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Molecular characterization of the heat shock transcription factors in switchgrass highlights PvHsf16 conferring cadmium stress

Gang Song[1], Hao Guan[2], Zhigang Fang[3], Yanling Ji[4], Jing Zhang[2*] and Bin Xu[2*]

[1] *Jiangsu Vocational College of Agriculture and Forestry, Jvrong 212400, China*
[2] *College of Agro-grassland Science, Nanjing Agricultural University, Nanjing 210095, China*
[3] *College of Life and Geographic Sciences, Kashi University, Kashi 844006, China*
[4] *College of Life Science, Nanjing Agricultural University, Nanjing 210095, China*
* Corresponding authors, E-mail: nauzj@njau.edu.cn; binxu@njau.edu.cn

Abstract

Heat shock transcription factors (Hsfs) play crucial roles in plants' responses to abiotic stress. However, the identification of *Hsf* family genes and their stress tolerance functions in switchgrass are less well studied. This study aimed to screen and identify *PvHsf* genes from the whole genome of switchgrass. A total of 51 *PvHsfs* were identified and categorized into three classes: Class A (*PvHsf01*–*PvHsf28*), Class B (*PvHsf29*–*PvHsf42*), and Class C (*PvHsf43*–*PvHsf51*). Collinearity analysis revealed that 46 *PvHsfs* had 42 collinearity relationships, and *PvHsf49* and *PvHsf50* were a pair of tandem duplicate genes. Developmental and stress-regulatory expression patterns of *PvHsfs* were also analyzed in various tissues and developmental stages or under NaCl, PEG, ABA, and $CdCl_2$ treatments. Nine *PvHsfs* were responsive to $CdCl_2$-treatment, including *PvHsf3*, *PvHsf4*, *PvHsf16*, *PvHsf19*, *PvHsf23*, *PvHsf24*, *PvHsf35*, *PvHsf44*, and *PvHsf45*, which were evaluated for their roles in cadmium (Cd) tolerance. By ectopic expressing these *PvHsf* genes in a Cd-sensitive yeast mutant, yeast cells expressing *PvHsf3* and *PvHsf16* exhibited higher Cd tolerance and the best result was by expressing *PvHsf16*. PvHsf16 was subcellularly localized in the nucleus. Expressing *PvHsf16* in Arabidopsis led to enhanced Cd tolerance with significantly higher Chl content, root length, and biomass compared to the WT plants under $CdCl_2$ treatment. Knowledge gained from this study will facilitate further functional analysis of *PvHsfs,* particularly *PvHsf16,* for molecular breeding of switchgrass and other related grass species.

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Introduction

Plant heat shock transcription factors (Hsfs) play pivotal roles in protecting against various stresses[1–3]. To date, genome-wide analysis of the Hsf family have been successfully characterized in Arabidopsis (*Arabidopsis thaliana*)[1], rice (*Oryza sativa*)[1], tomato (*Solanum lycopersicum*)[4], maize (*Zea mays*)[5], soybean (*Glycine max*)[6], peach (*Prunus persica*)[3], radish (*Raphanus sativus*)[7], and common bean (*Phaseolus vulgaris*)[8], etc. For example, Arabidopsis contains 21 *Hsf* genes that could be classified into three classes: class A (15 genes), class B (five genes), and class C (one gene)[1]. Soybeans had a much bigger *Hsf* family with 52 *Hsf* genes[6].

Besides conferring plant heat tolerance, *Hsf* genes also serve as regulators in other abiotic and biotic stress tolerances. For example, tomato HsfA1a serves as the master regulator of acquired thermotolerance that was irreplaceable by other Hsf[9], whereas tomato HsfB1 serves as a synergistic co-activator of HsfA1a[10]. Hsfs can recognize the conserved binding motifs (heat shock elements, HSEs) within the promoters of their responsive genes, including those coding for heat shock proteins (HSPs)[11]. For example, Arabidopsis HsfA1a could sense heat stress and pH changes by binding to the promoters of *HSP18.2* and *HSP70*[12]. Overexpressing *HsfA1b* resulted in increased water productivity and harvest index under both water-sufficient and water-limiting conditions in Arabidopsis[13]. Arabidopsis HsfA2 regulates multiple stress tolerances including salt, anoxia, osmotic, and submergence stresses[14]. Ectopic expressing two *Hsf* genes (*SaHsfA4a* and *SaHsfA4c*) of *Sedum alfredii* in yeast significantly enhanced its cadmium (Cd) tolerance[15]. Furthermore, HsfA4a in rice and wheat[16] and LpHSfa1a in tomato[17] were also involved in the regulation of Cd tolerance[17].

Cadmium (Cd) is a non-essential heavy metal trace element and toxic to both animals and plants[18]. Cd stress significantly reduces the seeds' germination and rooting rates, inhibits water and nutrient absorption and results in physiological and metabolic disorders, including decreased photosynthetic efficiency, inactivated enzymes, and developmental symptoms such as dwarfism, fading, and delayed growth phenomena[19,20]. Soil Cd pollution not only causes long-term adverse effects on crop growth but can also enter food chains posing significant health risks to humans. Phytoremediation by growing Cd-tolerant non-edible perennial tall grass species on Cd-polluted soil has been proposed as one effective option to mitigate the harm of Cd soil pollution.

Switchgrass (*Panicum virgatum*) is a perennial tall C4 grass that is native to North America. It serves as a model plant for bioenergy, ethanol, and methane production[21–23]. Previous studies have demonstrated that switchgrass has moderate tolerance to Cd toxicity[24–26], and overexpressing one HSP family gene (*PvBiP2*) improved both biomass yield and Cd tolerance in switchgrass[27]. Therefore, it was proposed that at least some *Hsf* genes are involved in switchgrass Cd tolerance. The objective of this study was to characterize *Hsf* family genes on a genome-wide scale and identify potential Cd-regulatory *Hsf* genes in switchgrass. The identified Cd-tolerant *Hsfs* can be utilized for the molecular breeding of Cd-sensitive grass species.

Materials and methods

Identification and sequence analysis of the *Hsf* family genes in switchgrass

The Hidden Maekov Model (HMM) profile of the Hsf domain (PF00447) was downloaded from the Protein family (Pfam) database

(http://pfam.xfam.org/) and used to search the Switchgrass Genome Database (http://phytozome.jgi.doe.gov/, *Panicum virgatum* v1.0, DOE-JGI). Based on the results, the specific HMM model file of the conserved domain of the Hsf protein was established, and the sequences containing the conserved Hsf domain were also used as query sequences to search against *Panicum virgatum* v1.0 database. All output genes with E-value ≤ 0.001 were collected and examined for the presence of the Hsf domain by Pfam and SMART (http://smart.embl-heidelberg.de/), and the incorrect putative genes were deleted. The non-redundant and identified genes were assigned as *P. virgatum Hsf* genes (*PvHsfs*).

The protein sequences, genomic sequences, intron numbers, and chromosomal location data of identified *PvHsfs* were derived from *Panicum virgatum* v1.0 database. The protein sequences were analyzed with WOLFPSORT program (www.genscript.com/wolf-psort.html) to obtain the number of amino acids, molecular weight, theoretical isoelectric points (pI) and instability index (considered as unstable if the value was > 40).

