Genome-Wide Analysis of the Heat Shock Transcription Factor Gene Family in *Brassica juncea*: Structure, Evolution, and Expression Profiles

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Heat shock transcription factor (HSF) is ubiquitous in the whole biological world and plays an important role in regulating growth and development and responses to environment stress. In this study, a total of 60 HSF transcription factors in *Brassica juncea* genome were identified and analyzed. Phylogenetic analysis showed that HSF genes were divided into three groups namely: A, B, and C, of which group A was further divided into nine subgroups (A1–A9). The analysis of gene structure and conserved motifs showed that some homologous genes are highly conserved. There was strong conservative microcollinearity among *Brassica rapa*, *B. juncea*, and *Brassica oleracea*, which provides a basis for studying the replication of gene families. Moreover, the results revealed that the promoter regions of *BjuHSF* genes were rich in *cis*-elements related to growth and development, hormone signal, and stress response. The prediction of protein interaction results showed that HSFs could interact with multiple transcription factors and proteins in the genome, while functional annotation revealed that *BjuHSF* genes were involved in many biological processes. The expression patterns of *BjuHSF* genes were analyzed by qPCR, and the results showed that these genes were closely linked to stress response, hormones, and development process. These results are a foundation for further analysis of the regulation mechanism of HSF gene family.

Keywords: heat shock transcription factor, *Brassica juncea*, evolutionary analysis, function annotation, expression pattern

Introduction

HHEAT SHOCK TRANSCRIPTION FACTOR (HSF) regulates heat shock response in cells of organisms. It is recognized and specifically combines with heat shock element (HSE) under heat stress (HS), which activates the transcription and expression of downstream heat shock protein (HSP) genes. It also plays a key role of resistance to high temperature stress and other life activities in plants (Guo *et al.*, 2016; Ohama *et al.*, 2017). Although HSF genes vary in size and sequence, their main regions in eukaryotic organisms are conserved. Typically, HSFs have five general or class-specific protein domains, including DNA-binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), nuclear export signal (NES), and c-terminal activator peptide motif (AHA). Among these, DBD is the most conservative, and it is the main area that combines with HSE. OD or HR-A/B region binds to HSP promoter, initiating transcription of HSP gene (Zhang *et al.*, 2020a).

HSF is found in various plants, and it has diverse family members. Previous studies revealed 78 HSFs in wheat (Zhou *et al.*, 2019), 35 in carrot (Huang *et al.*, 2015), and 25 in tea (Zhang *et al.*, 2020c). Based on the relationship between protein sequence, structure, and evolution, there are three types of members in the HSF family in plants: A, B, and C. All types of HSFs in various plants have great similarity (Zhu *et al.*, 2017b; Chen *et al.*, 2018). HSF in class A is mainly responsible for the regulation of heat shock gene expression; while HSF in class B has DNA-binding activity but has no heat shock-induced transcriptional activation activity, which may be a coregulator or inhibitor of genes in class A, and the functions of HSF in class C are unclear (Hu *et al.*, 2018). The number of HSF and the composition of each group are varied in different plants, which may be due to the difference in genome-wide replication events among species during the evolution process (Scharf *et al.*, 2012).

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Hitherto, research on HSF genes in plants has mainly focused on the role of HS response (Wang *et al.*, 2018; Bian *et al.*, 2020). The overexpression of *ZmHsfA2* improved the heat tolerance of *Arabidopsis thaliana* (Li *et al.*, 2019a). Research also reported that overexpression of *TaHsfC2a-B* in wheat triggered upregulation of expression levels of genes induced by drought, heat, and abscisic acid (ABA), which mediated plant heat tolerance through ABA signaling pathway (Hu *et al.*, 2018). Apart from HS, HSFs have shown to play a role in other abiotic stress responses. In *Arabidopsis*, overexpression of *AtHsfA6b* improves tolerance to salt and drought stress (Huang *et al.*, 2016). In addition, *AtHsfA7b* protein can directly bind to E-box motifs and/or HSEs to regulate a series of genes related to salt tolerance (Zang *et al.*, 2019). HSF genes also play an important role in plant growth and development. *TaHsfA2bs* with differential expression in sterile and fertile anthers in wheat suggest that they may be candidate genes involved in anther development (Ye *et al.*, 2020). *TaHsfA6f* are highly expressed in wheat leaves during reproduction period, while most *JcHsfs* were highly expressed in different tissues and developing seeds (Bi *et al.*, 2020; Zhang *et al.*, 2020b).

