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ORIGINAL PAPER

Genome-wide analysis of HSF family transcription factors and their responses to abiotic stresses in two Chinese cabbage varieties

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Abstract

Key message **HSF family transcription factors were analyzed in genome of Chinese cabbage. Chromosomal locations showed that duplication might result in expansion. Response to abiotic stresses was elucidated in Chinese cabbage varieties.**

Abstract The major heat shock factors regulating the heat stress response are heat shock transcription factors (HSFs), which interact with heat shock elements. In this study, Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) HSFs were comprehensively analyzed. A total of 52 *HSF* family genes were identified for phylogenetic relationships and motif analysis based on the genome sequence of Chinese cabbage. All HSFs were divided into classes A, B, and C. The chromosomal locations and gene duplications of these HSFs were also presented. Nine potential duplication events were found in Chinese cabbage chromosomes. Expression of three *HSF* genes in two varieties of Chinese cabbage using quantitative real-time PCR revealed that *BraHSF039* and *BraHSF043* were up-regulated under temperature and salt stresses treatments, and only *BraHSF043* gene was also down-regulated under salt stress in 'Lubaisanhao'. *BraHSF001* gene was down-regulated in the 'Lubaisanhao' variety under heat and cold stresses, under drought stress in 'Qingdao 87-114'. These results can serve as a foundation for further studies on HSFs in *Brassica*.

Keywords HSFs · Abiotic stress · Gene duplication · Quantitative real-time PCR · Chinese cabbage

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J. Ma and Z.-S. Xu contributed equally to this work.

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Introduction

Global warming reduces the production of various crops. Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) belonging to family Cruciferae is an economically important global vegetable, especially in Asia (Qi et al. 2010; Zhang et al. 2000). Thus, new accessions of Chinese cabbage resistant to heat stress shall be developed. 'Lubaisanhao' and 'Qingdao 87-114' are two important Chinese cabbage varieties with high yield, good quality, and disease resistance. 'Lubaisanhao' can be planted in relatively low-temperature environments, and 'Qingdao 87-114' can grow in Nanjing district, southern China (Li et al. 2013).

Abiotic stresses such as high salt, cold, heat, etc., can affect plant growth and crop yield (Yokotani et al. 2013). Transcription factors have been demonstrated to respond to stress in higher plants (Yamaguchi-Shinozaki and Shinozaki 2006; von Koskull-Döring et al. 2007; Zhuang et al. 2011; Cai et al. 2012). High temperature, one major abiotic stress, during reproductive stages can decrease seed setting and production by affecting pollen development and pollination (Zou et al. 2009; Jin et al. 2013). To survive high temperature, many heat-responsive genes combined with transcription factors are induced to protect plants from heat shock (HS) stress (Mittler et al. 2001; Rizhsky et al. 2002;

Chauhan et al. 2011). The most important regulators of heat-responsive genes are HS transcription factors (HSFs) (Wu 1995), which widely exist in both plants and animals to regulate the expression of HS proteins (HSPs). HSPs can act as molecular chaperones and play an essential role in HS response. They protect cells against heat damage and functions in protein folding as well as the intracellular distribution and degradation of other proteins (Schöffl et al. 1998; Hartl and Hayer-Hartl 2002; Morimoto et al. 1994).

Similar to other transcription factors, HSFs contain a highly conserved DNA-binding domain (DBD) at the N-terminus having a helix-turn-helix structure, which is the best preserved domain in evolution (Döring et al. 2000; Morimoto et al. 1994; Damberger et al. 1994; Vuister et al. 1994). The DBD can also mediate the trans-activating capability of HSFs by modulating interaction with other factors (Bulman et al. 2001; Hayashida et al. 2011). Thus, DBD is the most important domain in HSF sequences. Another important domain contained in HSFs is hydrophobic heptad repeats (HR-A and HR-B), which form a coiled coil that can mediate the oligomerization of HSFs (Scharf et al. 2012; Wu 1995; Sorger and Nelson 1989). HSFs also have a nuclear localization signal required for nuclear import, which is a cluster of basic amino acids rich in arginine and lysine residues at the C-terminus (Lyck et al. 1997). The nuclear export signal (NES) is located at the C-terminal of some plant HSFs (Heerklotz et al. 2001; Scharf et al. 2012).

In this study, we carried out genome analysis of 52 HSFs in Chinese cabbage and divided the 52 HSF sequences into three major classes (class A, class B, and class C) based on the sequences of DBD-containing proteins. The proportion of Chinese cabbage *HSF* family genes of the three classes, gene duplications, and chromosomal locations were identified. Quantitative real-time RT-PCR (qRT-PCR) was also performed for two varieties of Chinese cabbage under three stress treatments (38, 4 °C, salt and drought). The results can help elucidate the function of HSFs in Chinese cabbage.

