stresses, as well as in plant development. As the largest monopodial bamboo species in the world, how to adapt to various stresses under the background of global climate change is very important for the sustainable development of bamboo forest. However, our understanding of the function of Hsfs in moso bamboo (*Phyllostachys edulis*) is limited. In this study, a total of 22 non-redundant Hsf genes were identified in the moso bamboo genome. Structural characteristics and phylogenetic analysis revealed that members of the PheHsf family can be clustered into three classes (A, B and C). Furthermore, PheHsfs promoters contained a number of stress-, hormone- and development-related cis-acting elements. Transcriptome analysis indicated that most *PheHsfs* participate in rapid shoot growth and flower development in moso bamboo. Moreover, the expression patterns of all 12 members of class A were analyzed under various stresses (heat, drought, salt and cold treatment) through Figurereal-time quantitative polymerase chain reaction (qRT-PCR). Within the class A *PheHsf* members, *PheHsfA1a* was expressed mainly during moso bamboo development. Expression of four *PheHsfA4s* and one *PheHsfA2* (*PheHsfA4a-1*, *PheHsfA4a-2*, *PheHsfA4d-1*, *PheHsfA4d-2*, and *PheHsfA2a-2*) was up-regulated in response to various stresses. *PheHsfA2a-2*, *PheHsfA4d-1* and *PheHsfA4d-2* were strongly induced respectively by heat, drought and NaCl stress. Through co-expression analysis we found that two hub genes *PheHsfA4a-2* and *PheHsfA4a-1* were involved in a complex protein interaction network. Based on the prediction of protein interaction networks, five *PheHsfAs* (*PheHsfA4a-1*, *PheHsfA4a-2*, *PheHsfA4d-1*, *PheHsfA4d-2*, and *PheHsfA2a-2*) were predicted to play an important role in flower and shoot development and abiotic stress response of moso bamboo. This study provides an overview of the complexity of the PheHsf gene family and a basis for analyzing the functions of PheHsf genes of interest.

Keywords: moso bamboo; heat shock factor gene; abiotic stresses; co-expression

1. Introduction

Moso bamboo (*Phyllostachys edulis* (Carrière) J. Houzeau, synonym *Phyllostachys heterocycla* (Carrière) is a large woody bamboo of high ecological, economical and cultural value in Asia. Under suitable spring conditions, its shoot can grow from 0 to 20 m in 45–60 days [1]. Moso bamboo forest covers an area of 3.87 million hm^2, accounting for up to 70% of the Chinese bamboo forest area [2,3]. Because of its rapid growth and highly lignified culms, the annual economic value of moso bamboo production, including

timber and wood production, reaches 184 billion dollars [4]. Moreover, carbon sequestration in moso bamboo is two to four times greater than that of Chinese fir, making it an important global non-timber forest resource [5]. The growth of bamboo is dependent on natural precipitation and is vulnerable to high temperature and drought. Liu et al. [6] has shown that temperatures >40 °C and drought for >10 days during August result in severe losses in moso bamboo forests. Drought during spring can reduce moso bamboo shoot growth, yield and quality. From July to September, high temperatures and drought affect the sprouting phase of bamboo. These stresses affect the yield and quality of winter shoots, as well as new bamboo yield in the following year and the yield of wood during subsequent years [7]. Climate change has also been associated with more frequent high temperatures and drought, which in turn reduce the ecological and economical value of moso bamboo. Therefore, it is essential to elucidate the molecular mechanisms involved in heat and drought stress responses to improve stress tolerance in moso bamboo.

To survive high temperature and other stresses, plants have evolved a series of defense strategies [8,9]. Heat shock proteins (HSPs) act as molecular chaperones that protect cells against heat and other stress damage by preventing protein aggregation [10,11]. As the terminal components of the stress signal transduction chain, heat shock stress transcription factors (Hsfs) bind to the promoter regions of HSP genes to regulate transcription in response to stress [12,13], particularly heat stress [14]. Hsfs also contain a highly conserved DNA-binding domain (DBD) at their N-terminal and an oligomerization domain (OD or HR-A/B region) composed of two hydrophobic heptad repeats. Based on the amino acids of the HR-A/B region, plant Hsfs are grouped into three main classes (HsfA, HsfB and HsfC) [15]. Certain Hsfs contain a nuclear localization signal (NLS), a nuclear export signal (NES) and an activator motif (AHA). The AHA motif located at the C-terminus in class A Hsfs confers transcriptional activation. Moreover, a repressor domain (RD) that contains the tetrapeptide LFGV occurs at the C-terminal of class B Hsfs.

Recent studies have revealed that plant Hsfs plays important roles in generating responses to heat and other stimuli, as well as in organ development [16]. Class A HsfA1a is regarded as a master regulator and has a unique role in eliciting heat stress responses in tomato (*Solanum lycopersicum* L.) [17]. HsfA2 is functionally similar to HsfA1 in regulating thermotolerance, as well as serving as a key regulator in osmotic and oxidative stress responses [18–20]. The expression of *HsfA3* is induced in *Arabidopsis* by heat and drought stress, indicating that *HsfA3* might play a role in drought and heat stress signaling [21,22]. The thermotolerance of *Arabidopsis* with *hsfA3* T-DNA insertion mutants was decreased [23]. Moreover, the ectopic overexpression of *SlHsfA3* increases thermotolerance and salt hypersensitivity in germination in transgenic *Arabidopsis* [24]. The Arabidopsis mutant *athsfa4a* was more sensitive to dehydration. Furthermore, desiccation tolerance was rescued in *athsfa4a/BnHSFA4a* seeds to similar levels compared with those of Col-0 [25]. Transgenic chrysanthemum overexpressing *CmHSFA4* displayed enhanced salinity tolerance partly due to enhanced Na^+/K^+ ion and ROS homeostasis [26]. Interestingly, in rice, wheat and *Sedum Alfredii*, HsfA4a possibly confers cadmium tolerance [27,28]. In addition, AtHsfA9 plays a role in embryonic development and seed maturation in *Arabidopsis* [29]. In group B, the majority of HsfBs act as repressors due to the RD region [15]. However, AtHsfB1 act as repressors of the heat shock response under non-heat-stress conditions, but act as positive regulators of heat shock proteins under heat-stress conditions [30]. In group C, *FaHsfC1b* from *Festuca arundinacea* confers heat tolerance in *Arabidopsis* [31].

Although several Hsf family genes in *Arabidopsis* and other plant species have been characterized, functional analysis of those in moso bamboo has been limited. The completion of the draft genome sequence of moso bamboo has greatly facilitated the identification of Hsf family at the whole-genome level [32].

In this study, we describe the genome-wide identification and analysis of the PheHsf family of moso bamboo for the first time. In addition, expression patterns of *PheHsf* genes during development, as well as in response to various abiotic stresses, were also investigated. Our results will provide a foundation and valuable information for future functional analysis of PheHsfs.