The members of HSF gene family have been identified in *Arabidopsis*, rice, *Brassica rapa*, and *Brassica oleracea* (Lohani *et al.*, 2019; Zhang *et al.*, 2020c). However, there are no reports on HSF gene family of *B. juncea*. Stem mustard (*Brassica juncea* var. *Tumida* Tsen et Lee) is an important vegetable crop that belongs to the genus *Brassica* in the Cruciferae family. In China, where it originated from, the tumorous stem of stem mustard provides the raw material for processing pickled products. Presently, whole-genome sequencing of *B. juncea* has been conducted, and it provides an important reference for genetic breeding and identification of biological function of related genes. In this study, the HSF genes of *B. juncea* were explored. This included comprehensive analysis of the structural characteristics, chromosome location, evolutionary relationship, protein interaction, and tissue expression pattern of HSF gene family of *B. juncea*. The results of this study could provide a theoretical basis for further research on the functional identification of HSF genes in *B. juncea*.

Materials and Methods

Identification and screening of HSF genes in the *B. juncea*

The *B. juncea* protein sequences were downloaded from the *Brassica* genome database. The HSF family module sequence (HSF, PF00447) was downloaded from the Pfam database, HMMER software was used to retrieve the HSF protein sequence from the *B. juncea* genome sequence. Candidate proteins were further submitted to NCBI-CDD and Pfam for confirming the HSF domain. The molecular weight and isoelectric point (pI) of the HSF proteins were analyzed using online ExPASy software. The subcellular localization was predicted with WOLF PSORT and TargetP. The chromosomal location of *BjuHSF* genes was drawn using MapInspect software.

Phylogenetic and syntenic relationships among species

The HSF proteins in *Arabidopsis* and rice were downloaded from the TAIR and PlantTFDB, respectively. Multiple sequence alignments of all HSF protein sequences from *B. juncea*, *Arabidopsis* and rice were performed using Clustal X2.1. The result of multisequence coupling was analyzed with MEGA 5.0 software using the N-J method. The calibration parameter bootstrap was repeated 1000 times and the phylogenetic tree was constructed.

Sequence structure, conserved motifs, and promoter region analysis of the HSF family genes in *B. juncea*

The exon-intron structure and conserved motifs of *HSF* genes were analyzed by the GSDS server and MEME, respectively. Then the gene structure was drawn using the TBtools software (Chen *et al.*, 2020). The 1500 bp upstream of the *BjuHSF* gene' 5'UTR was downloaded from the *Brassica* genome database as its promoter sequence, and the *cis*-element analysis of the promoter sequences was analyzed using the PlantCARE.

Analysis of functional annotation and interaction network

Based on sequence similarity, the gene ontology (GO) annotations of the *BjuHSF* proteins were analyzed using the Blast2GO software. GO terms and annotations are assigned to each BLAST hit. In addition, the sequence homology of *Arabidopsis* and *BjuHSFs* was established by using the STRING program to establish the protein interaction network between *BjuHSFs* and other proteins in *B. juncea* genome.

Plant materials, abiotic treatments, and RNA extraction

The experimental samples used for the expression pattern analysis at different development stages were obtained from the four stages of the swelling stem of stem mustard variety "Fuza No.2." All samples were grown in pots in a controlled growth environment. For hormone treatment, plants for 4 weeks with five to six leaves were used in the experiments. The seedlings were sprayed separately with auxin (IAA) (10 μ M), ABA (75 μ M), salicylic acid (SA) (0.5 mM), and gibberellin (GA) (50 μ M) and placed for 24 h. For abiotic stress, all samples were harvested after 6 and 12 h of treatment with cold temperature treatment (4°C), drought treatment (10% PEG 8000), NaCl treatment (400 mM), and high-temperature treatment (38°C). All treatments were set to three biological replicates. The samples were frozen in liquid nitrogen immediately after collection and stored in -80°C refrigerator. Total RNA of all samples were extracted using a total RNA Kit (Tiangen, Beijing, China) and then using the PrimeScript RT Kit (TaKaRa, Dalian, China) with oligonucleotides (dT) and random primers for reverse transcription synthesis of cDNA.

Quantitative transcript analysis and qPCR validation

The transcript abundance of all genes in *B. juncea* during the stem swelling was obtained from the transcriptome

sequencing data that had been completed by our laboratory. The sequencing data have been submitted to NCBI under the accession number SRP151320. The expression abundance of *BjuHSF* gene in different developmental stages of the mustard was based on transcriptome data as reflected by FPKM value.

The expression level of selected *BjuHSF* genes was analyzed by qPCR with a Bio-Rad CFX96TM real-time PCR system (Bio-Rad, CA, USA). All experiments were performed in triplicate, and *UBC* was used as the internal control to normalize the gene expression (Li *et al.*, 2020). The relative expression levels of *BjuHSFs* were calculated using the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001). Primer pairs were designed using Primer Premier 6.0 software and shown in Supplementary Table S1. IRB approval was not applicable.