Materials and methods

Plant materials, growth conditions, and stress treatments

For RNA isolation, Chinese cabbage plants ('Lubaisanhao' and 'Qingdao 87-114' varieties) were grown in an incubator with a photoperiod of 14 h light and 10 h dark at 28 °C until they produced around four true leaves for subsequent stress treatments. For salt and drought treatments, four-leaf seedlings were incubated with NaCl (100 mM) and PEG 6000 (15 %). For temperature stress treatments, seedlings were exposed to 4 and 38 °C. After these different stress treatments, leaves were harvested at 0, 0.5, 1, 2, 4, 8 and 12 h, immediately frozen in liquid nitrogen, and then stored at −70 °C for RNA isolation.

Identification and analysis of *HSF* family genes in Chinese cabbage

The nucleotide and protein sequence of Chinese cabbage HSFs were identified based on the genome sequence (http://brassicadb.org) (Wang et al. 2011). The amino acid sequences of HSF proteins were aligned by the neighbor-joining method in ClustalX program (Chenna et al. 2003), and HSF protein-conserved motifs were defined by MEME suite (version 5.0) (Bailey et al. 2009). Then, a phylogenetic tree was constructed using MEGA (version 5.1) (Tamura et al. 2011). The theoretical isoelectric point (p*I*) was analyzed using ExPASy (Gasteiger et al. 2003). Protein statistics were analyzed using the Sequence Manipulation Suite (http://www.bio-soft.net/sms/). The RPSP program (http://biotech.ou.edu) was used to predict the solubility of recombinant proteins (Wilkinson and Harrison 1991). The database of the *Arabidopsis* HSF family was downloaded from the DATF web site (Guo et al. 2005). The *Populus trichocarpa* genome DNA database was downloaded from the DOE Joint Genome Institute web site (Tuskan et al. 2006). The rice HSF family database was downloaded from PlnTFDB (Riaño-Pachón et al. 2007). The synonymous substitution rate and synteny data were calculated by MicroSyn method (Cai et al. 2011).

RNA isolation and reverse transcription of cDNA

Total RNA was extracted using an RNA Simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The RNA concentration was reverse-transcribed, and first strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (TaKaRa, Dalian, China). cDNA was eventually diluted 20-fold for qRT-PCR analysis.

qRT-PCR analysis

Real-time RT-PCR was carried out using an Applied Biosystems 7500 Real-time PCR System with a SYBR Green Real-time PCR Kit (Novland, Shanghai, China). Each reaction contained 10 μL of 2× PCR Master Mix, 0.24 μL of NL *Taq* DNA polymerase, 2 μL of cDNA sample, and 500 nM gene-specific primer in a final volume of 20 μL. The PCR thermal cycle conditions were as follows: denaturing at 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 65 °C for 10 s. The experiments were repeated three times with independent

RNA samples of different Chinese cabbage varieties under different stress treatments. At the same time, the standard errors of mean among replicates and significant difference were calculated. In this study, three *HSF* family genes were selected to perform quantitative real-time PCR from three subgroups, respectively. The *BraHSF043* gene was selected from the class A; the *BraHSF001* gene was selected from the class B; the *BraHSF039* gene was selected from the class C (Supplementary Table 2). Primers were designed from unique regions of genes using Primer Premier5.0. The sequences of all primers are listed in Supplementary Table 1. All primers used for relative quantification were synthesized by Genscript Nanjing Inc. (Nanjing, China).

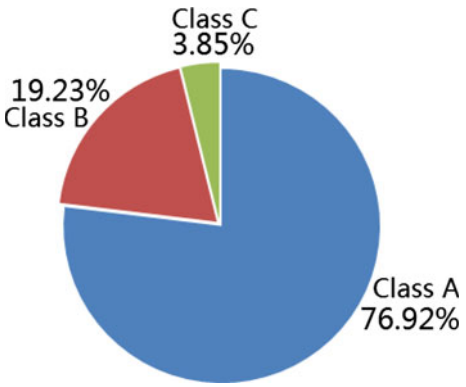

Fig. 1 Proportion of HSF classes in Chinese cabbage indicated in *three different colors* (color figure online)

Results

Identification and classification of *HSF* genes in Chinese cabbage

A total of 52 *HSF* genes were identified and described in Chinese cabbage, and the amino acid sequences were investigated by NCBI Blast program (Supplementary Tables 2–3). Each of the 52 genes contained an HSF DBD divided into three important representative classes (class A, class B, and class C). The proportion of each HSF class of Chinese cabbage was analyzed, and the results were as follows: the largest class was class A, having 76.92 % HSFs; the second largest class was class B, having 19.23 % HSFs; and the smallest class was class C, having 3.85 % HSFs (Fig. 1).