2. Materials and Methods

2.1. Database Searches for Hsf Genes in Moso Bamboo and Analyses of Physicochemical Characteristics

For more accurate identification of Hsf genes in Moso bamboo, multiple database searches were performed according Hou et al. [33]. First, Hsf mRNA sequences of *Oryza sativa* and *Brachypodium distachyon*, obtained from NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/) as query sequences to blast against the moso bamboo genome database. For the filtration step of the blast process, the Hsf genes were obtained by blast bamboo transcriptome using Hsf mRNA from some other species identified as query with loose *e*-value of <0.00001. Next, the protein sequences of putative genes obtained from the first step were blast against the NCBI non-redundant protein database with *e*-value of <0.0000000001 by Blast2GO to confirm the identification. The putative genes described as Hsf proteins or proteins belonging to Hsf family were kept, and genes described as other family proteins were abandoned. Then the HSF domain (PF00447) of the HSF family were researched in these putative Hsf proteins to reconfirm our data using the Pfam database (http://pfam.sanger.ac.uk/) and Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) with e-values <0.001. Only the gene models containing HSF domain were considered to belong to the Hsf family. Finally, the selected Hsfs were further screened using the full-length non-chimeric (FLNC) reads (http://www.forestrylab.org/db/PhePacBio/) [34]. The moso bamboo Hsf genome sequences, coding sequences, protein sequences, and putative novel or mis-annotated *Hsf* genes were obtained from moso bamboo genome database. The amino acid sequences of *Arabidopsis*, rice (*Oryza sativa*), and *B. distachyon* Hsf proteins were downloaded from Plant Transcription Factor Database v4.0 (PlantTFDB 4.0, http://planttfdb.cbi.pku.edu.cn/) and Heatster (http://www.cibiv.at/services/Hsf/) [35,36]. Molecular weights and theoretical isoelectric point (pI) were determined using ExPASY (http://web.expasy.org/compute_pi/). CELLO v2.5 Server (http://cello.life.nctu.edu.tw/) was used to predict the protein subcellular locations for candidate PheHsfs [37]

2.2. Phylogenetic Analysis

Arabidopsis and rice Hsf gene datasets [38] were used to classify the moso bamboo Hsf genes and predict their functional roles. Multiple sequence alignments of full-length Hsf proteins from Arabidopsis, rice, B. distachyon, and moso bamboo were performed using ClustalX 1.83 (http://www.clustal.org/) and two online programs (Clustal Omega and MUSCLE) [27,39–41]. An unrooted neighbor-joining (NJ) phylogenetic tree was constructed using MEGA7.0 with 1000 bootstrap replicates. Another phylogenetic tree with only the Moso bamboo Hsfs was also constructed using the amino acid sequences according to the same method.

2.3. Structural and Motif Analyses of PheHsf Genes

To confirm subgroup designation through phylogenetic analysis, the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) was used to illustrate exon-intron organization by aligning every cDNA sequence and its corresponding genomic DNA sequence [42]. The conserved motifs in the candidate PheHsf sequence were defined by MEME version 4.12.0 (http://meme-suite.org/tools/meme) [43] using the following parameters: number of repetitions = any, maximum number of motifs = 30, minimum width $\geq$4, maximum width $\leq$200, and only motifs with an e-value <0.01 were retained for further analysis. The DBD (DNA Binding domain) and HR-A/B domains were identified using Heatster (http://www.cibiv.at/services/Hsf/). NES domains in the PheHsfs were predicted with the NetNES 1.1 server software (http://www.cbs.dtu.dk/services/NetNES/). NLS domains were predicted using cNLS Mapper software (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). AHA domains were predicted according to the conserved motif sequence FWxxF/L, F/I/L [16].

2.4. Cis-Regulatory Element Analysis of PheHsf Genes

The 1500-bp sequence upstream from the initiation codon of each *PheHsf* gene was obtained from the moso bamboo genome database. These sequences were used to identify *cis*-acting regulatory elements with the online program Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.5. Plant Material

After the surface was sterilized with 1% formaldehyde, the moso bamboo seeds were germinated in Petri dishes (12-cm diameter) lined with filter paper and containing 10 mL of sterile water. After 4 days, the germinated seedlings were planted in vermiculite and watered with 1/2 Hoagland's nutrient medium weekly (all plants were grown in 16 h day/8 h night at 22 °C). Two-month-old seedlings were used for abiotic stress treatments. According to Cheng et al. [44], drought and stress was conducted by incubated seedlings with 20% (*m*/*v*) PEG6000 and 200 mM NaCl, respectively. Heat stress and low temperature treatments were respectively created by placing seedlings in a 42 °C and 4 °C lighted growth chamber according to Liu et al. [45]. The control seedlings were grown without any stress treatment. The second or third mature leaves were collected at 0 h, 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h after abiotic stress treatments. These materials were immediately frozen and stored in liquid nitrogen until total RNA extraction and real-time quantitative polymerase chain reaction (qRT-PCR).

2.6. RNA Isolation and Relative Expression Level Analysis of PheHsfs

For the tissue-specific expression analysis, the RPKM (the reads per kilobase of exon model per million mapped reads) values of *PheHsfs* were retrieved from transcriptome sequencing of developing flowers (four stages of flowering and leaves) and shoots of moso bamboo (winter shoots, six shoot heights, and culms) [1,46]. RPKM values were used to analyze the relative expression levels of the *PheHsf* genes. For the flower development samples, four developmental stages (F1: the floral buds begun to form; F2: the floral organs gradually matured; F3: the flowers were in full blossom; and F4: the embryo formation stage) were defined based on the anatomical structure of floral organs by Gao et al. [46]. For F1 and F2, the buds were collected, respectively. For F3 and F4, spikelets were collected, respectively. Leaves collected from non-flowering moso bamboo were defined as CK1. For the shoot development stage sample, four development stages of shoots were defined based on the continuing measurement of bamboo shoot height and anatomical changes by Li et al. [1]. The eight samples according to the four developmental stages of moso bamboo shoots were as follows: S1 (winter shoots), S2–S5 (0.5 m, 1 m, 3 m, and 6 m, early growth period), S6–S7 (9 m and 12 m, late growth), and CK (clum after leaf expansion, mature period). For S1–S7, the shoot tips of different heights were collected, respectively. For CK2, each top internode was cut from the top to 1/2, then each top internode was divided into basal, middle and top. After that, the samples were cut from the tissue located in the top part of the three internodes above and then mixed.

For qRT-PCR, *PheHsfs* primers were designed using Primer 3.0 (http://primer3.ut.ee/). Primer sequences, amplicon Length, amplification efficiency, and correlation coefficients are listed in Table S1, and their specificity was verified using the online tool Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) and the melting curves of PCR products. For every primer pair, a standard curve was constructed to calculate the gene-specific PCR efficiency from the 10-fold series dilution of the mix cDNA template. The R^2 (correlation coefficients) and slope values can be obtained from the standard curve. The following formula was used to calculate the corresponding PCR amplification efficiencies (E): $E = (10^{-1/\text{slope}} - 1) \times 100$ [47]. Tonoplast intrinsic protein 41 gene (*TIP41*) was used as an internal control [48]. The qRT-PCR reactions were conducted using a SYBR Green I master mix (Roche, Mannheim, Germany). The qRT-PCR conditions were as follows: 45 cycles

of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Three replicates were performed for each gene. Gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method [49].