Results

Identification of HSF family genes in *B. juncea*

To identify the HSF genes in *B. juncea*, the HSF domain of Pfam database was searched. A total of 60 HSF genes, referred to as *BjuHSF01* to *BjuHSF60* were identified. Based on the physical-chemical properties, location, solvent accessibility of the mutated residue, characteristics of gene name, login number, amino acid size, molecular weight, theoretical electric point, chromosomal location, as well as subcellular location are shown in Supplementary Table S2. The results showed that *BjuHSF* polypeptides had between 155 (*BjuHSF18*) and 553 (*BjuHSF57*) amino acids, with a molecular weight of between 18.36 (*BjuHSF18*) and 62.45 (*BjuHSF57*) kDa. The pI of most proteins was distributed in the range of 4–7, suggesting that they were acidic. This was an indication that they could play various roles in different microenvironments (Kiraga *et al.*, 2007). Analysis of subcellular localization showed that most of the genes were located in the nucleus, which was similar to HSF transcription factor genes reported in other species.

Phylogenetic and cluster analysis of HSF genes

To analyze the evolutionary relationship of HSF, the HSF proteins in *Arabidopsis*, rice, *B. rapa*, and *B. juncea* were selected for construction of phylogenetic trees. The results showed that 20 HSFs in *Arabidopsis*, 25 in rice, 35 in *B. rapa*, and 60 in *B. juncea* had obvious clustering phenomenon, as shown in Figure 1A. The family was divided into three groups (A, B, and C). Group A was further divided into nine subgroups (A1–A9), including A2 subgroups of genes not found in *B. juncea*. Figure 1B, shows that group A had 40 genes in *B. juncea*, 14 in *Arabidopsis*, 13 in rice, and 23 in *B. rapa*. Further, there were 14 genes of *B. juncea* in A1 subgroup. Group B had 16 genes of *B. juncea*, while group C had at least 4 genes of *B. juncea*. From a genetic relationship perspective, there were 32 pairs of direct homologous genes among species, such as *BjuHSF29* and *BrHSF30*, *BjuHSF35* and *BrHSF16*, as well as *BjuHSF32* and *BrHSF15*, were in between *B. juncea* and *B. rapa*. Results also showed that HSF of *B. juncea* preferentially clustered with orthologous genes of *B. rapa*, followed by *Arabidopsis*, and finally with rice. This indicated that it is consistent with evolutionary relationship of species.

Chromosomal distribution, collinear analysis, and duplication of *BjuHSFs*

Based on the chromosomal genome information of *B. juncea*, MapInspect software was used to map the chromosome distribution of 60 HSF family members (Fig. 2). The results showed that except for *BjuHSF10*, *BjuHSF29*, *BjuHSF31*, *BjuHSF38*, *BjuHSF41*, *BjuHSF47*, and *BjuHSF50*, which were located in unanchored contigs, the remaining 53 HSF genes were nonuniform and distributed in 18 chromosomes. The number of chromosomal distribution genes in subgenome A was more than that in subgenome B. The chromosomes in subgenome A had 32 genes, accounting for about 60%, while subgenome B had 21 *BjuHSF* genes, which was equivalent to about 40%. The largest number of genes were ChrA03 (9 genes), followed by ChrA01, ChrB07, and ChrB02, having 7, 6, and 4 genes, respectively. The least numbers having one gene were ChrA05, ChrA06, and ChrB03. Some HSF genes clustered in similar or identical regions on the chromosomes, which could be attributed to chromosome tandem duplication. Examples include: *BjuHSF27*, *BjuHSF60*, *BjuHSF35*, and *BjuHSF32* on ChrA03; *BjuHSF33* and *BjuHSF34* on ChrB02; and *BjuHSF24* and *BjuHSF25* on ChrB07. Segmental and tandem duplication events are the main causes of gene-family expansion. This study revealed that a large number of HSF gene pairs had cross collinearity, and more fragments were duplicated on each chromosome (Fig. 3). All the protein-coding sequences in the genomic region containing HSF in pairs and cycles were compared. These results are presented in Figure 4, and where a strongly conserved microcollinearity among *B. rapa*, *B. juncea*, and *B. oleracea* was revealed. These results show that tandem and segmental duplication play a significant role in the generation of gene families.

Analysis of HSF family gene structure and conserved motifs in *B. juncea*

The exon–intron structure in *B. juncea* was identified, and the results are shown in Figure 5. These genes had at least one intron except for *BjuHSF59* and *BjuHSF60*. *BjuHSF50* had the longest intron sequence and was of about 4000 bp. Each group had similar gene structures, indicating that the gene structures of HSF genes were closely related and highly conserved. The length of introns in this family of genes had a certain degree of variation, like other plants such as potato (Tang *et al.*, 2016) and carnation (Li *et al.*, 2019b). The conserved motifs were identified by MEME program. The distribution of the 15 conserved motifs was further analyzed and the results showed that none of HSFs contained all the 15 conserved motifs, and all genes were deficient of conserved motifs in varying degrees, with each gene containing 4–11 conserved motifs (Fig. 5C and Supplementary Table S3). Motifs 9 and 5 were unique to *BjuHSF01*–*BjuHSF14* and *BjuHSF41*–*BjuHSF56*, respectively. Except for *BjuHSF36*, which did not have motif 2, the three conservative motifs (motifs 1, 2, and 3) were found among the other 59 *BjuHSF* proteins. This indicates that these three motifs are widely distributed and have the strongest conservatism in the protein sequence of *BjuHSFs*. There was a highly conserved intron–exon distribution structure between gene pairs, which provides a basis for the study of gene family replication.