Multiple sequence alignment and phylogenetic analysis of Hsf proteins

The full amino acid sequences of Hsf members from three plant species, including *A. thaliana*, rice, and switchgrass were aligned using CLUSTALW program. The full amino acid sequences of Arabidopsis and rice were downloaded from the TAIR database (www.arabidopsis.org) and the RGAP database (http://rice.plantbiology.msu.edu), respectively. An unrooted neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 7.0 software with the bootstrap test replicated 1,000 times pair-wise deletion in a Poisson model.

Gene structure and conserved motif analysis of PvHsfs

Exton and intron organization of *PvHsfs* was illustrated with Gene Structure Display Server program (GSDS, https://gsds.gao-lab.org/Gsds_help.php). The identification of PvHsfs conserved motifs was performed using MEME program (http://meme-suite.org/tools/meme) and the TBtools program[28] (https://github.com/CJ-Chen/TBtools/releases) was used to visualize the analysis results.

Chromosomal locations and gene duplication analysis of *PvHsfs*

The GFF3 file containing the positional and gene structure information of switchgrass was downloaded from Switchgrass Genome Database. TBtools software was selected to map the *PvHsfs* on distinct chromosomes[28]. The gene duplication events of *PvHsfs* were analyzed by using the Multiple Collinearity Scan toolkit (MCScanX).

Heterologous expression of *PvHsfs* in yeast

Saccharomyces cerevisiae yeast mutant strain Δ*ycf1*[27,29], that was sensitive to Cd stress, was used to screen *PvHsfs*. The differentially expressed *PvHsfs* were selected based on the transcriptome data was sourced from previous work[30] and qPCR results obtained in this study. In our previous work, switchgrass seedlings were exposed to a 50 μmol·L^{-1} $CdCl_2$ hydroponic solution for durations of 0, 6, and 24 h. Subsequently, root samples were collected for RNA-seq transcriptome analysis[30]. The CDS of selected *PvHsfs* were cloned into the pGAD426 vector and then transformed to the yeast strain using the Frozen-EZ Yeast Transformation Kit (Zymo Research, USA). The transformed yeast strains were grown in the synthetic dropout uracil (SD/-URA) medium. The pGAD426-*GUS* plasmid was used as the negative control. For Cd tolerance assessment, the transformed Δycf1 yeast solution was spotted on solidified SD/-URA medium containing 100 μM $CdCl_2$ and incubated at 28 °C for 3 d.

Subcellular localization gene transformation analysis

The coding sequences (CDS) of the *PvHsf16* were amplified with gene-specific primers (Supplementary Table S1) and cloned into the pEarleyGate103 and then transformed into *Agrobacterium tumefaciens* strain '*AGL1*'. Then, the resuspended *Agrobacterium* at OD_{600} = 0.6 in 1/4 MS solution was injected into *Nicotiana benthamiana* leaves. The transformed leaves were incubated in the dark for 12 h and then moved to a normal growth environment for 3 d, and then examined the GFP fluorescence signal using a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss SAS, Jena, Germany). The *AGL1* cell harboring pEarleyGate103- *PvHsf16* was used to transform Arabidopsis ecotype 'Columbia' using the floral dip method. Transgenic lines were selected through basta resistance and PCR confirmation and the T2 homozygous lines were used for salt tolerance analysis.

Plant material and stress treatments

An elite line 'HR8'of switchgrass was used as the plant material[31]. The method of seedling cultivation was the same as a previous study[30]. In brief, the hydroponic-grown plants with five fully expanded leaves were treated with 200 mM NaCl, 200 g·L^{-1} PEG, 0.1 mM ABA, and 50 μM $CdCl_2$, respectively. In all treatments, seven uniform plants were grown in each beaker, plants in each beaker were treated as one replicate, and there were three replicates for each treatment. The second leaves from the top were sampled at designated time points, such as 0, 1, 3, 6, 12, and 24 h, and the collected samples were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

Seeds of wild type (WT) and *PvHsf16*-overexpression (OE lines) Arabidopsis were germinated and grown on 1/2 MS medium for 5 d, followed by transfer to 1/2 MS medium containing 90 mM and 180 mM $CdCl_2$ for an additional 10 d. After this the Chl content, root length, and biomass under each treatment were measured. Four biological replicates were used for measuring these physiological and morphological indicators. The 1/2 MS medium without $CdCl_2$ was used as non-stress control. Chl of Arabidopsis leaves was extracted by dimethyl sulfoxide (DMSO) and the absorbance values of the extraction solution were measured at wavelengths of 663 and 645 nm using a spectrophotometer (Biochrom Ltd, Cambridge, UK)[32].

qRT-PCR analysis

The procedure for mRNA isolation, gDNA digestion, first-strand cDNA synthesis, and the qRT-PCR reaction was the same as described previously[33]. Relative expression levels of *PvHsfs* were calculated using the $2^{-\Delta\Delta C_T}$ method with *PvFTSH4* as the reference gene. Primers used for PCR reaction are listed in Supplementary Table S1.

Statistical analysis

Data were statistically analyzed by the LSD and Duncan test at a significance level of 0.05 using PASW Statistics software (Version 18.0, SPSS Inc., Chicago, IL, USA). Data are expressed as means ± standard error (SE).

Results

Genome-wide identification and gene duplication of *PvHsf* genes in switchgrass

A total of 51 *PvHsfs*, named *PvHsf 01–PvHsf 51*, were identified in the switchgrass genome (Fig. 1, Supplementary Table S2). Fifty-one *PvHsf* genes were distributed unevenly among the 16 chromosomes (Chr) from Chr01K to Chr09K and Chr01N to Chr09N, while Chr08K and Chr08N had no *PvHsf* gene (Fig. 1). There were seven