2.7. Co-Expression Network and Protein Interactions of PheHsfs

The expression correlation of the PheHsfs was calculated by Pearson correlation coefficient (PCC; R-value) using gene expression RPKM values from the high-throughput transcriptome data in R. Expression correlation data were used for the correlation network, and co-expressed gene pairs were filtered with a PCC cut-off of 0.85 as previously described [50]. Cytoscape version 3.4.0 were used to analyze and visualize the network [51].

For the protein interaction networks, the homolog Hsf proteins in rice were constructed by STRING (http://stringdb.org/) using an option value >0.7. The homolog proteins of the determined interactive rice proteins were identified in moso bamboo by BLASTP analysis.

3. Results

3.1. The Hsf Family Genes in Moso Bamboo

To identify Hsf genes in moso bamboo, a total of 41 candidate PheHsf genes were retrieved from the annotation in the Bamboo Genome Database. From these, 19 candidate PheHsf genes with incomplete HSF domains were considered as PheHsf-like genes, which were not selected for subsequent analysis in this study. Twenty-two putative moso bamboo PheHsf genes containing full HSF domains (PF00447) were confirmed by searching the Pfam and the Conserved Domain Databases. However, the CDS sequence for PheHsfA5, PheHsfB4a-1 and PheHsfB4c-1 contained 42 bases (from 268 to 309 base), 102 bases (233 to 336) and 123 base (256 to 377) inserts, respectively, when compared to the cloned cDNA sequence (Fasta S3). For this analysis, the CDS and amino acid sequences of these three genes are based on the cDNA sequences. The CDS and amino acid sequences are listed in supplementary files (Fasta S1 and S2). The identified 22 PheHsf genes were distributed among 22 scaffolds (Table 1). The sequences of 22 PheHsf genes were named according to the corresponding relationship among in rice, *B. distachyon* and moso bamboo. The number of amino acid (aa) sequences of PheHsf proteins ranged from 247 (PheHsfC1b-1) to 679 (PheHsfA1a), the predicted isoelectric points (pI) varied between 4.70 (PheHsfA6a) and 9.81 (PheHsfB4c-1), and the molecular weight (MW) ranged from 26.77 kDa (PheHsfC1b-1) to 75.05 kDa (PheHsfA1a) (Table 1).

3.2. Phylogenetic Relationships and Multiple Sequence Alignment of PheHsf Genes

To predict the potential function, an unrooted phylogenetic tree was constructed from an alignment of 94 full length Hsf protein sequences from different species (23 AtHsf, 25 OsHsf, 24 BdHsf, and 22 PheHsf) (Figure 1). Because the alignment results of Hsf proteins in moso bamboo using ClustalX 1.83, MUSCLE, and Clustal Omega were similar, we employed the results of ClustalX 1.83 and MEGA7.0 to illustrate the phylogenetic relationships of the PheHsf family. The PheHsf genes were also grouped into three subgroups, including class A (PheHsfA1a, PheHsfA2a-1, PheHsfA2a-2, PheHsfA4a-1, PheHsfA4a-2, PheHsfA4d-1, PheHsfA4d-2, PheHsfA6b-2, PheHsfA6b-1, PheHsfA6a, PheHsfA5, and PheHsfA7a), class B (PheHsfB1, PheHsfB2a, PheHsfB2c, PheHsfB4c-1, PheHsfB4c-2, PheHsfB4a-2, and PheHsfB4a-1), and class C (PheHsfC1b-1, PheHsfC1b-2, and PheHsfC2b), which is similar to that described in Arabidopsis [52], rice [38], and *B. distachyon* [53]. In moso bamboo, class A was the largest and consisted of 12 members from six subclasses, however, three subclasses (A3, A8, and A9) in this class were not detected. There were seven members in class B from subclasses B1, B2, and B4, but no members in subclass B3. Class C was the smallest, with only three members.

Table 1. Overview of *PheHsf* genes in moso bamboo.

Name	Gene ID	Scaffolds	Number (aa)	MW (kDa)	PI	CELLO Localization (Reliability)
PheHsfA4a-1	PH01000018G0140	PH01000018	443	49.07	5.22	Nuclear (3.717)
PheHsfB2c	PH01000314G0470	PH01000314	382	40.12	4.86	Nuclear (2.907)
PheHsfC1b-1	PH01000333G0160	PH01000333	247	26.77	8.71	Cytoplasmic (1.219) Mitochondrial (1.205) Chloroplast (1.221)
PheHsfA1a	PH01000371G0730	PH01000371	679	75.05	6.06	Nuclear (2.682)
PheHsfB4a-1	PH01000700G0690	PH01000700	385	41.99	8.87	Cytoplasmic (1.061) Nuclear (1.481)
PheHsfC1b-2	PH01000701G0030	PH01000701	296	32.24	8.84	Cytoplasmic (1.557)
PheHsfA4a-2	PH01000746G0370	PH01000746	444	49.50	5.36	Nuclear (3.999)
PheHsfA4d-1	PH01000814G0580	PH01000814	554	61.26	4.95	Nuclear (2.593)
PheHsfC2b	PH01000849G0330	PH01000849	276	29.22	7.46	Extracellular (1.087)
PheHsfA5	PH01001018G0530	PH01001018	499	55.33	5.21	Nuclear (3.607)
PheHsfA7a	PH01001028G0180	PH01001028	381	42.51	5.94	Cytoplasmic (1.751)
PheHsfB4c-2	PH01001228G0360	PH01001228	276	29.72	8.34	Nuclear (3.293)
PheHsfB4c-1	PH01001554G0080	PH01001554	420	45.70	9.81	Nuclear (1.699)
PheHsfA4d-2	PH01002437G0340	PH01002437	453	51.34	5.18	Nuclear (4.154)
PheHsfB4a-2	PH01004959G0060	PH01004959	275	30.24	8.37	Chloroplast (1.119) Nuclear (1.604)
PheHsfB2a	PH01000000G3800	PH01000000	292	31.22	4.98	Nuclear (3.984)
PheHsfA6b-2	PH01000081G0140	PH01000081	362	41.44	4.97	Nuclear (3.466)
PheHsfA2a-2	PH01000174G0590	PH01000174	342	38.47	5.32	Nuclear (4.565)
PheHsfA6a	PH01000194G0800	PH01000194	378	42.59	4.70	Nuclear (1.785)
PheHsfA6b-1	PH01000208G0690	PH01000208	348	40.04	4.89	Cytoplasmic (2.207) Nuclear (2.182)
PheHsfA2a-1	PH01003916G0010	PH01003916	358	40.31	5.49	Nuclear (3.869)
PheHsfB1	PH01000149G1320	PH01000149	297	32.42	8.55	Nuclear (3.609)

Gene ID: refer to Bamboo Genome Datebase (http://202.127.18.221/bamboo/index.php); MW: molecular weight represents the predicted weights of PheHsf proteins; PI: represents the predicted isoelectric point of PheHsf proteins.