FIG. 1. Phylogenetic analysis of HSF proteins among *Brassica juncea*, *Brassica rapa*, *Arabidopsis thaliana*, and *Oryza sativa* by MEGA7.0. (A) The full-length HSF domain protein sequences from *B. juncea* (BjuHSF), *B. rapa* (BrHSF), *Arabidopsis* (AtHSF), and *O. sativa* (OsHSF), each of group was aligned by ClustalX. (B) HSF family members of *B. juncea*, *B. rapa*, *Arabidopsis*, and *O. sativa*. HSF, heat shock transcription factor.

FIG. 2. Chromosome location of *BjuHSF* genes. Chromosome numbers are indicated at the *top* of each bar. The scale bar on the *left* indicates the size of chromosomes.

Analysis of the cis-regulatory elements BjuHSFs promoter region

Cis-regulatory elements in the promoter region can regulate the transcriptional activity of genes. The function and expression potential of genes can therefore be understood by analyzing the composition of *cis*-elements. The promoter sequences of *BjuHSFs* were analyzed using the online software PlantCARE. The results showed the existence of 28 *cis*-elements, including 14 plant growth and development elements, 7 hormone response elements, and 7 stress response elements (Fig. 6). The G-box occupied the largest proportion of all elements, followed by ABA response element (ABRE). During growth and development, it was found that the G-box and Box4 had 191 and 89 elements, respectively. Among the hormone-related elements, there were 170, 66, 34, and 21 involved into abscisic acid (ABRE), jasmonic acid (CGTCA-motif), GA (P-box, GARE-motif, TATC-box), and SA response elements (TCA-element), respectively. A total of 121 anaerobic induction elements (ARE), 45 stress response elements (STRE), 23 drought induction elements (MBS), and 25 low temperature induction elements (LTR) were detected related to abiotic and biotic stress.

Functional annotation for the BjuHSF genes

Functional annotation analysis for BjuHSFs was performed *via* Blast2GO in the GO database. All the BjuHSFs are annotated and a total of 383 annotations were obtained (Fig. 7 and Supplementary Table S4). The genes having the highest annotation information were BjuHSF39 and BjuHSF38, with 12 annotations, respectively, while the genes with the least annotation information were BjuHSF07

and BjuHSF32 with only two annotations. The genes having six annotations account for the most with 19 genes. The annotation results were classified into 14 function terms, which were in turn assigned into three ontology categories, namely biological process (BP), cellular component (CC), and molecular function (MF). Within BP, the top two groups were “cellular process” and “metabolic process” and all the HSF genes were annotated by these two categories. In the MF category, 60 genes were related to “transcription regulator activity” and “binding.” Under the CC category, the top three groups were “cell part,” “organelle,” and “cell.” This indicates that BjuHSF genes may be involved in different growth and development processes of *B. juncea*.

Interaction network analysis of BjuHSF proteins

To explore the regulatory relationship of HSF transcription factors in *B. juncea*, an interaction network was constructed according to the orthologs in *Arabidopsis*. A total of 37 BjuHSF proteins and 60 proteins in *B. juncea* genome were revealed in the complex interaction network map (Fig. 8). Two proteins belonging to A1 subgroup (BjuHSF13 and BjuHSF14), two belonging to A9 subgroup (BjuHSF39 and BjuHSF40), and four belonging to B group (BjuHSF53, 54, 55, and 56) exhibited significant correlations with DREB and HSP proteins. This suggests that these proteins function together to regulate some BPs. It has been reported that HSF interacts with DREB, HSP, and other genes to regulate plant heat tolerance. Both *DREB2A* and *DREB2C* have been shown to regulate *HSFA3* and enhance heat resistance (Schramm *et al.*, 2008; Chen *et al.*, 2010). Overexpression

FIG. 3. Analysis of the successive duplications of the HSF genes in the *B. juncea* genome. Among them, 18 chromosomes and four contigs were arranged in a circle with a single color. The gray line connected a pair of duplicate genes generated by doubling the genome. The black line connecting the chromosomes represented the syntenic region of the BjuHSF genes.

of *OsDREB2B* in rice improves plant resistance to high temperature and promoted the expression of *HSF* and *HSP* genes (Matsukura *et al.*, 2010).