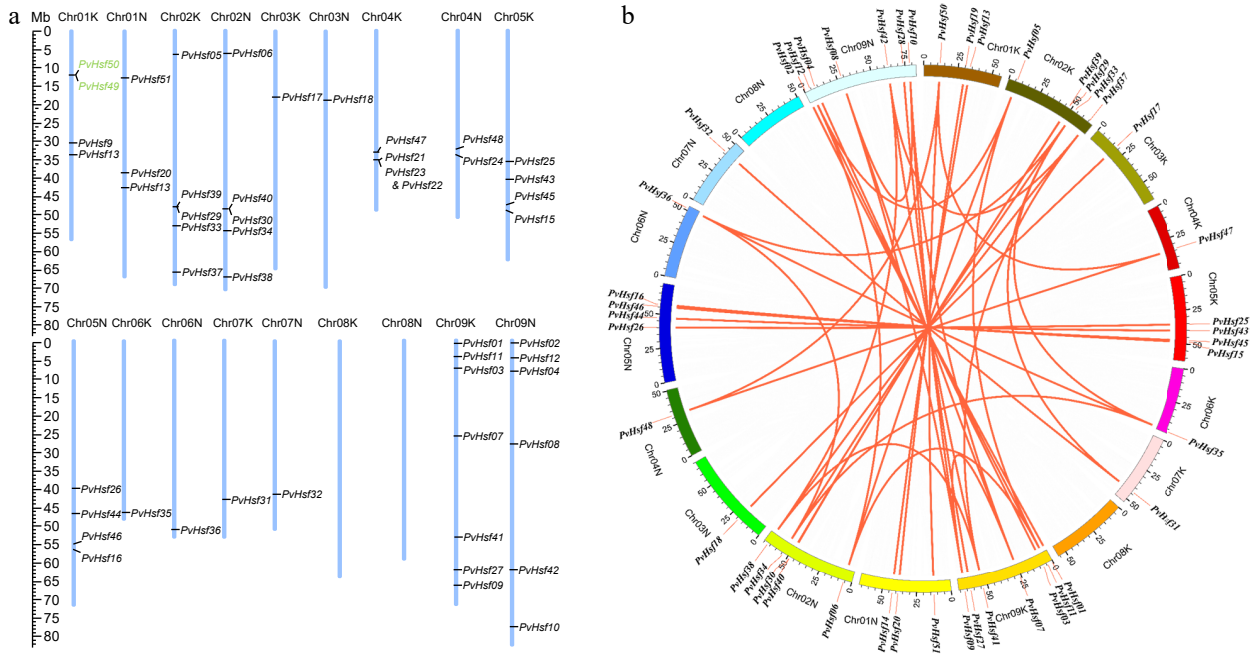

Fig. 1 Chromosomal distribution and circos diagram for the chromosomal distribution and interchromosomal relationships of *Hsf* genes in switchgrass. (a) The scale bar on the left indicates the length (Mb) of switchgrass chromosomes. (b) Gray lines indicate all synteny blocks in the switchgrass genome, and the red lines indicate duplicated Hsf gene pairs. The chromosome number is indicated at the center of each chromosome.

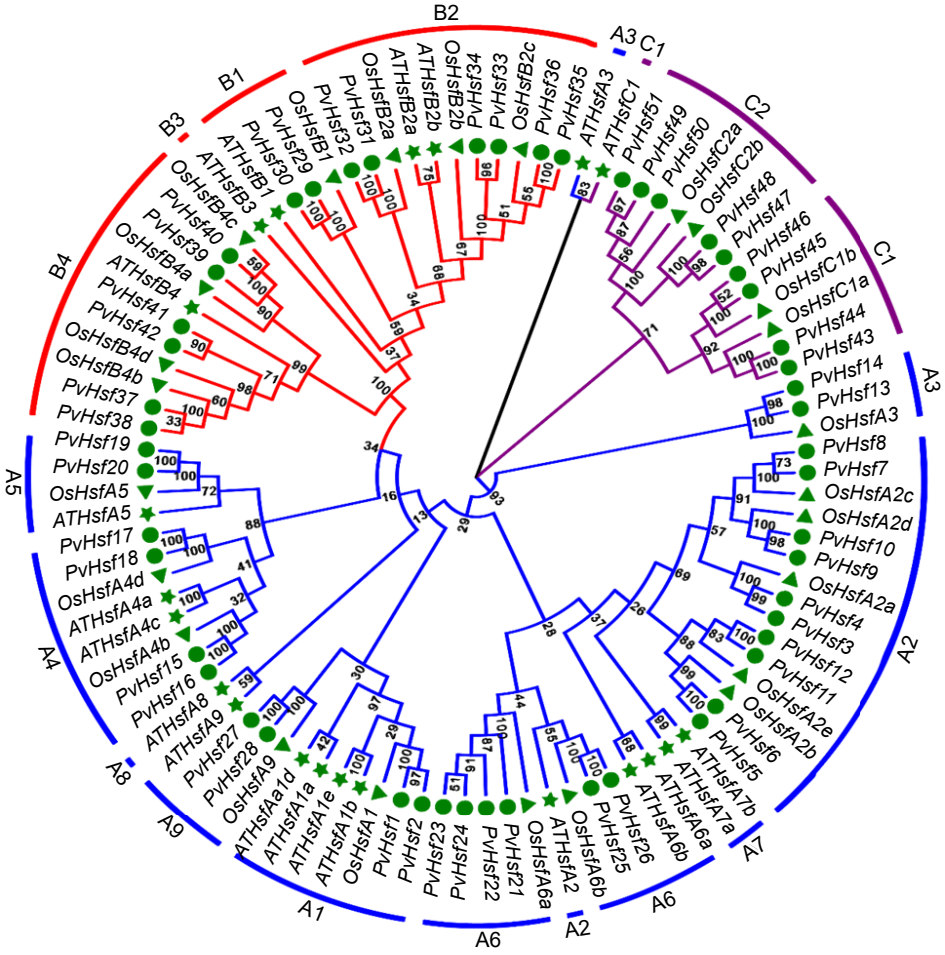

Fig. 2 Unrooted phylogenetic tree of switchgrass, *Arabidopsis*, and rice Hsf proteins. The 97 Hsf proteins' alignments and unrooted phylogenetic tree was based on the Neighbor-joining method with 1,000 bootstrap replicates and P-distance method performed by MEGA 7.0. Hsf proteins of switchgrass, rice, and *Arabidopsis* are marked with green circles, triangles, and stars, respectively. The 15 distinct groups are divided by different circular arcs with the color of blue, red, and purple. Gene ID and amino acid sequences of AtHsfs and OsHsfs are shown in Supplementary Table S3.

PvHsf genes localized on Chr09K and Chr09N, respectively, while only one *PvHsf* on Chr03K, Chr06K, Chr07K, Chr03N, Chr06N, Chr07N (Fig. 1). The *PvHsf* proteins ranged from 207 (PvHsf 13) to 562 amino acids (PvHsf 32) in length with predicted isoelectric points (pI) varying from 4.8 (PvHsf 06) to 11.84 (PvHsf 50) and molecular weight (MW) from 22.1 kDa (PvHsf 13) to 61.9 kDa (PvHsf 32) (Supplementary Table S2). *PvHsf49* and *PvHsf50* were found as a pair of tandem duplication genes located on Chr01K (Fig. 1b). And 46 genes from 42 groups exhibiting replication relationships: some genes occurred from multiple duplication events (e.g., *PvHsf09* is paired with *PvHsf07*, *PvHsf08*, and *PvHsf10*); and some might only experience a single duplication (e.g., the pair of *PvHsf17* and *PvHsf18*, and the pair of *PvHsf45* and *PvHsf46*).

Phylogenetic analysis and multiple sequence alignment of PvHsf proteins

To investigate the classification and the evolutionary characteristics of the PvHsf proteins, an unrooted phylogenetic tree was generated by using the 51 PvHsfs, 21 AtHsfs from *Arabidopsis*, and 25 OsHsfs from rice (Fig. 2, Supplementary Table S3). According to this phylogenetic analysis, the PvHsfs were clustered into three classes, namely A, B (PvHsf29–PvHsf42), and C (PvHsf43–PvHsf51). The class A PvHsf was the largest group and consisted of 28 members, including PvHsf01-PvHsf28, which can be further classified into nine subclasses. In class A, no switchgrass was identified as orthologous to *Arabidopsis* HsfA7 and HsfA8. Class B consisted of 14 members that can be classified into three subclasses, and no switchgrass gene was found to be orthologous to the Arabidopsis HsfB3. Class C only contained nine members comprising two subclasses (Fig. 2, Supplementary Table S3). This result implied gene expansion as well as gene deletion in a rarer case occurred during the evolutionary process in switchgrass.