3.3. Structure and Motif Analyses of PheHsf Genes

To better understand the gene structure diversity of PheHsfs, we compared the intron-exon arrangements and the conserved motifs (Figure S1). The number of introns in the Hsf genes of moso bamboo ranged from zero to three. Most of the PheHsfs (19/22) contained one to two introns (Figure S1b). Three introns were found in PheHsf4, whereas none were detected in PheHsfB4a-1 and PheHsfB4c-1.

Based on the known information on functional domains of Hsfs in some model plants [15,54], the sequence and positions of similar domains were identified in the PheHsfs by sequence alignment. As shown in Table 2, five conserved domains (DBD, HR-A/B, NLS, NES, and AHA) were identified. The DBD domain comprised of three α-helices (α1–3) and four β-sheets (β1–4) were found in all PheHsfs (Figure S2a). HR-A/B domain is critical for one Hsf interacting with other Hsfs to form trimer [15]. All class A PheHsfs have a 21 amino-acid (aa) insertion between HR-A and HR-B regions; class C PheHsfs have seven aa insertions; all class B PheHsfs have no insertion (Figure S2b). NLS and NES domain function in the assembly of a nuclear import complex and the receptor-mediated export in complex with the NES receptor [15]. The majority of the PheHsfs showed the presence of a NES and/or NLS domain. In addition, the activation domain AHA was found in all class A members but not in classes B and C (Table 2).

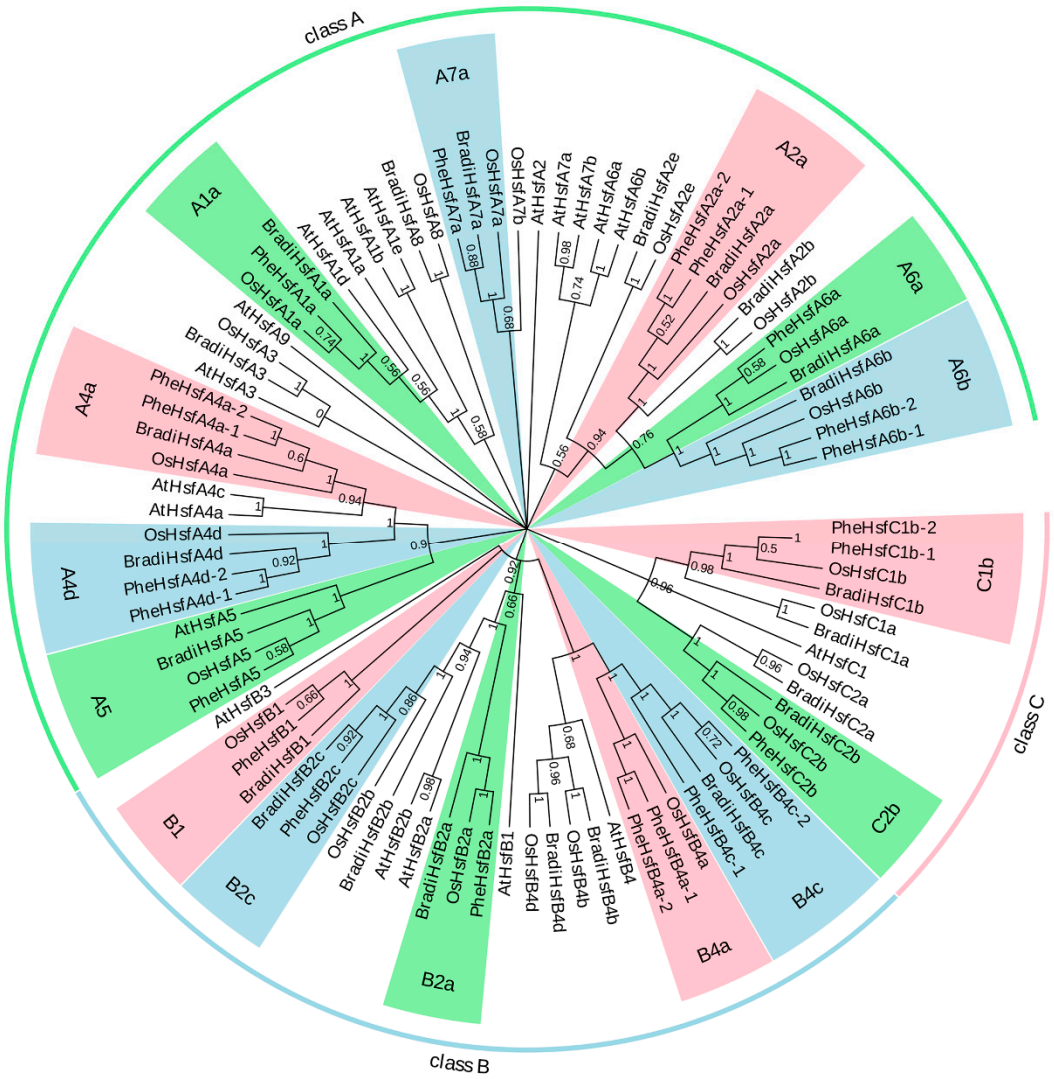

Figure 1. Phylogenetic relationship of PheHsf, AtHsf and OsHsf proteins. Neighbor-joining method and MEGA 7.0 software were used for phylogenetic analysis of Hsf proteins from *Phyllostachys edulis* (22 PheHsfs), *Arabidopsis thaliana* (23 AtHsfs), *Oryza sativa* (25 OsHsfs), and *Brachypodium distachyon* (24 BdHsfs). The names of subclass are shown outside of the circle. Branch lines of subclass are colored, indicating different Hsf subclasses.

A MEME motif search revealed a total of 19 motifs in the PheHsfs (Figure S1c). Three motifs (1, 2, and 4) constituting the DBD domain were identified. Motif 3 and 5 for the OD domain were detected in class A and C, and motif 6 for the OD domain was observed in class B. Motif 6 and motif 11, motif 8, and motif 17 (NSL) were observed in class A, class B and C, respectively. In general, the structure of the PheHsf proteins is conserved in moso bamboo. Furthermore, motif 7 represented the AHA domain close to the PheHsfA C-terminus (Figure S1c and Table 2).

Table 2. Functional domains of PheHsfs.