Analysis of the expression of BjuHSF genes

Previous studies have shown that subgroups A1 and A7 play roles in the development and abiotic stresses in plants. In *Arabidopsis*, overexpression *HsfA1a* positively affects tolerance of multiple stresses by inducing the expression of *HSP* (Qian *et al.*, 2014). For example, *AtHsfA7b* positively regulates salt tolerance of chrysanthemum and *Arabidopsis*

(Li *et al.*, 2018; Zang *et al.*, 2019). *HbHsfA7a* was significantly upregulated under low-temperature stress in the other hardy rubber (Li *et al.*, 2019c). Promoter analysis also showed that A1 and A7 may participate in the regulation of development and abiotic stresses in *B. juncea*. Therefore, qPCR was hereby used to verify the expression patterns of BjuHSF genes in A1 and A7 with reference to previous studies.

Transcript abundance of BjuHSF in stem developmental stages. The expression patterns of HSF family genes were analyzed based on transcriptome data in stem developmental stages. A comparison of the expression abundance in

FIG. 4. Analysis of collinearity among *B. rapa*, *B. juncea*, and *Brassica oleracea* genomes. Colored circular rectangles denote the chromosomes of three cruciferous plants. The black curves represent gene pairs that are collinear with some BjuHSF genes.

FIG. 5. Phylogenetic relationship, exon-intron structure and conserved motifs analysis of HSF genes in *B. juncea*. (A) The phylogenetic tree of BjuHSFs created by MEGA 7.0. (B) The exon-intron structure of BjuHSFs genes. Boxes and lines represent exons and introns, respectively. (C) Conserved motifs predicted in BjuHSF proteins. The 15 motifs were identified by MEME program, with each number of colored box representing a different motif. Color images are available online.