Multiple sequence alignment analysis of 51 PvHsf proteins showed that every *P. virgatum* Hsf protein contained the DBD domain, including three α helices and four β sheets at N-terminal (Fig. 3). The DBD domain of PvHsfs is highly conserved, with 42 PvHsfs composed of 90 aa and the rest nine PvHsfs of 83 to 94 aa due to insertion and deletions (InDels) in different positions (Fig. 3). For example, PvHsf47 missed seven aa in the α1 helical structure, while PvHsf21contained eight additional aa inserted between the α1 helix and the β1 fold. These InDels occurred during the evolutionary process resulting in genetic variations besides Hsf expansion in switchgrass.

Structure and motif analysis of PvHsf proteins

To study the structural diversity of *PvHsf* genes, the exon/intron organization of each *PvHsf* gene was analyzed by comparing its CDS to its full gene sequence. As shown in Fig. 4, the number of introns

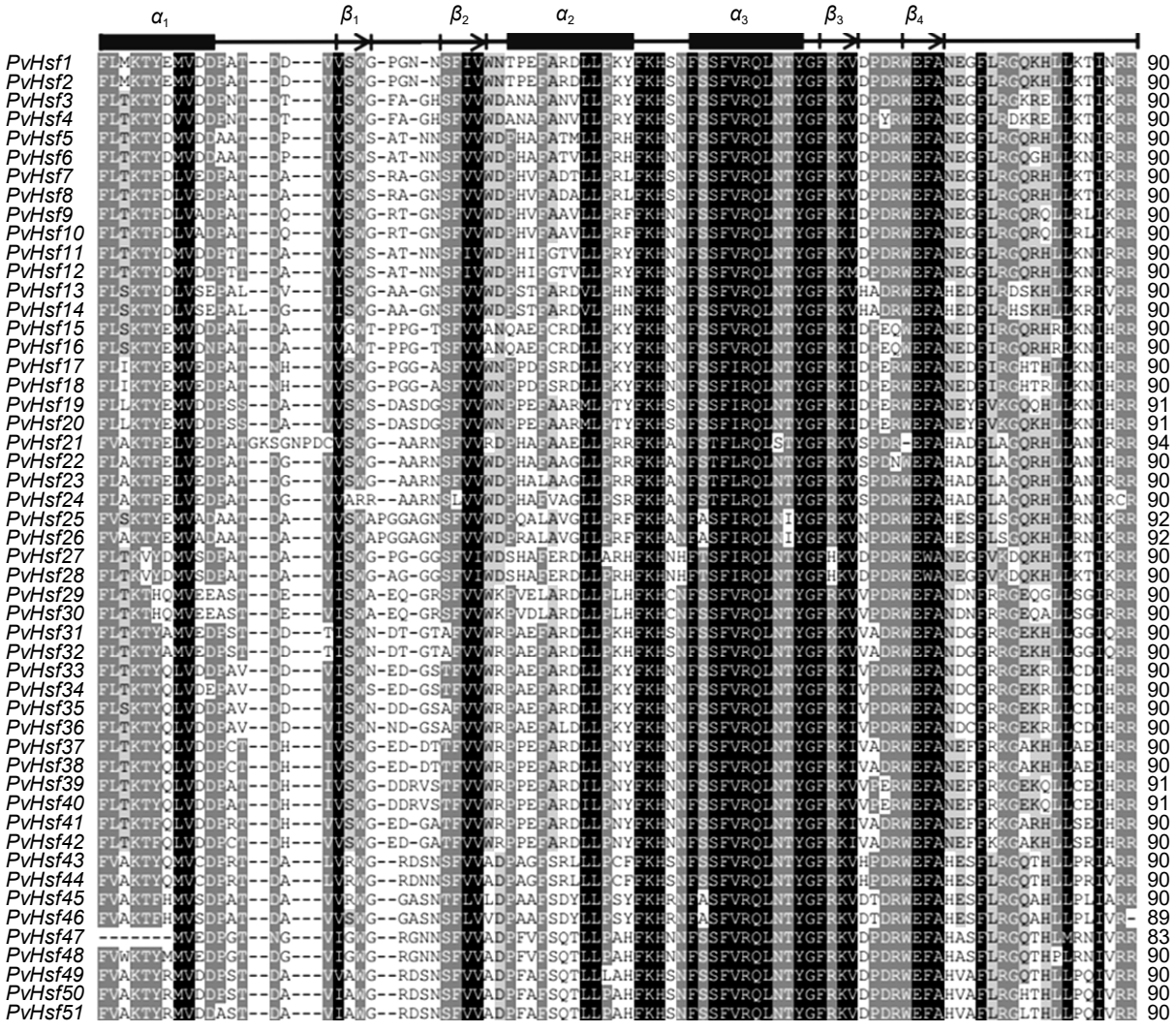

Fig. 3 Multiple sequence alignment of the DBD domains of Hsfs in switchgrass. The secondary structure elements of DBD (α1-β1-β2-α2-α3-β3-β4) are shown above the alignment. Rectangles represent α-helices and arrows represent β-sheets.