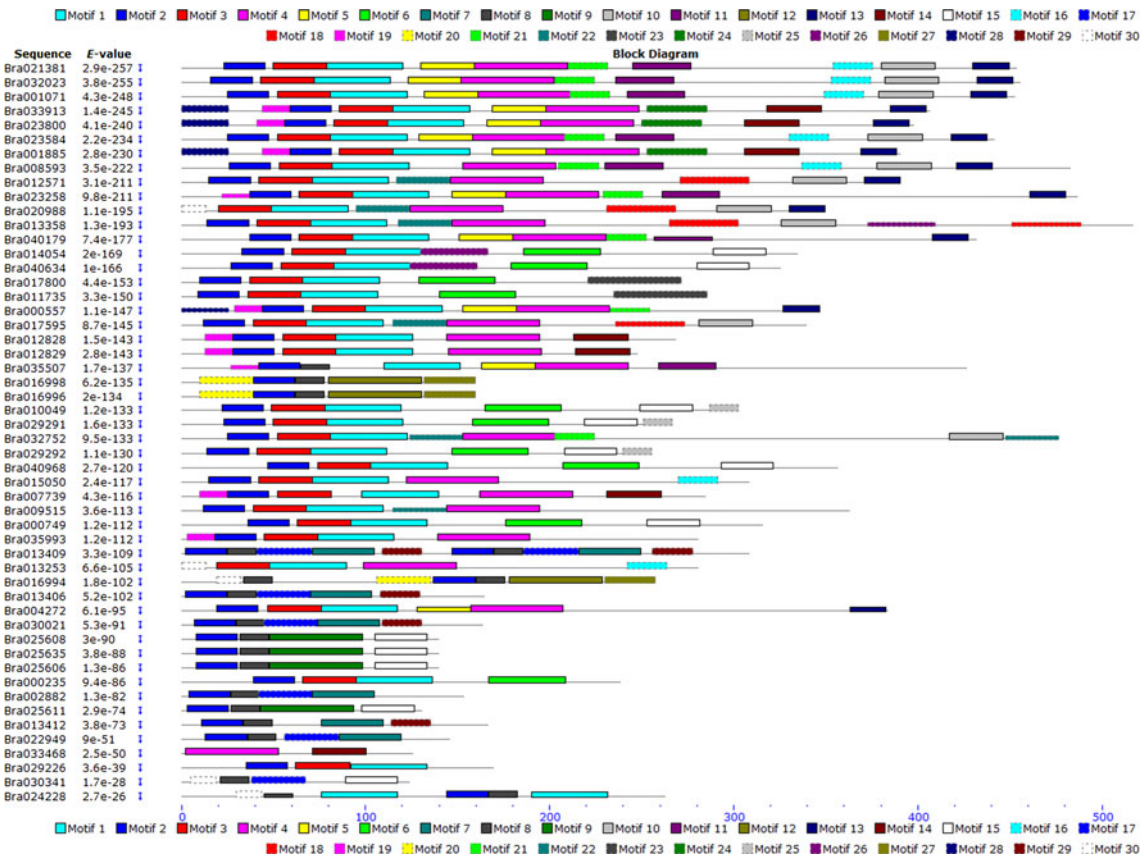

Fig. 2 Common motif of HSF family proteins in Chinese cabbage. HSF domains are represented by *striped boxes* (color figure online)

Fig. 3 Sequence logos of HSF domains in Chinese cabbage. The overall height of the stack indicates the level of sequence conservation. The height of residues within the stack indicates the relative frequency of each residue at that position

Characterization of deduced amino acid sequences of HSFs of Chinese cabbage and *Arabidopsis*

The physical and chemical characteristics of three classes of HSFs from Chinese cabbage were analyzed using the programs ExPASy (http://cn.expasy.org/) and RPSP (http://biotech.ou.edu). The theoretical p*I* of classes B and C was >5.0, but that of class A was >4.0 (Supplementary Table 4). The percentage of aliphatic amino acids was about twice the percentage of aromatic amino acids. The percentage of positive amino acids was almost as large as the percentage of negative amino acids. The percentage of positive amino acids of most HSFs from Chinese cabbage was a little more than the percentage of negative amino

acids. Theoretical prediction of protein solubility plays an important part in the structure and stability of a protein. The percentage of insoluble recombinant protein of most HSFs in class B was >80 %, which was larger than those of the other two groups; only three HSFs in class B were <80 % (BraHSF016, 63.9 %; BraHSF018, 72.3 %; and BraHSF026, 75.0 %). However, the percentage of insoluble recombinant protein of HSFs in class A ranged within 50–70 %, and only a few were >80 %. Furthermore, only two HSFs in class C were about 80 % (BraHSF021, 78.5 %; BraHSF039, 80.3 %).