0	0	0	2	0	0	0	1	8	1	0	0	2	0	6	0	1	1	0	0	0	0	2	1	0	0	BjuHSF01		
0	0	2	0	0	0	0	0	9	0	1	0	0	0	8	0	2	1	0	1	0	2	0	0	1	0	0	1	BjuHSF02
0	1	2	1	0	0	0	1	4	0	0	0	1	2	4	0	0	0	0	0	0	0	0	1	1	1	0	0	BjuHSF03
0	0	2	1	0	0	0	0	8	1	0	0	0	0	6	0	1	1	0	0	0	2	0	1	2	0	0	1	BjuHSF04
0	0	0	1	0	0	0	1	5	0	0	0	0	0	4	0	2	0	0	0	0	6	0	1	1	1	0	0	BjuHSF05
1	0	1	1	0	0	0	0	6	1	2	0	0	3	7	0	2	1	0	1	0	2	0	0	1	2	0	1	BjuHSF06
1	0	1	0	0	1	0	1	7	1	1	1	2	3	5	0	1	0	0	0	0	0	0	1	0	0	1	0	BjuHSF07
0	0	0	0	1	0	0	0	4	0	0	0	0	0	3	1	1	0	0	0	0	0	0	0	1	0	1	0	BjuHSF08
0	0	0	0	0	0	0	0	2	1	2	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	BjuHSF09
1	0	2	1	1	0	0	0	13	0	0	0	1	0	12	0	1	0	0	0	0	3	0	0	1	0	0	1	BjuHSF10
0	0	0	0	1	2	0	0	4	1	0	0	0	0	4	0	2	0	0	1	0	2	0	0	1	1	0	1	BjuHSF11
1	0	1	0	0	0	0	1	2	0	0	1	1	0	3	0	0	0	0	1	1	1	0	0	1	0	0	0	BjuHSF12
0	0	6	0	0	0	0	1	3	0	1	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	1	0	BjuHSF13
1	0	3	0	0	0	0	1	3	4	0	1	0	0	4	0	0	0	0	1	0	4	3	0	1	1	2	0	BjuHSF14
0	0	1	1	0	0	0	0	6	0	0	0	2	1	3	1	0	1	0	1	0	4	0	0	3	1	0	1	BjuHSF15
0	0	3	0	2	0	0	0	2	2	0	0	0	1	2	1	0	0	0	0	0	3	0	1	1	0	1	0	BjuHSF16
0	1	1	0	0	0	1	0	0	1	0	1	0	2	0	0	1	2	0	0	0	3	0	1	1	0	0	0	BjuHSF17
0	1	0	0	0	0	0	1	5	1	1	1	0	1	6	1	2	1	0	1	0	3	0	0	1	1	1	1	BjuHSF18
1	0	2	0	1	0	0	0	0	1	0	0	1	3	1	0	0	0	0	0	0	1	0	0	0	0	0	1	BjuHSF19
0	0	0	0	0	0	0	1	0	0	0	0	1	0	2	0	2	0	0	0	0	3	1	0	1	0	2	0	BjuHSF20
0	0	5	0	0	0	0	2	3	3	0	0	1	0	2	1	1	0	0	1	0	4	0	0	0	0	0	1	BjuHSF21
0	0	2	0	0	0	0	0	1	0	0	0	1	2	1	0	0	0	0	0	0	0	1	0	0	0	0	1	BjuHSF22
0	0	4	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	BjuHSF23
1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	BjuHSF24
1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	BjuHSF25
0	0	2	1	0	0	0	0	0	3	0	1	0	0	0	0	1	2	0	2	0	1	0	0	1	0	0	1	BjuHSF26
0	1	2	0	0	0	0	0	5	0	0	1	1	0	3	0	3	0	0	1	1	4	0	0	0	0	0	1	BjuHSF27
2	0	2	1	0	0	0	1	2	1	0	0	0	2	2	0	1	0	0	1	0	1	0	1	0	0	0	0	BjuHSF28
0	0	0	0	0	0	0	1	11	2	0	1	1	0	11	0	0	0	0	0	0	2	1	0	1	0	1	0	BjuHSF29
0	0	4	0	0	1	0	0	4	1	0	0	1	0	3	1	3	0	0	0	0	3	1	1	0	0	0	0	BjuHSF30
0	0	3	0	0	0	0	0	3	1	0	0	1	0	3	1	2	0	0	1	0	5	1	1	1	0	0	0	BjuHSF31
0	0	2	0	0	1	0	0	3	2	0	1	2	0	4	0	1	0	0	1	0	5	0	2	1	0	0	1	BjuHSF32
0	0	2	1	0	0	0	0	3	0	0	0	1	0	3	1	0	1	0	0	0	6	0	1	0	0	1	2	BjuHSF33
0	0	4	0	1	0	0	0	3	0	0	1	3	1	2	1	1	0	0	0	0	5	3	1	0	0	0	0	BjuHSF34
0	1	0	0	0	0	0	0	6	1	0	1	2	1	6	1	2	1	0	0	0	6	2	1	1	0	0	0	BjuHSF35
0	0	2	0	1	1	0	1	0	2	0	0	0	0	0	1	0	0	2	0	0	2	0	0	2	0	0	1	BjuHSF36
0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	4	1	0	1	1	0	1	BjuHSF37
0	1	2	0	0	0	0	0	0	1	0	1	0	0	0	0	0	2	0	0	0	5	3	0	1	0	0	0	BjuHSF38
1	0	1	1	0	0	0	1	4	1	0	0	0	1	3	0	2	1	0	0	0	6	1	0	1	0	0	0	BjuHSF39
1	0	0	0	0	1	0	0	3	1	0	1	0	0	2	0	2	1	0	0	0	0	0	1	0	1	3	0	BjuHSF40
0	1	4	0	0	1	0	0	1	1	0	2	0	1	1	0	0	0	1	0	0	0	0	0	0	0	1	1	BjuHSF41
0	0	3	0	1	0	0	0	7	0	0	0	0	1	6	1	2	0	0	2	0	1	0	1	2	0	1	0	BjuHSF42
1	0	2	0	0	0	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0	0	0	0	2	0	0	0	BjuHSF43
0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	2	1	BjuHSF44
0	0	1	0	0	0	0	0	6	2	0	0	0	0	4	0	2	1	0	0	0	1	1	0	1	0	0	0	BjuHSF45
2	0	3	0	0	0	0	0	1	0	0	0	0	0	1	0	5	0	0	0	0	2	1	0	5	0	0	0	BjuHSF46
1	0	2	0	2	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	BjuHSF47
2	1	0	0	1	0	0	1	4	0	1	0	0	0	5	0	1	0	0	0	1	2	0	0	1	0	1	0	BjuHSF48
3	1	0	0	0	0	0	1	1	2	2	1	0	1	1	0	2	1	0	0	1	0	1	0	1	0	0	0	BjuHSF49
0	0	2	2	0	0	0	0	6	0	2	0	1	1	7	0	0	0	0	1	0	3	2	1	0	1	0	0	BjuHSF50
0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0	0	3	0	0	2	0	0	0	BjuHSF51
0	0	1	1	0	0	0	0	2	0	0	1	2	1	2	0	0	0	0	1	0	3	1	0	1	0	1	0	BjuHSF52
0	0	0	0	1	0	0	1	0	2	0	1	0	1	0	0	2	0	0	0	1	0	0	1	0	0	1	3	BjuHSF53
0	0	1	0	0	0	0	1	2	0	0	0	0	2	2	0	1	2	0	0	0	1	0	1	0	0	0	0	BjuHSF54
1	0	0	0	0	0	1	0	3	0	0	0	0	2	1	0	1	1	0	0	0	2	0	0	0	1	1	0	BjuHSF55
0	0	0	0	0	1	0	1	1	2	0	0	0	2	1	0	1	0	0	0	0	1	0	0	0	1	1	0	BjuHSF56
0	0	1	0	0	0	0	0	2	0	0	2	0	0	1	0	2	0	0	0	0	0	0	0	1	1	0	0	BjuHSF57
0	0	2	0	0	1	0	1	2	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	BjuHSF58
1	0	0	0	0	0	0	0	4	0	0	1	0	2	3	0	2	0	1	0	0	0	0	0	0	0	2	0	BjuHSF59
0	0	2	0	0	0	0	1	4	1	0	1	0	2	3	0	2	0	0	0	0	1	0	2	0	0	0	0	BjuHSF60
AE-box	ATCT-motif	Box 4	CAT-box	circadian	GA-motif	Gap-box	GATA-motif	G-Box	GT1-motif	I-box	MRE	O2-site	TCT-motif	ABRE	AuxRR-core	CGTCA-motif	P-box	TATC-box	TCA-element	GARE-motif	ARE	LTR	MBS					