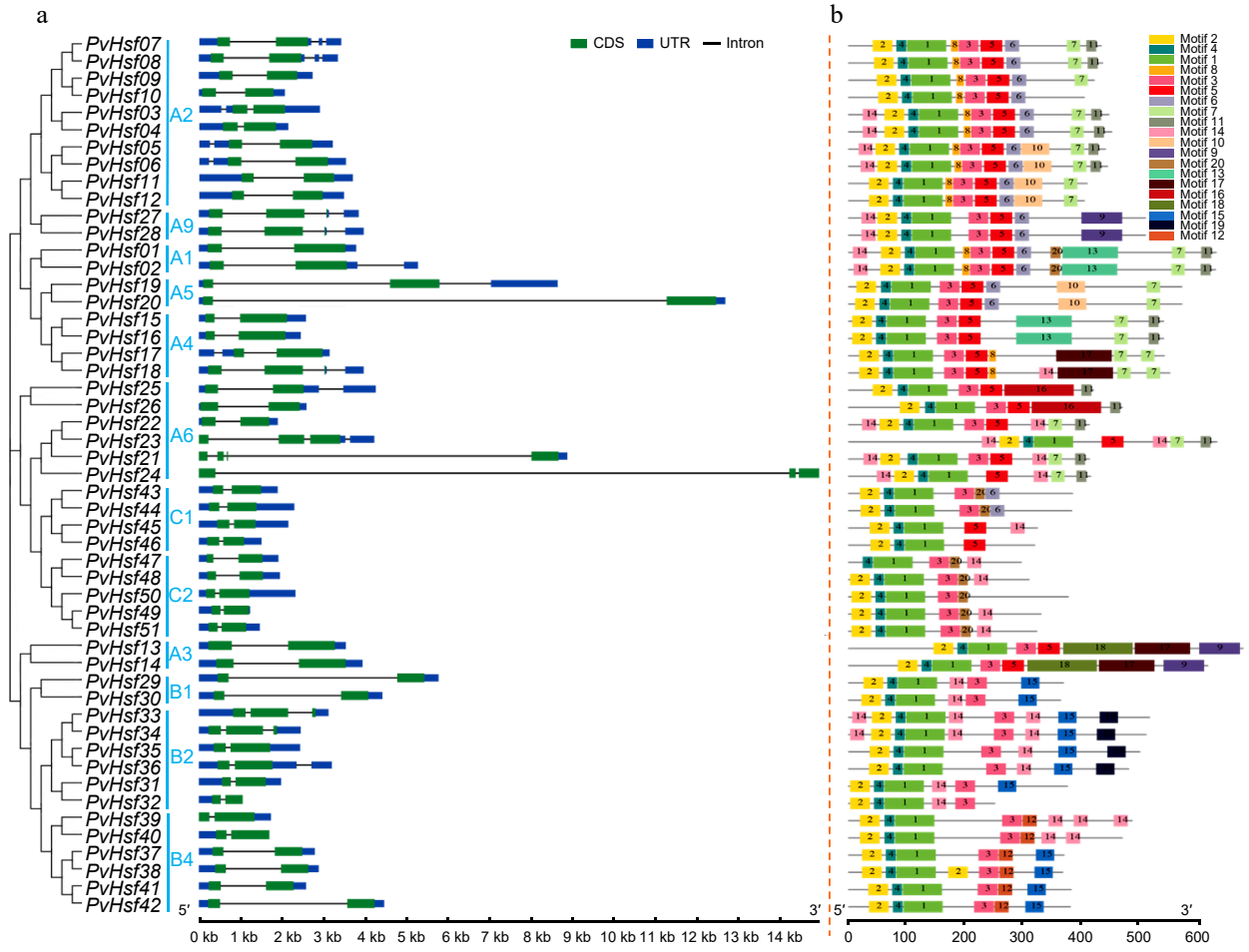

Fig. 4 Phylogenetic relationship, exon-intron structure, and conserved motifs of the Hsf family in switchgrass.

of 51 *PvHsfs* varied from one to three, while all nine class C (*HsfC*) genes, 11 *HsfB*s, and 15 *HsfC*s contained only one intron.

Twenty motifs were detected in PvHsf proteins (Fig. 4b, Supplementary Table S4). The DBD domains, composed of motif 4, motif 1, and motif 2, were the most conserved ones in nearly all of the 51 PvHsf members with only one exception for PvHsf47 in the absence of motif 2. The Coiled-Coil motif (motif 3), a unique motif forming the HR-A/B regions within the three classes, was found in most PvHsf members except PvHsf23, PvHsf24, PvHsf45, and PvHsf46. The AHA motif (motif 7), NLS motif (motif 6), and NES motif (motif 11) were found in most class A members but not in classes B and C (Fig. 4b).

Expression profile of PvHsfs at different developmental stages and in various organs

The RNA-seq data (FPKM values) of 51 *PvHsf* genes at seven developmental stages (days after pollination, DAP) and in 14 organs were downloaded from the PviUTs database[34]. According to the cluster analysis of FPKM values, the expression profiles of 51 *PvHsf* genes were divided into three distinct groups on the heatmap of developmental stages or of various organs (Fig. 5). All 16 *PvHsf* genes in group 1 showed high expression levels at initial developmental stages (0–10 d after pollination, DAP), 18 genes in group 2 were highly expressed at 15–20 or 25 DAP and the expression of 17 genes in group 3 were consistently upregulated with the peak at 30 DAP (Fig. 5a). The differential expressions at developmental stages indicated that some *PvHsfs* might be involved in seed development. There were 16 *PvHsfs* clustered into group 1 because of high expression level in floral organs, 21 *PvHsfs* in group 3 (i.e., *PvHsf 27/28/14/45/17/18/43/46*) were highly expressed in vascular tissue (Fig. 5b). Specifically, the expression level of *PvHsf51* was highest at 30 DAP and was also highly expressed in roots.

qRT-PCR expression analysis of *PvHsf* genes under abiotic stresses

The expression patterns of *PvHsf* genes were analyzed after NaCl, PEG, ABA, and $CdCl_2$ treatment. Nearly all 51 *PvHsf* genes' expression levels increased after NaCl, PEG, and ABA treatments, but only a few increased after the $CdCl_2$ treatment (Fig. 6). For example, *PvHsf09* and *PvHsf10* in group 1 were highly expressed after 1 h of treatment, and the 40 *PvHsfs* in group 2 reaching peak value after 12 h and the remaining nine genes were continuously up-regulated by NaCl treatment (Fig. 6a). Under 24 h of PEG treatment, a total of 48 *PvHsf* genes in group 1 were up-regulated to their peak expression at 6 or 12 h, while the remaining three *PvHsf* genes, such as *PvHsf37/38/42*, in group 2 were consistently up-regulated (Fig. 6b). *PvHsf24*, the only gene in group 2, was consistently up-regulated by 24 h after ABA treatment (Fig. 6c). *PvHsf09* and *PvHsf10* in group 1 were rapidly induced after 1 h of ABA treatment (Fig. 6c). The remaining 48 *PvHsf* genes were clustered into group 3, and their expression levels were up-regulated to their peak at 6 h of treatment (Fig. 6c). For $CdCl_2$ treatment, the relative expressions of 51 *PvHsf* genes were clustered into two groups, namely group 1 and group 2. Thirteen *PvHsfs* in group 1 were up-regulated, including *PvHsf3*, *4*, *7*, *8*, *19*, *21*, *23*, *24*, *30*, *35*, *36*, *44*, and *45*, while *PvHsfs* in group 2 were down-regulated by $CdCl_2$ treatment (Fig. 6d).