Analysis of conserved domains (motifs) and phylogenetic relationship of BraHSFs

All motifs were identified by MEME using the complete amino acid sequences of 52 Chinese cabbage HSFs (Figs. 2, 3). Thirty motifs were identified on the 52 HSFs. Most HSFs have motifs 1, 2, and 3, all located at the N-terminal of HSFs. By contrast, motifs 10, 13, and 16 are located at the C-terminus. Moreover, motif 4 was located at the middle of most Chinese cabbage HSFs.

A neighbor-joining tree for phylogenetic analysis was constructed to reveal the phylogenetic relationship of these *Brassica* HSFs, and phylogenetic analysis was performed based on the full-length amino acid sequences of the N-terminal domains of HSFs. These *Brassica* HSFs were classified into three classes according to the classified *Arabidopsis* HSFs, namely, class A, class B, and class C (Fig. 4). Class A had the highest number of 40 HSFs; by contrast, only two HSFs were classified into class C. Moreover, class B had 10 HSF members. Similarly, other plants such as rice, *Arabidopsis*, and poplar also shared the same classification of HSFs. Class A contained the highest number of HSFs and class C contained the lowest number.

We summarized the HSFs of Chinese cabbage, *Arabidopsis*, rice, and poplar with numbers 52, 21, 25, and 28, respectively (Table 1). A total of 52 HSF family genes were identified in Chinese cabbage in these studies. By contrast, only 21, 25, and 28 HSF genes (about half the number of HSF genes in Chinese cabbage) were found in *Arabidopsis*, rice, and poplar, respectively. Class A was the largest group among the three classes, and class C had the lowest number of HSF genes in four plants.

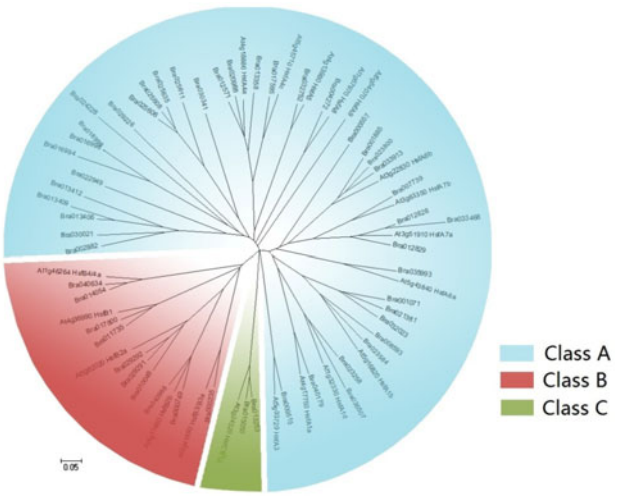

Fig. 4 An unrooted phylogenetic tree of HSFs in Chinese cabbage. The amino acid sequences of the DBD were aligned using ClustalW. The phylogenetic tree was constructed using MEGA5 (color figure online)

Mapping and duplication events of *HSF* genes in Chinese cabbage chromosomes

The Chinese cabbage *HSF* family genes were unevenly distributed in all 10 chromosomes (Fig. 4 and Supplementary Tables 2–3). A total of 12 HSF genes from all three different classes were located in chromosome 03, which had the highest number of *BraHSF* genes. Nine *HSF* genes, the second maximum number, were located in

Table 1 Summary of HSFs of Chinese cabbage, *Arabidopsis*, poplar, and rice

Plant classification	*B. rapa* subsp. *Pekinensis* number	*A. thaliana* number	*O. sativa* number	*P. trichocarpa* number
Class A	40	13	12	15
Class B	10	5	8	12
Class C	2	1	4	1
Soloist	0	2	1	0
Total HSF family factors	52	21	25	28
Total genes	41,174	27,228	40,577	45,654
Percentage of HSF genes (%)	0.1263	0.0771	0.0616	0.06133
Transcription factors	3,580	2,437	2,798	2,758
Genome size (Mb)	485	125	430	480
Average number of HSF family factors per Mb	0.1072	0.168	0.0581	0.0583

chromosome 01, and 10 *HSF* genes were located in chromosome 04. Chromosome 04 only had class A *BraHSF* family genes. By contrast, chromosomes 05 and 06 only had one *BraHSF* family gene, respectively, which had the lowest number of genes among all 10 chromosomes.