Gene	DBD	HR-A/B	NLS	AHA	RD	NES
PheHsfA1a	1–84	121–171	(201) ANKKRRLPKQ	(410) SFWEQFLVA	nd	nd
PheHsfA2a-1	39–128	151–202	(237) ISKKRRRRID	(314) DDFWEDLLHE	nd	nd
PheHsfA2a-2	40–129	156–197	(238) ISKKRRRRID	(315) DDFWEDLLHE	nd	(165) LLM
PheHsfA4a-1	14–103	124–165	(204) DHHRKKRRLPKPISF	(379) GFWQQFLTE	nd	nd
PheHsfA4a-2	14–103	124–172	(204) DHHRKKRRLPKPISF	(380) GFWQQFLTE	nd	nd
PheHsfA4d-1	13–96	112–140	(199) FSKKRRAPKI	(363) LFWERFLTE	nd	nd
PheHsfA4d-2	16–105	120–149	(208) FSKKRRVPKI	(382) LFWERFLTE	nd	(252) MELAL
PheHsfA5	30–134	152–179	(241) FQKKRRLTGL	(446) KFWEQFLTE	nd	nd
PheHsfA6a	46–135	165–206	(247) ISKKRRRPID	(319) DDFWAELLVE	nd	(288) LENLAL
PheHsfA6b-1	45–134	163–204	(133) LLKMIKRRRLLYY	(318) DDFWEELLNE	nd	nd
PheHsfA6b-2	45–134	163–204	(135) KMIKRRRPLS	(318) EDFWEELLNE	nd	nd
PheHsfA7a	74–165	193–227	(166) KNIKRRRASK	(329) DDVWEELDAI	nd	nd
PheHsfB1	30–117	176–205	(253) EDATRKRKRCEEAAARERPFKMIRI	nd	(246) KLFGVLL	nd
PheHsfB2a	7–100	160–183	nd	nd	(203) TLFGVTI	(264) LDVLALSL
PheHsfB2c	29–118	199–232	nd	nd	(304) RLFGVSI	nd
PheHsfB4a-1	19–112	110–139	(237) RKRLLQEQPPTSPEWKRSMV	nd	(215) KLFGVNL	nd
PheHsfB4a-2	19–103	201–230	(347) RKRSLQEQPPTSPDWKRSMV	nd	(325) KLFGVDL	nd
PheHsfB4c-1	26–116	256–285	nd	nd	(323) KLFGVHI	nd
PheHsfB4c-2	26–116	124–143	nd	nd	(213) KLFGVHL	(251) LESDDLSL
PheHsfC1b-1	25–114	136–165	(209) PGKKRRIGAE	nd	nd	nd
PheHsfC1b-2	72–161	186–213	(257) TPGKRRRIG	nd	nd	nd
PheHsfC2b	12–105	140–176	nd	nd	nd	(173) LKV

DBD: DNA-binding domain; HR–A/B: OD (oligomerisation domain); NLS: Nuclear localization signal; AHA: Activator motifs; RD: Tetrapeptid motif LFGV as core of repressor domain; NES: Nuclear export signal; nd, no motifs detectable by sequence similarity search.

3.4. Cis-Regulatory Element Analysis in Promoters of PheHsfs

To predict the biological function of PheHsfs, the 1500 bp upstream sequence from the translation start sites of PheHsf genes were analyzed using the PlantCARE database. The results show that the promoter of each PheHsf has several cis-regulatory elements such as phytohormone- (abscisic acid, jasmonic acid and gibberellic acid), abiotic stress- (low temperature, heat stress, drought, and fungal elicitor), and developmental process-related elements. Figure S3 shows that the ABA-responsive element (ABRE), the MeJA-responsive element (CGTCA-motif), and SA-responsive element (TCA-element) were found in the promoters of 17, 16, and 11 PheHsf genes, respectively. The promoters of 12 and 10 PheHsf genes contained the HSE and LTR, respectively. MYB-binding sites involved in drought inducibility (MBS), fungal elicitor-responsive elements (Box-W1) and defense- and stress-responsive elements (TC-rich) were found in 17, 15 and 8 PheHsf genes, respectively. Additionally, meristem expression (CAT-box), meristem-specific activation (CCGTCC-box), and endosperm expression (Skn-1_motif) motifs were found in the 13, 10 and 18 PheHsf genes, respectively. These findings indicate that PheHsfs might be associated with different transcriptional regulatory mechanisms for developmental, hormone and stress processes.

3.5. Expression Pattern of the PheHsf Genes in Shoot and Flower Development

Based on the RNA-Seq data of different flowering developmental stages [43] and the internodes of shoots at different heights [1], a heat map was constructed according to the RPKM of 22 PheHsfs (Figure 2). During four flowering developmental stages of moso bamboo, *PheHsf* genes could be classified into four groups (A, B, C, and D) according to their relative expression levels (Figure 2a). Most of the *PheHsfs* were highly expressed (RPKM > 10) in at least one stage, and only four PheHsf genes were expressed at low levels (RPKM < 2) in at least two stages during floral development (Table S2). Four members (*PheHsfB4a-1*, PheHsf15, *PheHsf12*, and *PheHsfB4c-1*) of group C had higher transcript accumulation during two earlier stages (F1 and F2) and were downregulated at two later stages (F3 and F4 stage). Group D consisted of 10 PheHsf genes (*PheHsfA4a-1*, *PheHsfB2c*, *PheHsfA1a*, *PheHsfA4a-2*, *PheHsfC2b*, *PheHsfA7a*, *PheHsfB2a*, *PheHsfA2a-2*, *PheHsfA6b-1*, and *PheHsfB1*), and their expression levels steadily increased at the F3 and F4 stages. Group B comprised of six *PheHsfs* (*PheHsfA4d-1*, *PheHsfA4d-2*, *PheHsfA5*, *PheHsfA6b-2*, *PheHsfA6a*, and *PheHsfA2a-1*), showing higher transcript accumulation at two later stages (F3 and F4) and in the leaves. Group A only had two genes, *PheHsfC1b-1* and *PheHsfC1b-2*, with expression levels six times and three times higher in leaves than the four stages of floral development, respectively. During shoot growth, Most *PheHsfs* (19/22)

showed very low expression levels (RPKM < 8) at all seven stages, and only two genes (*PheHsfA1a* and *PheHsfA6b-2*) had higher expression levels (RPKM > 20) in at least one stage of bamboo shoot growth (Table S3). Based on the expression profiles, the *PheHsfs* were classified into four groups (Figure 2b). Among these, *PheHsfA1a* and *PheHsfA2a-2* were clustered into the same group with continuous down-regulated expression from the S1 to S7 stage. However, *PheHsfA6b-2* and *PheHsfB2c* were clustered in the same group, which showed twin peaks during the S2 and S5 stage, respectively.

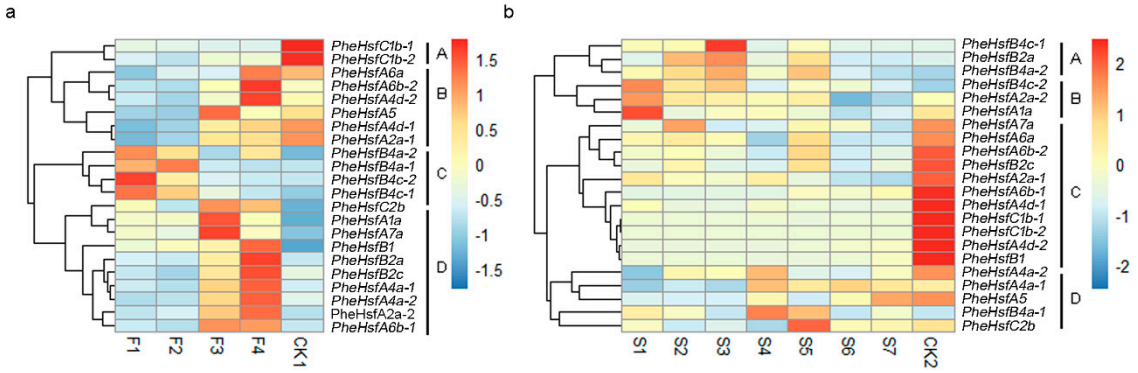

Figure 2. Expression pattern of *PheHsfs* in developmental flowers and shoots of moso bamboo. (**a**) The expression profile of *PheHsfs* in different stages of flowering. F1–F4: The four stages of developmental flowers (the floral buds begun to form, the floral organs gradually matured but did not undergo flowering, the flowers were in full blossom, and the embryo formation stage); CK1: leaves. (**b**) The expression profile of *PheHsfs* in different stages of shoots. S1: winter shoot; S2–S7: different heights of shoots (0.5 m, 1 m, 3 m, 6 m, 9 m and 12 m), and CK2 (culms after leaf expansion). All the samples had one repeat. The heatmap was pictured using R based on the RPKM of *Phehsfs* in each sample. Details of the RPKM are shown in Tables S2 and S3.