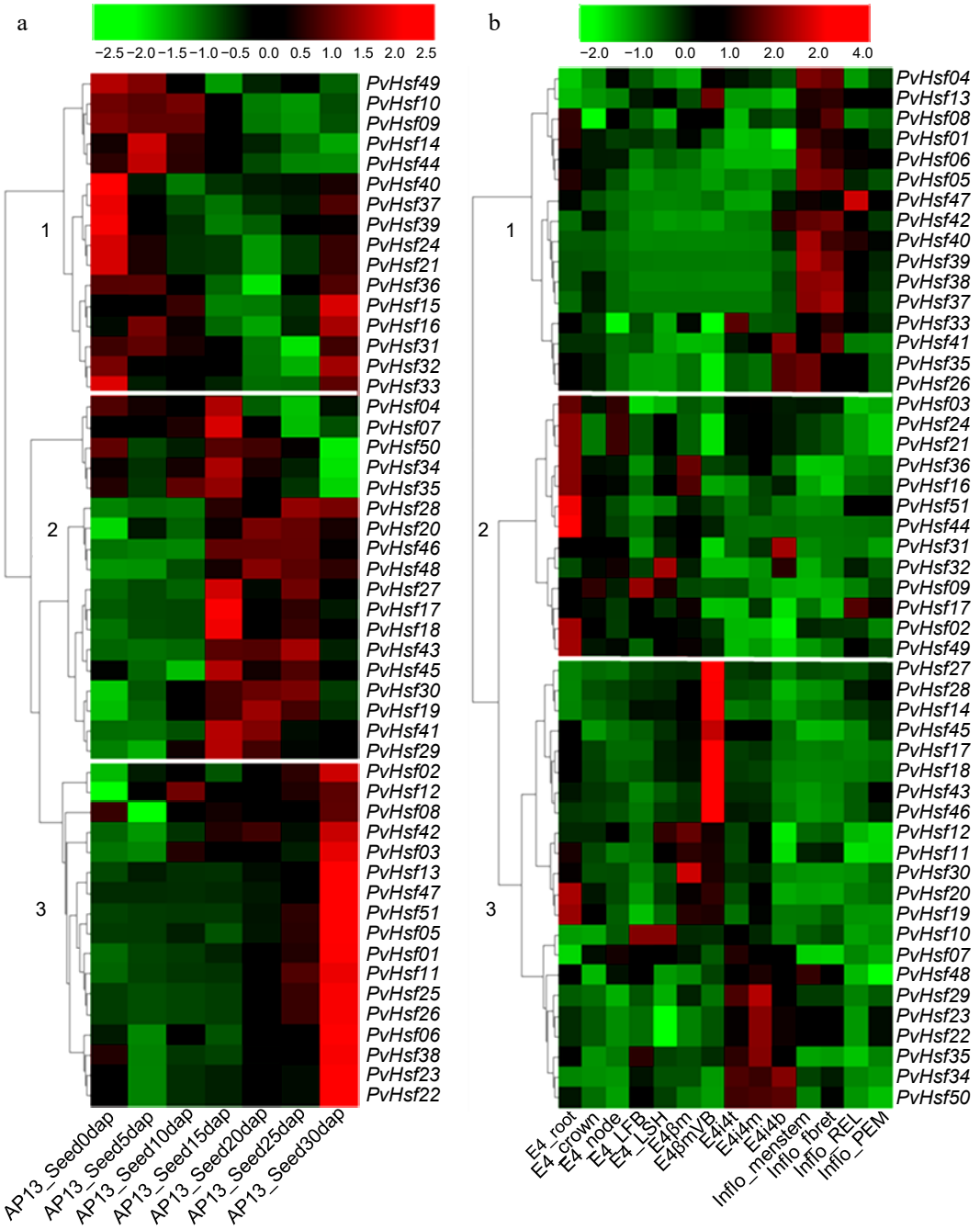

Fig. 5 The expression of *PvHsf* genes in different tissues, (a) organs, and (b) developmental stages in switchgrass. (a) Relative expression of *PvHsf* genes in floral organs days after pollination (DAP). (b) Relative expression of *PvHsf* genes in root, crown, node, leaf, leaf sheath, vascular tissues, stem, etc.

Identification of *PvHsfs* as a potential positive regulator in Cd stress

To screen potential *PvHsfs* regulating Cd tolerance, $CdCl_2$-inducible genes were expressed in yeast mutant strains Δ*ycf1* that was susceptible to Cd stress. The differentially expressed PvHsfs were selected based on the transcriptome data was sourced from previous work[31] and qPCR results obtained in this study (Fig. 6d). As shown in Fig. 7, the Δ*ycf1* cells harboring *PvHsf16* and *PvHsf3* exhibited remarkably enhanced growth when compared with the *GUS* control on the SD/-URA agar medium with 100 μM $CdCl_2$. For the rest *PvHsf* genes, no significant effect was observed on Cd tolerance of the yeast mutants compared to the *GUS* control (Fig. 7), indicating that *PvHsf16* and *PvHsf3* might positively regulate Cd-tolerance.

Subcellular localization and functional characterization of *PvHsf16*

The potential function of PvHsf16 in Cd-tolerance was then evaluated. When expressed in *N. benthamiana*, the fluorescent signal of PvHsf16-GFP merged with the nuclear marker signal, indicating that PvHsf16 was subcellularly localized to the nucleus (Fig. 8a). Eleven transgenic Arabidopsis with overexpressed *PvHsf16* (abbreviated as OE lines) were then generated and three liens, e.g., OE-5/-6/-8 with the highest expression levels of the *PvHsf16* were selected for further analysis (Supplementary Fig. S1). By growing the wildtype (WT) and OE lines in a plate containing 1/2 MS with supplementation of 0, 90 μM, and 180 μM $CdCl_2$, the OE lines demonstrated similar growth trait under 1/2 MS, but enhanced Cd tolerance as