FIG. 6. *Cis*-regulatory elements analysis in the promoter region of *BjuHSF* genes. *Cis*-acting elements are divided into three categories based on functional annotation: plant growth and development, phytohormone responsive, or abiotic and biotic stresses-related *cis*-acting elements.

FIG. 8. Interaction network of BjuHSF factors in *B. juncea* according to orthologs in *Arabidopsis*.

FIG. 9. Expression profiles of *BjuHSF* genes by the analysis of transcriptome data of different developmental stages of mustard. The color scale bar on the right side of the heat map corresponds to the value converted by $\log_2^{\text{FPKM}}$.

FIG. 10. Relative expression of *BjuHSF* genes at different developmental stages. The color scale bar on the right side of the heat map corresponds to the value of relative expression.

transcriptome data at different development stages showed that the corresponding expression levels were detected for 57 genes except for *BjuHSF24*, *BjuHSF28*, and *BjuHSF36*. The expression pattern of these genes was as shown in Figure 9. For subgroup A1, with exception of *BjuHSF06* and *BjuHSF07*, low expression levels were recorded, while other genes had high expression levels. It is noteworthy that the overall expression of A6 subgroup was low. In subgroup A7, the expression level of *BjuHSF* gene in the late stage was higher than in the early stage. In group B, the expression levels of *BjuHSF45* and *BjuHSF50* were lower, while the expression levels of *BjuHSF41–BjuHSF44* genes were higher in the late stage than in the early stage. In group C, the overall expression of *BjuHSFs* was higher. The trend of gene expression was similar among some subgroups.

Quantitative expression of the *BjuHSF* genes at different stages of stem swelling. Figure 10 shows that the 14 *HSF* genes in the A1 subgroup had transcriptional activity in the four stages of stem swelling. During S1 and S2, the *HSF* gene expression was low, while those of S3 and S4 were high. The expression levels of *BjuHSF03*, *BjuHSF11*, and *BjuHSF14* were higher in the S3 stage, while the expression levels of *BjuHSF09*, *BjuHSF10*, and *BjuHSF11* were higher in the S4 stage. However, the expression levels of *BjuHSF06* and *BjuHSF07* were low during the four development periods, without significant changes. The expression pattern of qPCR was consistent with that of transcriptome data.

Expression of *BjuHSFs* in response to the treatment with phytohormone. Figure 11 shows that the *BjuHSFs* in A1 subgroup had different expression patterns under different

FIG. 11. Expression profiles of *BjuHSFs* in response to treatment with GA, ABA, SA, and IAA. Relative expression values were normalized by log2 transform to represent color scores. ABA, abscisic acid; GA, gibberellin; IAA, auxin; SA, salicylic acid

hormone treatments. In addition to *BjuHSF06*, *BjuHSF12*, and *BjuHSF14*, the expression of the other 11 genes were significantly upregulated during different hormone treatments. Under GA treatment, the expression of *BjuHSF01*, *BjuHSF03*, *BjuHSF08*, and *BjuHSF09* was significantly upregulated. The upregulation of *BjuHSF03*, *BjuHSF07*, *BjuHSF08*, and *BjuHSF09* was obvious under ABA hormone treatment. Under SA treatment, other genes were significantly upregulated except for *BjuHSF06*, *BjuHSF07*, *BjuHSF12*, and *BjuHSF14*. Under IAA treatment, the genes were significantly upregulated except for *BjuHSF12* and *BjuHSF14*.

The expression levels of *BjuHSF* gene under different abiotic stresses. The expression of genes in A1 subgroup (*BjuHSF01–BjuHSF14*) and A7 subgroup (*BjuHSF29–BjuHSF35*) were analyzed using qPCR to determine the response to stress. Figure 12 depicts that 21 genes were expressed in trace amounts in the *B. juncea* without stress treatment, and these transcription factors revealed different expression patterns under different stresses with most of the genes being upregulated. The variation range of gene expression was largest under HS, especially for members of subgroup A7. The expression of *BjuHSF30* and *BjuHSF31* genes was significantly enhanced. Under NaCl stress for 6 h, the expression levels of *BjuHSF04*, *BjuHSF06*, and *BjuHSF12* were significantly increased, while the expression levels of other genes were not significant. The expression levels of *BjuHSF04*, *BjuHSF05*, and *BjuHSF06* increased significantly under 12 h of cold stress. *BjuHSF30* expression increased significantly under dry stress for 12 h.