For different periods of flower and shoot development in moso bamboo, the *PheHsfA1a* and *PheHsfA6b-2* genes showed high transcript accumulation in all 11 stages, whereas four genes (*PheHsfB4a-1*, *PheHsfA4d-1*, *PheHsfA7a*, and *PheHsfB4a-2*) exhibited extremely low transcript accumulation at all stages. Moreover, the other 15 *PheHsfs* depicted significantly higher transcript levels during flower development than during shoot growth. These findings indicate that these genes have different regulatory roles in moso bamboo flower development and shoot growth.

3.6. PheHsfAs Expression in Moso Bamboo in Response to Various Stresses

The expression of 12 PheHsfAs were analyzed by qRT-PCR under four abiotic stresses: high temperature, cold, drought and salt (Figure 3). The expression levels of all members of subclass A were upregulated (>2 folds) after at least one treatment (Table S4, Figure 3b). For heat stress, eight PheHsfAs were upregulated (>2 folds). PheHsfA4d-1, PheHsfA2a-2, and PheHsfA6b-1 rapidly responded to high temperatures, showing upregulation up to ~nine-fold within 0–1 h of 42 °C (Table S4) treatment. Among these, PheHsfA2a-2 (~22-fold higher than the control) was the most strongly induced gene. For cold stress, the expression levels of seven PheHsfAs (PheHsfA4a-1, PheHsfA1a, PheHsfA4a-2, PheHsfA4d-2, PheHsfA4d-2, PheHsfA5 and PheHsfA2a-2) were at least two-fold greater than the control for at least one of the time points. For drought and salt stress, eight PheHsfAs (PheHsfA4a-1, PheHsfA4a-2, PheHsfA4d-1, PheHsfA4d-2, PheHsfA5, PheHsfA7a, and PheHsfA6a) were upregulated. The expression level of PheHsfA4d-2 was ~20-fold higher than control after 3 h of 20% PEG treatment. The expression level of PheHsfA4d-1 was ~70-fold higher than the control after 3 h of 200 mM NaCl treatment (Table S4). Among the PheHsfA genes, five genes (PheHsfA4a-1, PheHsfA4a-2, PheHsfA4d-1, PheHsfA4d-2, and PheHsfA2a-2) were upregulated relative to the control treatment. (Figure 3b). These findings indicate that these genes might play vital roles in different stress response pathways.

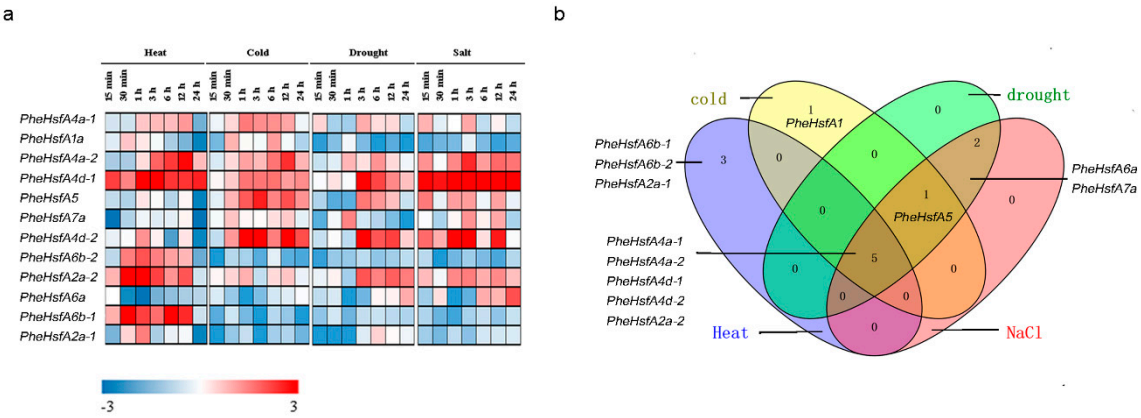

Figure 3. Expression analysis of PheHsfAs under different abiotic stress treatments in moso bamboo. (**a**) Heat map representation for the expression patterns of PheHsfAs after 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h of heat, cold, drought, and salt stresses: expression levels under stress vs. control; the expression results were obtained by qRT-PCR. The different colors correspond to log2 transformed value. (**b**) Venn diagram showing the number of overlapping PheHsfAs that are up-regulated > two-fold under abiotic stress: heat, cold, salt, and drought. Details of the expression data are shown in Table S4.

3.7. Expression Correlation and Interaction Networks

To further investigate the PheHsf proteins and how they interact with each other, a co-expression network was constructed using expression values of PheHsf genes during shoot and flower development. The connecting gene with PCC magnitude >0.85 was recognized as strongly co-expressed genes [36]. The result showed that 18 PheHsf genes and 38 correlations in a co-expression network with PPC >0.85 cutoffs were obtained (Figure 4a). Genes with stronger correlation might play roles as interacting partners in similar biological pathways. Based on the results, *PheHsfA4a-1* and *PheHsfA4a-2* were recognized as hub genes as nodes with 10 and 9 connectivity in the whole network, respectively, which had more connectivity than other PheHsf genes. Both *PheHsfA4a-1* and *PheHsfA4a-2* had an up-regulated expression in response to different stress treatments (heat stress, cold, drought and salt). These results indicated that the two *PheHsfA4s* with a greater role in shoot and flower development also had an important role in stress responses.

To identify the two PheHsfA4a-associated proteins and protein complexes, prediction networks were built with STRING (http://www.string-db.org/) based on the interaction network of rice orthologous genes (Figure 4b). Because the rice orthologous gene of both *PheHsfA4a-1* and *PheHsfA4a-2* were *OsHsfA4a*, the identified moso bamboo proteins predicted to participate in the interaction network with *PheHsfA4a-1* and *PheHsfA4a-2* were the same. They interacted directly with 10 identified proteins, including five HSP70 proteins, one HSP90 protein, and four MAPK proteins. Because *PheHsfA2a-2* (~22-fold higher than the control), *PheHsfA4d-2* (~20-fold higher than the control) and *PheHsfA4d-1* (~79-fold higher than the control) were strongly induced by heat, drought and NaCl stress, respectively. We also identified the two PheHsfA4d- and *PheHsfA2a-2*- associated proteins and protein complexes, and PheHsfA4ds and *PheHsfA2a-2* were also predicted to interact with HSP70 proteins, HSP90 protein, and MAPK proteins (Figure 4c,d).