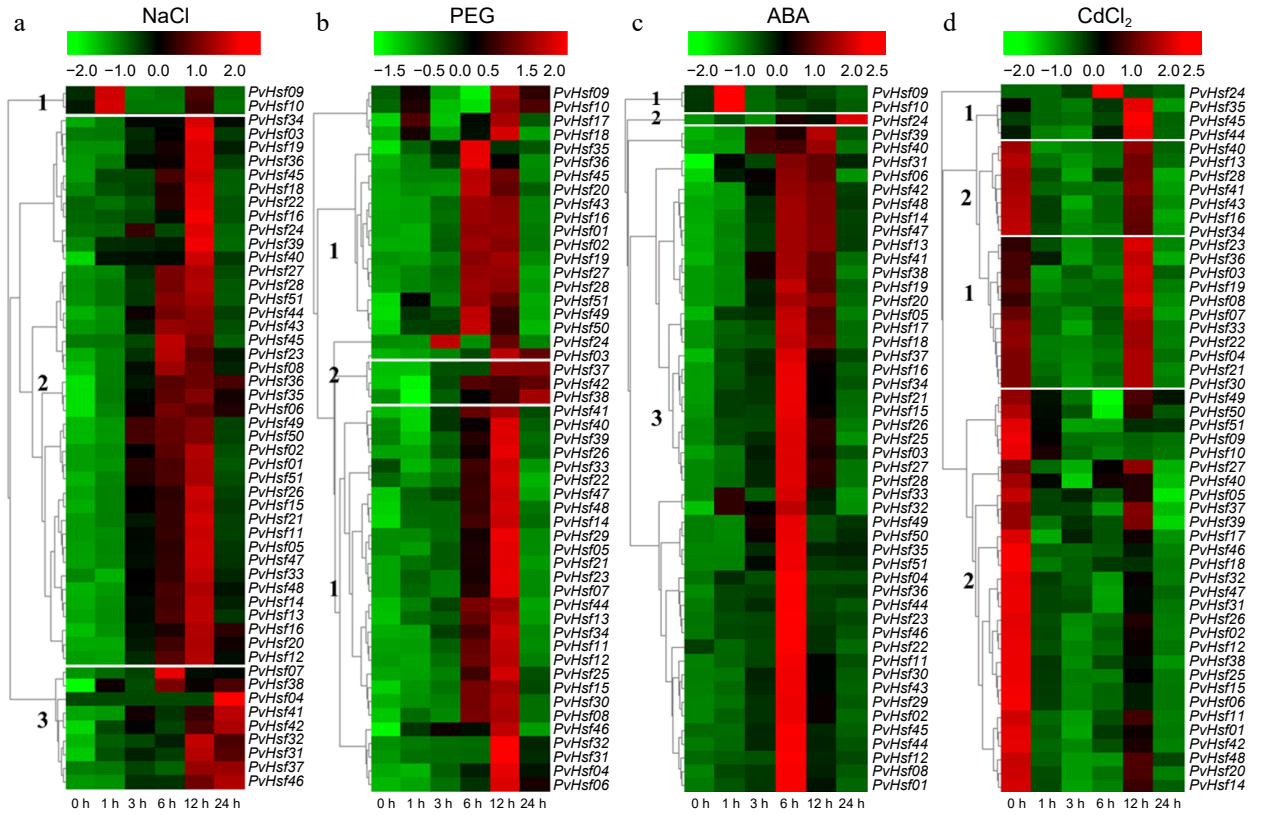

Fig. 6 qRT-PCR analysis of the *PvHsf* gene expression in switchgrass leaves in response to (a) NaCl stress, (b) PEG stress, (c) ABA stress, and (d) $CdCl_2$ stress. The expression of PvHsfs was analyzed at 0, 1, 3, 6, 12, and 24 h under stress treatment.

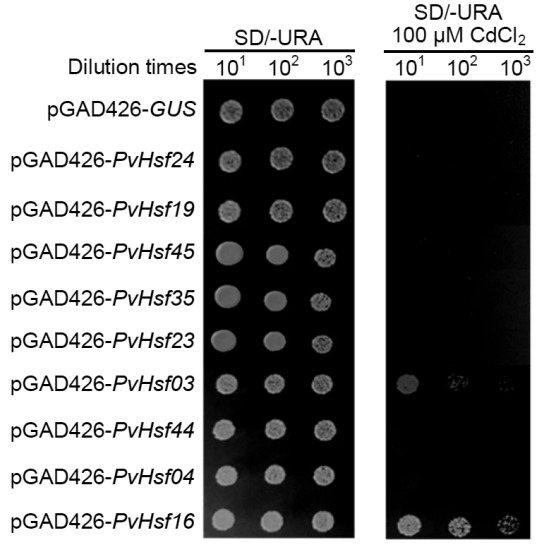

Fig. 7 Functional evaluation of *PvHsfs* in Cd-tolerance using Cd-sensitive yeast.

evidenced by the significantly higher Chl content, root length, and biomass than the WT plants under $CdCl_2$ treatment (Fig. 8b–e).

Discussion

Cd is one of the most toxic heavy metals in the environment, and its pollution has become more severe over the past decade[18,35]. Establishing vegetation cover on Cd-contaminated land is an effective strategy for phytoremediation of Cd-contaminated soil[36]. Switchgrass (*Panicum virgatum* L.) is a C4 perennial grass species that has been identified as a prime candidate crop for phytoextraction of heavy metals because of its broad climatic tolerance, rapid growth rate, and high biomass yield on marginal lands[24–26,35]. Previous studies have shown that switchgrass exhibited relative tolerance to Cd stress, despite growth inhibition by Cd[27,31]. The Hsfs family enables plants to withstand various abiotic stresses, such as heat, drought, salt, and Cd[37,38]. The availability of genome databases has facilitated the genome-wide characterization of Hsf and other gene families[39,40]. This study presented the first genome-wide overview of the *Hsf* gene family in switchgrass and identified potential *PvHsf* genes positively regulating switchgrass Cd-tolerance.

The *Hsf* gene family were found in most eukaryotes but experienced dramatic expansion in the sessile plants. *Drosophila melanogaster* and *Caenorhabditis elegans* contain only one member each, while yeast has only one Hsf and three Hsf-related proteins. In higher plants, the Hsf family comprises a much larger family of genes derived from a complex, plant-specific superfamily found across various species[3–8]. For example, Arabidopsis contains 21 Hsf members, rice contains 25[1,41], and soybean contains 52 Hsf members[6]. Although Hsfs vary in sequence size, eukaryotic Hsfs generally exhibit a typical structure that includes a conserved DNA binding domain (DBD) and an oligomerization domain (OD or HR-A/B). The DBD is a highly conserved region characterized by a helix-turn-helix structure, allowing it to specifically recognize and bind to heat shock elements (HSE) in the promoters of target genes, thereby activating the transcription and expression of stress resistance genes[41]. The OD domain, also known as HR-A/B, consists of two hydrophobic heptapeptide repeat regions and possesses a helical coiled-coil spatial structure. Therefore, we predicted the PvHsfs using the conserved DBD and OD domain and identified 52 *PvHsf* genes through genome-wide analysis.

Plant Hsfs can be divided into three classes, A B, and C based on the number of inserted amino acids in the HR-A/B region[42]. In