Several large chromosomal fragments lacked *BraHSF* family genes. For example, no *HSF* genes were present in the long arm of chromosome 05 and the short arm of chromosomes 06, 07, 09, and 10. Furthermore, no *HSF* genes were found at about 20 Mb of the long arm of chromosomes 02 and 06, at 14 Mb of the long arm of chromosome 07, and at 10 Mb of chromosome 08. Different from the absent *BraHSF* gene fragments, some gene clusters existed in several chromosomes, e.g., A01, A03, and A04. Several *HSF* genes all belonging to class A were clustered within a short distance. A segment ($\sim$2.8 kb) on A01 contained three *HSF* genes, and two similar segments located at the long and short arms of A04 contained three clustered HSF genes.

Some potential duplicate genes were predicted according to the synonymous substitution rate and synteny data (Supplementary Tables 5–6). Nine potential duplication events were marked in Fig. 5. Eight pairs of duplicate genes belonged to class A, and only one pair was from class B. Interestingly, two appearances of three *HSF* genes pairwise duplicated with each other were found among A01, A03, and A05, as well as A01, A03, and A08. The special duplication involved the following genes: *BraHSF003/BraHSF024/BraHSF043* and *BraHSF008/BraHSF020/BraHSF037*.

Expression analysis of *BraHSF* family genes under stress treatments in two Chinese cabbage varieties

Three selected genes [*BraHSF001* (class B), *BraHSF039* (class C), and *BraHSF043* (class A)] were chosen from three classes for real-time gene expression analysis to confirm the stress responsiveness to abiotic stress in two Chinese cabbage varieties. Four stress treatments: high temperature, low temperature, high salt, and drought were examined in *HSF* genes in the leaves of Chinese cabbage. The results are shown in Fig. 6a–d. Most of these *BraHSF* genes showed up-regulated expression under four different stress treatments. The *BraHSF001* gene was down-regulated under cold and heat stress treatments in 'Lubaisanhao'. The *BraHSF043* gene was also down-regulated under salt stress in 'Lubaisanhao'. However, in 'Qingdao 87-114', the *BraHSF001* was down-regulated under drought stress. Other genes were up-regulated at different levels under the four stress treatments in the two varieties of Chinese cabbage. The expression profiles of two varieties under these four treatments were not the same.

Discussion

Chinese cabbage is an important vegetable in eastern Asia. Abiotic stresses are major factors limiting vegetable production because of global population increase and modern industries, which influence the balance of the ecological environment. Abiotic stresses especially heat stress

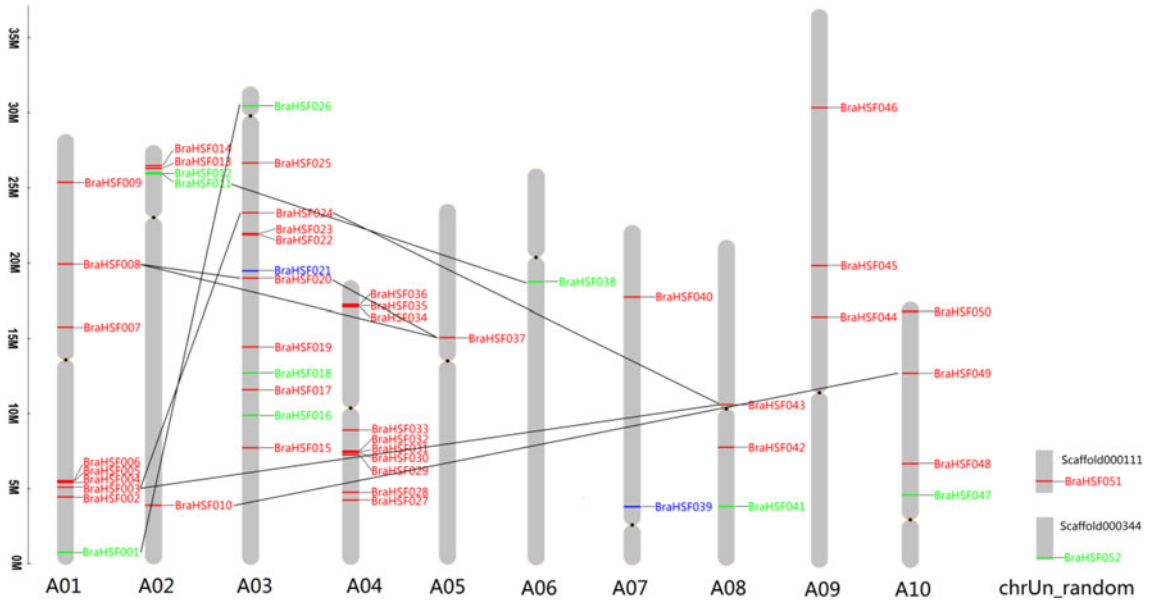

Fig. 5 Chromosomal locations and predicted clusters of *HSF* genes in Chinese cabbage