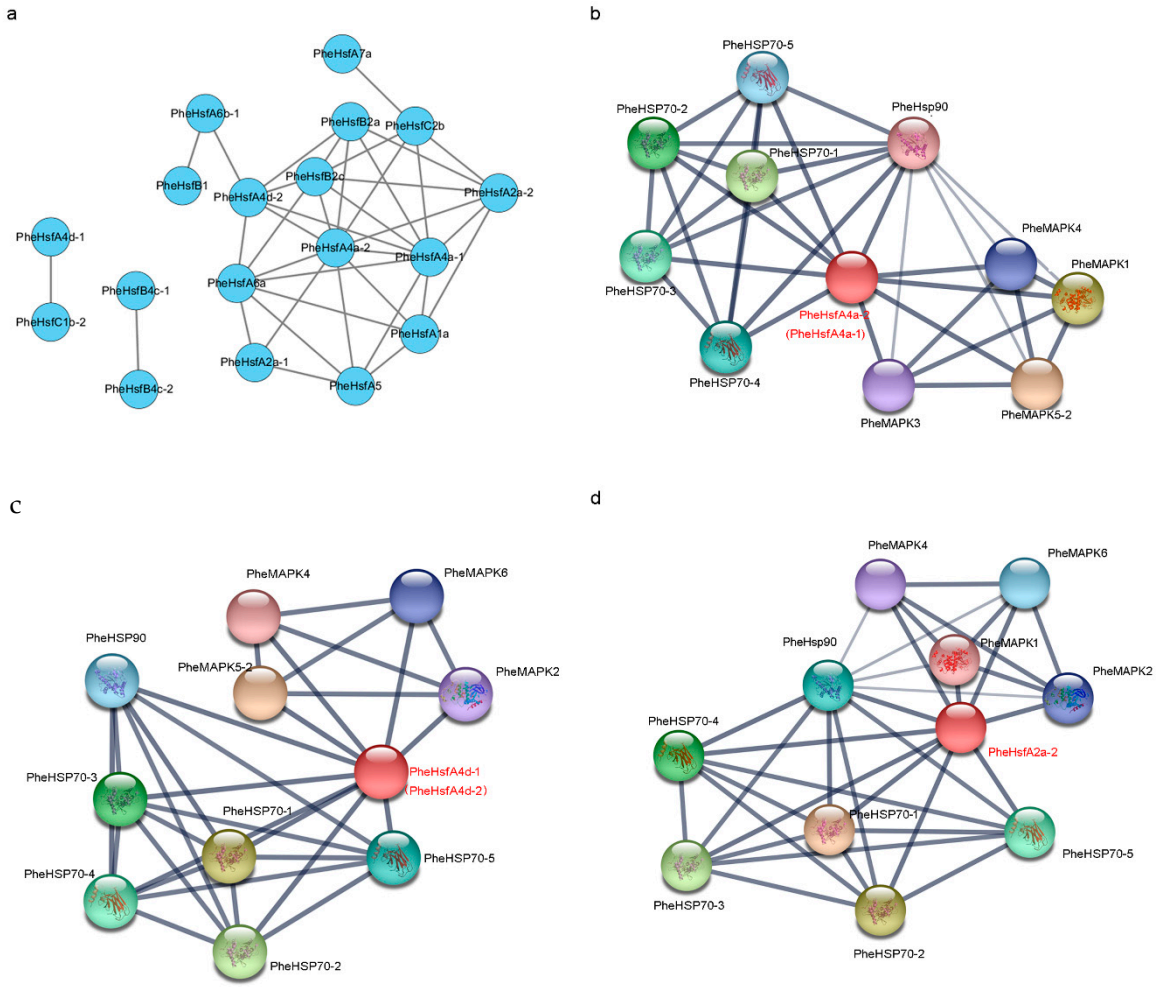

Figure 4. Co-expression network and interaction network of selected PheHsf genes in moso bamboo. (**a**) The model was built based on RPKM of RNA-seq of shoot and flower development. (**b**–**d**) Interaction network of PheHsfA4as, PheHsfA4ds and PheHsfA2a-2 in moso bamboo, respectively. Colored balls (protein nodes) in the network were used as a visual aid to indicate different input proteins and predicted interactors. Protein nodes which are enlarged indicate the availability of 3D protein structure information. Gray lines connect proteins which are associated by recurring textmining evidence. Line thickness indicates the strength of data support.

4. Discussion

4.1. Characterization of the Moso Bamboo Hsf Genes Family

Hsf genes play essential roles in plant development and in responding to various stress conditions [55,56]. To explore the functions of *PheHsf*s in moso bamboo for the first time, the present study identified a total of 22 PheHsf genes according to the moso bamboo genome database and FLNC reads database [32,34,57].

We found that the moso bamboo has a similar number of *Hsf*s as rice, *B. distachyon*, maize, and *A. thaliana* (22–25). This partially accounts for the support of Hsf conservation during evolution. Phylogenetic analysis of Hsfs in moso bamboo, *O. sativa*, *B. distachyon*, and *A. thaliana* indicated that PheHsfs have a higher degree of sequence similarity with OsHsfs and BdHsfs than AtHsfs, which coincides with the evolutionary relationships among the four species. All three *Hsf* classes (A, B, and C) were identified in the three monocots and one dicot, implying that the Hsf genes originated prior to the divergence of monocots and dicots.

In the investigation of conserved Hsf domains and intron-exon structures, all 22 of the PheHsfs contain the necessary (DBD and OD) and/or specific protein domains (NLS, NES, and AHA). The hydrophobic core of DBD domain ensures precise positioning and highly selective interaction with heat stress promoter elements [15]. The OD of plant Hsfs confers distinct patterns of specificity for hetero oligomerization [15]. These AHA motifs, which are located in the C-terminal, are characterized by aromatic (W, F, Y), large hydrophobic (L, I, V) and acidic (E, D) amino acid residues [15]. Those domains might be essential for functional conservation [56]. Twenty of twenty-two PheHsf genes have one intron in their DBD domain (Figure S1), which is an evolutionarily conserved intron [13]. However, no intron was identified in *PheHsfB4a-1* and *PheHsfB4c-1* of subclass B4, which is different from subclass B4 of rice and *B. distachyon* [53].

4.2. Cis-Regulatory Element Analysis in the Promoters of PheHsfs

Previous studies have illustrated the key roles of *Hsf*s in developmental processes and stress tolerance through their regulation of target genes [58]. The number and form of *cis*-elements in the promoter region might play an essential function in the regulation of gene expression in relation to metabolic pathways [59]. The in silico survey of putative *cis*-elements using PlantCARE showed that 12 of the 22 *PheHsfs* promoters contained HSEs. This implies that *PheHsfs* might regulate themselves [55]. Additionally, the promoter region of *PheHsfA1a* contained more types of development-related *cis*-elements (Figure S3), which coincides with its constitutive expression during shoot and flower development in moso bamboo (Figure 2).