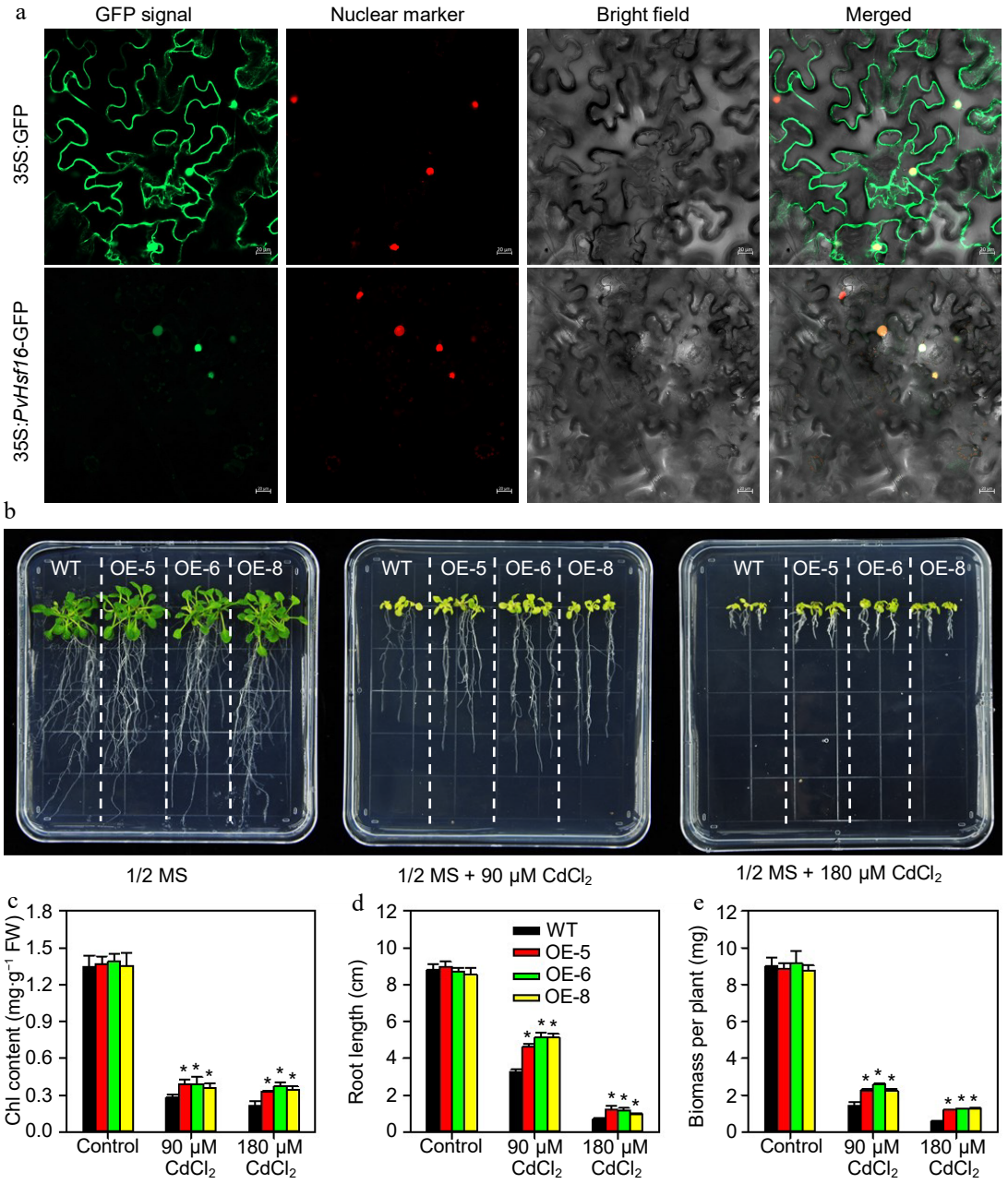

Fig. 8 Subcellular localization of *PvHsf16* and analysis of transgenic Arabidopsis over-expressing *PvHsf16*. (a) Subcellular localization of *PvHsf16*-GFP fusion protein in leaves of *N. benthamiana*. (b) Phenotypes of *PvHsf16*-overexpressed *Arabidopsis* (OE) lines under $CdCl_2$ stress. (c)–(e) Chl content, root length, and biomass of *PvHsf16*-OE lines under $CdCl_2$ stress. Data (n = 4) of transgenic lines marked with * in (c)–(e) represent significant difference at $p \leq 0.05$ compared to WT under each treatment.

switchgrass, we were also able to divide PvHsfs into class A–C, namely PvHsfAs, PvHsfBs, and PvHsfCs. Gene features, including exon-intron distribution, gene length, and GC content, significantly influence evolutionary events such as whole genome duplication (WGD). Numerous studies suggest that introns play a crucial role in gene expression and evolutionary processes. Most *Hsf* members of the Rosaceae family possess only one intron[39]. However, introns vary from zero to two in *Brassica oleracea*[43] and from zero to five in *B. napus*[44]. In this study, the number of introns in PvHsfs ranges from zero to three in switchgrass. For instance, *Hsf18/23/27/28* in class A and *Hsf33/34* in class B each contain two introns, while only *PvHsf21* in class A possesses three introns. Notably, all *PvHsfs* in class C contain solely one intron. As previously mentioned, class C *Hsfs* are the smallest families[1]. These reports provide substantial evidence for the tight conservation of class C and moderate conservation of class B members; however, evolutionary events are most frequently observed among class A members. Moreover, even though the DBD domain of PvHsfs is highly conserved, nine PvHsfs had deleted and inserted aa in different positions of the DBD domain. It is still unknown whether and how InDels in the DBD domains might affect the DNA-binding preference and binding efficiency of these Hsfs, which is worth further study into how these variations might contribute to the plasticity and successful adaptation of switchgrass in different environmental niches.

Plants frequently encounter various abiotic stresses, such as high temperature, drought, salinity, and heavy metal stress. Plant Hsf transcription factors are crucial for the response to abiotic stress. For example, Arabidopsis AtHsfA1a and AtHsfA1b mitigate high-temperature damage by regulating the synthesis of heat shock proteins (Hsp)[45]. In plants such as rice and strawberries, genes

including *OsHsfA2*, *OsHsfA4*, *OsHsfA7*, *OsHsfA9*, *OsHsfB2b*, and *FvHsfA2a* are activated by high salinity stress, thereby improving salt tolerance[46,47]. AtHsfA1b not only mediates the expression of the galactinol synthase gene *GolS1*, but also regulates downstream drought-responsive elements *DREB2A* and *DREB2C*, thereby enhancing plant drought resistance[48]. For instance, AtHsfA3 directly interacts with the *DREB2A* and *DREB2C* elements in the promoters of *Hsp18.1*-CI, *Hsp26.5*-II, and *Hsp70*, and thereby activating their promoter activities[49]. Regarding heavy metal (i.e., Cd) stress regulation, the identification of Hsf family members in switchgrass remains limited. In this study, it was found that the expressions of *PvHsf* genes are directly involved in abiotic stress signaling pathways. The transcriptions of *PvHsf* genes were regulated by NaCl, PEG, ABA, and $CdCl_2$ treatment. Notably, the expression of most *Hsf* genes suddenly decreased under abiotic stress at 24 h compared to 12 h. This sudden decrease could likely be attributed to regulatory feedback mechanisms that activate after an initial response to stress, leading to a reduction in *Hsf* gene expression as part of a negative feedback loop. Consequently, more sampling times should be incorporated between 12 and 24 h to further investigate the expression patterns of *Hsfs* under these abiotic stress conditions. Notably, nine $CdCl_2$-inducible *PvHsfs*, including *PvHsf3*, *PvHsf4*, *PvHsf16*, *PvHsf19*, *PvHsf23*, *PvHsf24*, *PvHsf35*, *PvHsf44*, and *PvHsf45*. Their potential regulatory role in Cd-tolerance was tested using the yeast heterologous system and two potential regulatory genes *PvHsf3* and *PvHsf16* identified. It is notable that the result of the yeast system is only preliminary that relies on the precondition of an existence of a conserved signaling pathway across plants and yeast. Therefore, it is necessary to further test the selected genes' function in plants. The ectopic expression of *PvHsf16* in Arabidopsis further confirmed its role in plant Cd-tolerance. Analyzing Cd concentrations in diverse tissues of transgenic Arabidopsis is crucial for elucidating the regulatory role of *PvHsf16* in Cd stress. Genetic transformations of *PvHsf16* are currently being performed, specifically overexpression and RNA interference, in switchgrass. Subsequently, alterations in biomass, reactive oxygen species content, and Cd accumulation in transgenic switchgrass will be assessed to investigate how *PvHsf16* regulates Cd stress. Together, these results imply that *PvHsf16* could be used as a candidate gene for Cd- tolerance in the molecular breeding of switchgrass.

Conclusions

Through genome-wide Hsf gene family analysis, a total of 51 *PvHsf* genes were identified and characterized in switchgrass. This analysis led to the identification of two genes, *PvHsf16* and *PvHsf3*, potentially positively regulating switchgrass Cd tolerance. Knowledge gained from this study will facilitate further functional analysis of *PvHsfs,* particularly *PvHsf16,* for understanding the mechanism of Cd tolerance and for molecular breeding of switchgrass and related grass species.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Zhang J, Xu B; data analysis: Song G, Guan H, Fang Z, Ji Y; draft manuscript preparation: Zhang J, Xu B. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Conflict of interest

The authors declare that they have no conflict of interest.

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