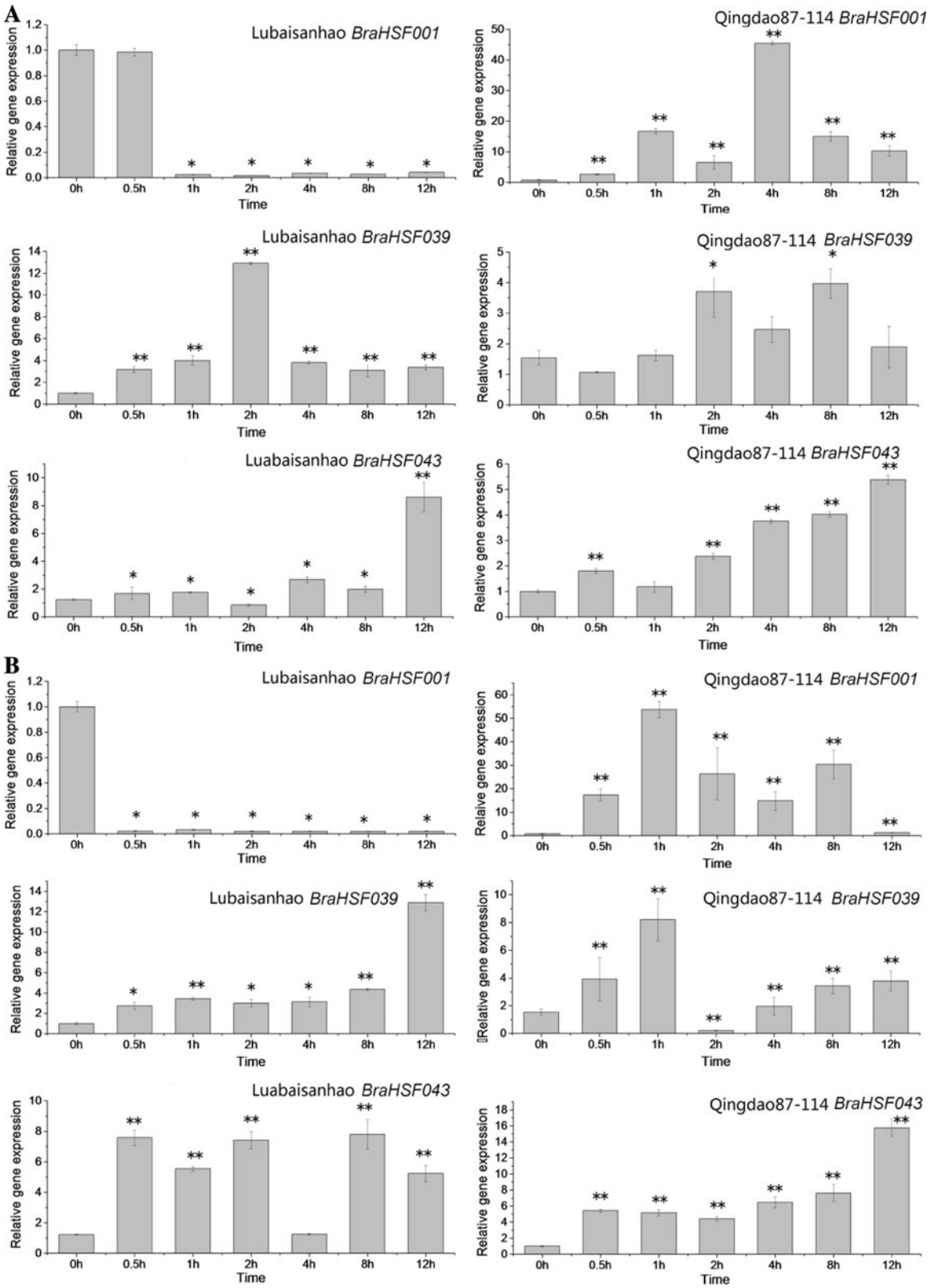

Fig. 6 Expression patterns of *HSF* genes in Chinese cabbage under heat, cold, salt and drought treatments. **a** Expression patterns at 38 °C. **b** Expression patterns at 4 °C. **c** Expression patterns under salt treatment. **d** Expression patterns under drought treatment. The values with significant differences between the control (0 h) and stress treatments are indicated by *asterisks* (*$P \leq 0.05$, **$P \leq 0.01$)