Discussion

The HSFs family is one of the largest transcription factors in plants, which can specifically identify HSE *cis*-element (5'-AGAAAnnTTCT-3') stored in HS induced gene promoters (Ohama *et al.*, 2017). Previous studies indicate that HSF genes are involved in plant growth and development and play key roles in the control of plant responses to abiotic stresses, biotic stresses, and phytohormones (Lin *et al.*,

2018; Lohani *et al.*, 2019). Presently, there are no reports on HSF genes in *B. juncea*.

In this study, we identified a total of 60 HSF genes in *B. juncea*. Based on the phylogenetic tree and homology analysis, they were grouped into three classes (A, B, and C) consistent with those for *Arabidopsis* (Guo *et al.*, 2008), tomato (Tang *et al.*, 2016), and tea (Liu *et al.*, 2016). There were 32 pairs of orthologous genes in *B. rapa* and *B. juncea* of HSF, which are clustered preferentially with orthologous genes of *B. rapa*, followed by *Arabidopsis*, and finally rice. This indicates that *B. juncea* of the same genus dicotyledon is more closely related to *B. rapa* and *Arabidopsis* than rice. Furthermore, *Brassica* crops are more closely related and that the direct homologous genes derived from a common ancestor genes existed before the differential separation of monocotyledons and dicotyledons (Hardison, 1996).

B. juncea (AABB, 2n=36) is an allotetraploid species derived from the hybridization between diploid species *B. rapa* (AA, 2n=20) and *Brassica nigra* (BB, 2n=16) (Zhang *et al.*, 2014; Li *et al.*, 2019d). Comparative genomic analysis confirmed that *B. rapa* of *Arabidopsis* differentiation has genome tripling, gene duplication, deletion, and rearrangement (Lysak *et al.*, 2005; Song *et al.*, 2014; Lohani *et al.*, 2019). Collinearity analysis of *B. rapa* and *B. oleracea* genomes revealed that the number of collinear blocks and homologous gene pairs of *B. rapa* and *B. oleracea* genomes was higher than that of *B. juncea* and *B. oleracea*. It is worth noting that A2 subgroup was not found in mustard. On the contrary, the number of members of A9 subgroup increased to two, compared to one for *Arabidopsis* and *B. rapa* (Fig. 1B). This indicates that gene loss and replication occur at different stages of evolution, leading to the diversity of HSF (Zhou *et al.*, 2019). Previous studies revealed that members of gene families can spread out into clusters on chromosomes by tandem repeats, or can spread out across different chromosomes by repetition (Steven *et al.*, 2005; Zhu *et al.*, 2017a). In this study, some *BjuHSFs* were scattered in clusters of tandem repeats on chromosomes, suggesting that they may have experienced gene expansion events during evolution.

FIG. 12. Expression profiles of *BjuHSFs* in response to treatment with cold temperature, heat temperature, drought, and salt stress. Relative expression values were normalized by log2 transform to represent color scores.

Numerous studies have revealed that most genes in HSF family are involved in plant growth and development and also can be induced by heat or other abiotic stresses. For example, eight *RsHSF* genes in radish were highly expressed in leaves, roots, cortex, cambium, and xyloid. The expression pattern of 12 *RsHSF* genes was also changed under heat, salt, and heavy metals (Tang *et al.*, 2019). Studies revealed the roles of *HSFA1* in the osmosis, salt stress, and the oxidative stress response in *Arabidopsis* (Liu *et al.*, 2011). The current study exhibited that *BjuHSF01*, *BjuHSF02*, *BjuHSF03*, and *BjuHSF10* were significantly upregulated under treatment of ABA hormones. These four genes also had many ABRE promoter elements. The expression levels of *BjuHSF01*, *BjuHSF02*, *BjuHSF03*, and *BjuHSF10* were relatively high during growth and development, and contained more G-box photosensitive elements that regulate growth and development. It is speculated that the expression difference of *BjuHSF* under different stresses may be related to the difference in promoter *cis*-regulatory elements. During the heat shock induction process, the expression of *BjuHSF30* and *BjuHSF31* genes was significantly enhanced, but no HSE element was detected in the promoter region of *BjuHSF*. This suggests that the expression of these heat-related *BjuHSF* genes may not be directly induced by HS, which is consistent with the results of previous studies (Tang *et al.*, 2016; Li *et al.*, 2018). Based on the above results, it was concluded that the specific function of the HSF gene family in *B. juncea* requires further functional verification. In summary, the HSF family was identified and analyzed systematically in this study, which provides a theoretical basis for future exploration of the function and evolution of HSF genes.

Authors' Contributions

Conceptualization: M.L. and H.T.; data curation: F.X., Y.L., Y.Z., and Y.W.; formal analysis: Y.L. and Y.Z.; investigation: Y.L., Q.C., and L.G.; writing—original draft: M.L., and F.X.; writing—editing: X.W. All authors read and approved the final article.

Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4

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