4.3. PheHsfAs Involvement in Development Processes

The 22 *PheHsfs* exhibited diverse expression patterns during shoot and flower development of moso bamboo under normal conditions. *PheHsfA1a* was upregulated during shoot and flower development and was constitutively expressed in different tissues (Figure 2). These findings are similar to that in *Arabidopsis* [60] and *Salix suchowensis* [61]. Under normal conditions, class A1 *Hsfs* of *Arabidopsis* are involved in housekeeping processes, and *SsuHsf-A1a* of *Salix suchowensis* are constitutively expressed in different tissues [60,61]. In rice, nearly all classA members showed high expression levels in all tissues [45,62]. In this study, nearly all the *PheHsfAs* (except *PheHsfA7a*) were found to show high transcription levels in the leaves, culms, and the four stages of flower development, similar to that in rice. Only two members of *PheHsfAs* (*PheHsfA1a* and *PheHsfA6b-2*) have high transcription levels in the seven stages of shoot development. This indicated that the function of *PheHsfAs* is conserved and/or specific in regulating flower development and shoot growth in moso bamboo.

4.4. PheHsfAs are Involved in Stress Responses

Under heat or other stress conditions, plant Hsfs regulate the transcription of target genes (*Hsps* and other stress-inducible genes) to enhance stress resistance. Recent genome-wide expression profile analyses showed that most of the Hsf genes are upregulated after heat, cold, drought, and salt stress [56,58,60]. An increase in the number of Hsf genes has been shown to improve plant stress tolerance [54,63]. In moso bamboo, Zhao et al. identified seven and two *PheHsfs* that were upregulated after 0.5 h and 8 h of high light stress (1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$), suggesting that these play vital roles both in response to short-term (0.5 h) and mid-term (8 h) high light [64]. However, the regulatory roles of Hsfs in response to other abiotic stresses are unclear. Classes B and C do not harbor AHA motifs, which are essential for the activity of class A Hsfs [16]. Therefore, class A PheHsf genes were selected to further study their response patterns under stress and hormone treatments.

Under heat treatment, 9 of 12 *OsHsfAs* and 9 of 13 *BdHsfAs* were upregulated in rice and *B. distachyon*, respectively [59,62]. In our study, 8 of 12 *PheHsfAs* were found to be induced by high temperature. Of these, *PheHsfA4d-1*, *PheHsfA2a*-2 and *PheHsfA6b* responded more quickly to heat stress than others. *PheHsfA2a*-2 showed stronger induced under-heat stress conditions,

which is similar to that of its homologous genes in rice, Arabidopsis, and *B. distachyon* [18,45,53]. These findings suggest that *PheHsfA2a*-2 plays an important role in response to heat stress of moso bamboo. However, *PheHsfA2a-1*, which is the most similar to *PheHsfA2a*-2 and also belongs to A2a-type PheHsfs, was slightly upregulated 1 h after heat stress application. *PheHsfA4d-2*, which is the most similar to *PheHsfA4d-1*, was less upregulated by heat and salt stress compared to *PheHsfA4d-1*, but showed higher relative expression levels than *PheHsfA4d-1* with cold and drought stresses.

HsfA4a of wheat (*Triticum aestivum*) and rice conferred cadmium tolerance in yeast and plants, but other Hsfs with similar structure (OsHsfA4d, AtHsfA4a, and AtHsfA4c) did not [27]. HsfA4a of Arabidopsis and chrysanthemum (chrysanthemum cultivar 'Jinba') confers salt and oxidative stress [26,65]. In this study, *PheHsfA4a-1*, *PheHsfA4a-2*, *PheHsfA4d-1* and *PheHsfA4d-2* were upregulated in response to these four abiotic stress (heat, cold, drought and salt). These findings indicate that the three *PheHsfA4s* could act as potential "nodes" for connecting the above four abiotic stresses.

4.5. Expression Correlation and Interaction Networks

In addition, the expression values of PheHsf genes in shoot and flower developmental stages were used to identify potential underlying co-expression networks using Cytoscape. Based on their degree of connectivity, the hub genes *PheHsfA4a-1* and *PheHsfA4a-2* were identified to play a regulatory role and correlated with other PheHsfs in the complex feedback network. According to the prediction of five PheHsf proteins (four PheHsfA4s and one PheHsfA2a-2) interaction networks, the interactive proteins might include MAPKs and heat shock proteins. MPK5 protein in rice acts as a positive regulator of drought, salt and cold tolerance; is involved in disease resistance and abiotic stress tolerance signaling pathways; and also negatively modulates pathogenesis-related (PR) gene expression and broad-spectrum disease resistance [66]. The MPK1 protein in rice acts downstream of heterotrimeric G protein alpha subunit and small GTPase RAC1 and may regulate the expression of various genes involved in biotic and abiotic stress response [67]. Base on the above information and our results, four PheHsfA4s and one PheHsfA2a-2 may play a very important role in shoot and flower development and stress response.

5. Conclusions

In this study, 22 PheHsf genes in moso bamboo were identified for the first time. These genes could be classified into three classes (A, B and C) according to the comparison of the phylogenetic relationships with *O. sativa*, *B. distachyon* and *A. thaliana* Hsf genes. Expression analyses revealed that two hub genes, *PheHsfA4a-1* and *PheHsfA4a-2*, might act as a potential "node" for crosstalk between developmental processes and abiotic stress responses. Furthermore, *PheHsfA2a-2*, *PheHsfA4d-1* and *PheHsfA4d-2* might also act as essential parts in response to stress. These results provide insights into the responses of *PheHsfAs* to abiotic stresses treatments, although their underlying molecular mechanism requires further study.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/2/100/s1, Figure S1: Phylogenetic tree and gene structure of PheHsf genes, Figure S2: Multiple sequence alignment of the DBD domains and OD region of the PheHsf proteins, Figure S3: Various cis-acting elements in PheHsf genes promoter regions, Table S1: Primer sequences used in gene expression with qRT-PCR experiments, Table S2: The relative level of gene expression of PheHsf genes during developmental flowers (F1 to F4) and leaves (CK)(RPKM), Table S3: The relative level of gene expression of PheHsf genes during growing shoots (S1–S7) and clum (CK)(RPKM), Table S4: Expression analysis of *PheHsfAs* under different abiotic stress in moso bamboo, Fasta S1: PheHsfs and PheHsfs-like CDS sequence, Fasta S2: PheHsfs and PheHsfs-like protein sequence, Fasta S3: The cloned cDNA sequence of PheHsfA5, PheHsfB4a-1 and PheHsfB4c-1.

Author Contributions: X.L. and J.G. designed the experiments; X.L. and J.L. (Juan Li) performed the tissue and organ collection; L.X. and X.L. performed the experiments; Z.C. formal analysis; J.L. (Jun Liu) and S.M. assisted in equipment maintenance and sample collection; L.X. writing—original draft preparation; X.L. and D.H. writing—review and editing; J.G. review and funding acquisition. L.X. and X.L. contributed equally in this work and should be considered first co-authors.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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