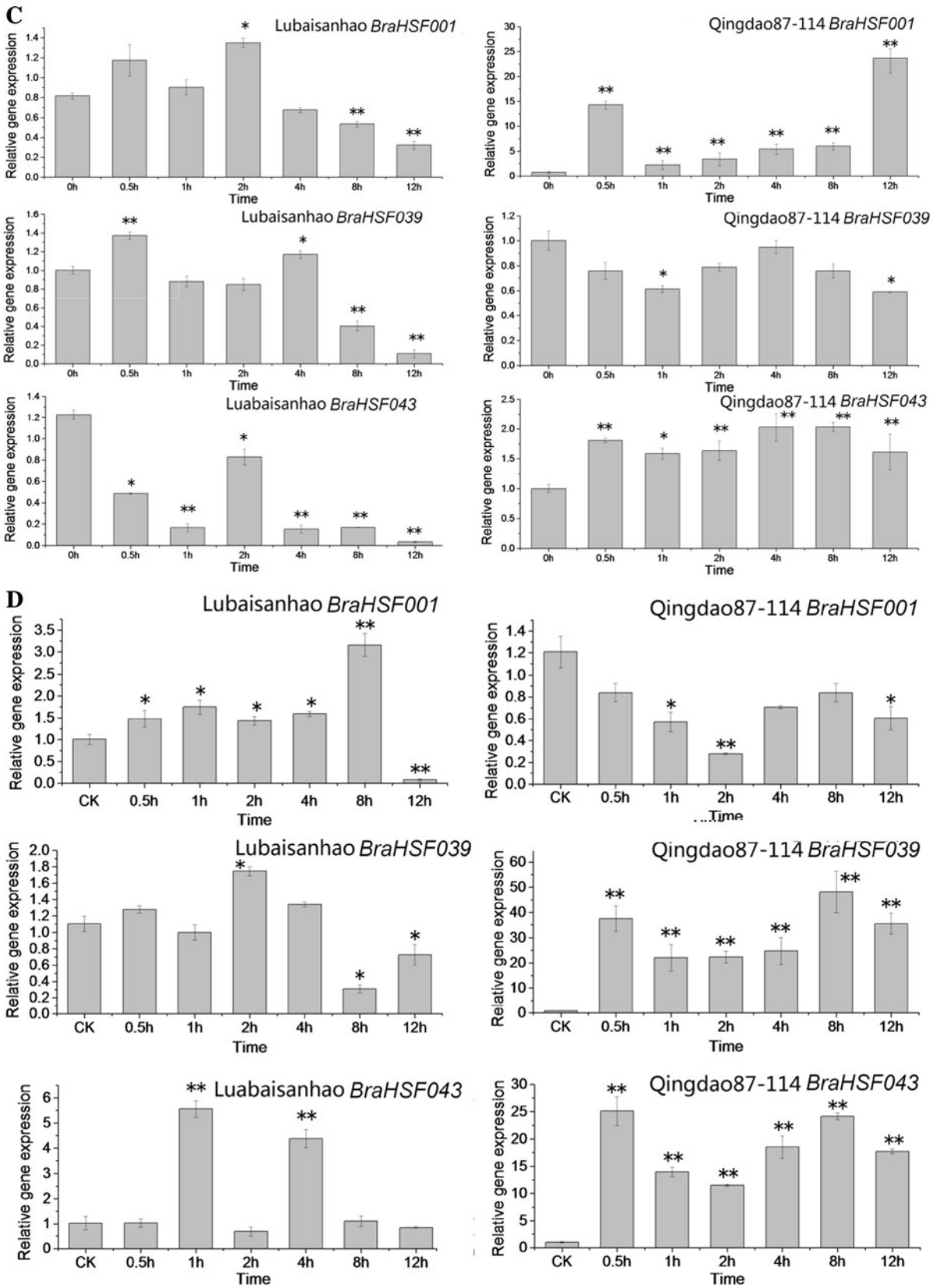

Fig. 6 continued

increasingly harm vegetable production and quality. Most kinds of vegetables including Chinese cabbage have low heat resistance, leading to the off-season at the end of spring and the beginning of summer.

Heat tolerance can be manifested in many aspects, including growth characteristics, cell and tissue structure, protein metabolism, as well as other physiological and biochemical processes (Rowe et al. 2013; Scharf et al. 2012; Mang et al. 2012). HSF is an important transcription factor family in plants responsible for plant high-temperature tolerance and interactions with HS elements in the promoter region of *HSF* genes (Nover et al. 2001; Åkerfelt et al. 2010; Hayashida et al. 2011). *HSF* genes are originally found in yeast but are also found in the polytene chromosomes of *Drosophila*, mammals, and plants (Sorger and Pelham 1988; Wiederrecht et al. 1988; Clos et al. 1990; Rabindran et al. 1991; Scharf et al. 1993). Based on *HSF* genes discovered so far, in-depth research and new technologies enable the identification of more *HSF* genes in various organisms. Previous research have revealed 21 HSFs in *Arabidopsis*, 25 in rice, 28 in *Populus*, 16 in *Medicago*, and 25 in maize (Miller and Mittler 2006; Guo et al. 2008; Lin et al. 2011; Wang et al. 2012). The involvement and reaction of HSFs under high temperature and other stress treatments were comprehensively investigated in *Arabidopsis*, rice, and tomato (Guo et al. 2008; Chauhan et al. 2011; Yoshida et al. 2011; Hahn et al. 2011; Mishra et al. 2002). Nevertheless, few reports on HSFs in Chinese cabbage have been reported. In this study, 52 *BraHSF* genes were thoroughly analyzed for phylogenetic relationships, motifs, gene locations in chromosomes and duplication events. We also investigated the expression profiles of *HSF* genes in two varieties of Chinese cabbage under three different stress treatments. Results showed that *BraHSF* genes had various characteristics and complicated expression mechanisms.

The Chinese cabbage genome size was twice as large as that of *Arabidopsis*, but HSFs in Chinese cabbage were two-fold greater than that in *Arabidopsis* (Table 1). *BraHSF* genes were distributed in all 10 Chinese cabbage chromosomes, and two special *HSF* genes were located at scaffold000111 and scaffold000344. However, the distribution of *BraHSF* genes was not even (Fig. 4). Some *BraHSF* genes in the short and long arms of chromosomes were also unevenly distributed. Previous studies have shown that gene duplication events play an important role in gene expansion and arrangement. We found nine potential duplication events of *BraHSF* genes on *Brassica* chromosomes, and all nine duplication events occurred within the same classes of *BraHSF* genes based on the phylogenetic tree classified into three classes. For example, *BraHSF001* and *BraHSF026* belonging to class B had a potential duplication relationship; *BraHSF010* and *BraHSF049* from class A also had the same manifestation. Interestingly, we found two appearances of three *HSF* genes pairwise duplicated with each other: *BraHSF003*/*BraHSF024*/*BraHSF043* and *BraHSF008*/*BraHSF020*/*BraHSF037*. Clusters of *BraHSF* genes were also found in Chinese cabbage chromosomes, such as *BraHSF004*, *BraHSF005*, and *BraHSF006* in the short arm of chromosome 01, as well as *BraHSF030*, *BraHSF031*, and *BraHSF032* in the long arm of chromosome 04. All these clustered spots occurred in class A. These results indicated that the network and duplication events of *HSF* genes of Chinese cabbage led to the expansion of *BraHSF* genes in Chinese cabbage chromosomes.

qRT-PCR was used to compare the expression of *HSF* genes in the leaves of Chinese cabbage under four different stress (heat, cold, salt and drought) treatments. Under salt and drought stresses, the expression of three genes from three different classes of the 'Lubaisanhao' variety was normal. The expression of all three genes had two obvious peaks, yet *BraHSF001* and *BraHSF039* had more similar expression profiles. However, different expression profiles appeared in the 'Qingdao 87-114' variety; *BraHSF043* was similar to *BraHSF039*, with a high expression level throughout the entire stress-treatment period. *BraHSF001* had two obvious peaks with the highest at 12 h. Meanwhile, several differences were observed between the two varieties under heat stress treatment. *BraHSF001* expression was down-regulated in the 'Lubaisanhao' variety but up-regulated in the 'Qingdao 87-114' variety, with a peak at the middle period (4 h). *BraHSF039* expression had one peak in the middle period (2 h) in the 'Lubaisanhao' variety but had two peaks in the 'Qingdao 87-114' variety. *BraHSF001* gene of the two varieties had the same expression profile, i.e., up-regulated with a peak at the last period (12 h). A complicated situation emerged after cold stress treatment. Except for *BraHSF001* in the 'Lubaisanhao' variety, the expression of all other genes was up-regulated to different extents. *BraHSF001* and *BraHSF039* in the 'Qingdao 87-114' variety were rapidly up-regulated with a peak at 1 h. *BraHSF039* expression in the 'Lubaisanhao' variety and *BraHSF043* expression in the 'Qingdao 87-114' variety slowly increased until reaching the peak at 12 h. *BraHSF043* always showed a relatively high expression level, but the phase suddenly declined at 4 h and then increased. The involvement of these *HSF* genes in response to different stresses revealed that the modulation of *HSF* genes in Chinese cabbage was complicated and requires further study.

Author contribution Conceived and designed the experiments: ASX. Performed the experiments: JM. Analyzed the data: JM, ZSX, FW, GFT, MYL, ASX. Contributed reagents/materials/analysis tools: ASX. Wrote the

paper: JM, ASX. Revised the paper: JM, ZSX, GFT, ASX. All authors read and approved the final manuscript and have no conflicts of interest with regard to this research or its funding.

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