Systematic Analysis of Hsf Family Genes in the *Brassica napus* Genome Reveals Novel Responses to Heat, Drought and High CO₂ Stresses

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Drought and heat stress are major causes of lost plant crop yield. In the future, high levels of CO₂, in combination of other abiotic stress factors, will become a novel source of stress. Little is known of the mechanisms involved in the acclimation responses of plants to this combination of abiotic stress factors, though it has been demonstrated that heat shock transcription factors (Hsfs) are involved in plant response to various abiotic stresses. In this study, we performed a genome-wide identification and a systematic analysis of genes in the Hsf gene family in *Brassica napus*. A total of 64 genes encoding Hsf proteins were identified and classified into 3 major classes: A, B and C. We found that, unlike in other eudicots, the A9 subclass is absent in rapeseed. Further gene structure analysis revealed a loss of the only intron in the DBD domain for BnaHsf63 and -64 within class C, which is evolutionarily conserved in all Hsf genes. Transcription profile results demonstrated that most BnaHsf family genes are upregulated by both drought and heat conditions, while some are responded to a high CO₂ treatment. According to the combined RNA-seq and qRT-PCR analysis, the A1E/A4A/A7 subclasses were upregulated by both drought and heat treatments. Members in class C seemed to be predominantly induced only by drought. Among BnaHsf genes, the A2/A3/B2 subclasses were regulated by all three abiotic stresses. Members in A2/B2 subclasses were upregulated by drought and heat treatments, but were downregulated under high CO₂ conditions. While the A3 subclass was upregulated by all the three abiotic stresses. Various stress-related cis-acting elements, enriched in promoter regions, were correlated with the transcriptional response of BnaHsfs to these abiotic stresses. Further study of these novel groups of multifunctional BnaHsf genes will improve our understanding of plant acclimation response to abiotic stresses, and may be useful for improving the abiotic stress resistance of crop varieties.

**Keywords:** Hsf gene family, abiotic stress, high CO₂, gene expression, *Brassica napus*
INTRODUCTION

Environmental stresses, such as drought and heat, cause substantial loss to plant growth and production (Hu and Xiong, 2014; Fang and Xiong, 2015; Pereira, 2016; Zhu, 2016). Increasing CO$_2$ levels result in lower concentrations of zinc, iron and protein for most C3 crops, and threaten human food sources; these results suggest that high CO$_2$ concentrations (hereafter abbreviated to high CO$_2$) may be a novel stress in the future (Myers et al., 2014). However, the basic molecular mechanisms driving plant responses to high CO$_2$ remain elusive (Becklin et al., 2017). To cope with abiotic stresses, plants have evolved diverse adaptive strategies and signaling mechanisms. Transcription factors play crucial regulatory roles in the signal transduction process under these stresses (Hu and Xiong, 2014; Yoshida et al., 2014; Song et al., 2016). Among the transcription factors, heat shock transcription factors (Hsfs) serve as the terminal components in the signal transduction chain mediating the activation of genes responsive to heat and other stresses by recognizing palindromic binding motifs conserved in promoters of heat stress inducible genes called heat stress elements (HSEs: 5′-AGAAnnTTCT-3′) (von Koskull-Döring et al., 2007; Scharf et al., 2012; Guo et al., 2016).

As many other transcription factors, Hsf proteins possess a conserved modular structure (von Koskull-Döring et al., 2007; Scharf et al., 2012). The highly-structured DNA-binding domain (DBD) is located close to the N-terminal of all Hsfs and is responsible for selective interaction with HSE. An oligomerization domain (OD or HR-A/B region) is connected to the DBD by a flexible linker of variable length (15-80 amino acid residues) with a bipartite heptad pattern of hydrophobic amino acid residues, leading to the formation of a coiled-coil domain for protein interaction of Hsfs. Based on the length of the flexible linker and the number of amino acid residues inserted into the HR-A/B region, plant Hsfs are grouped into three main classes (HsfA, -B, and -C) (von Koskull-Döring et al., 2007; Scharf et al., 2012; Guo et al., 2016). The nuclear localization signal (NLS) and the nuclear export signal (NES) control the intracellular distribution of Hsfs between nucleus and cytoplasm. AHA motifs are usually present in the HsfA subfamily and confer transcriptional activator function. However, HsfB members, except HsfB5, are characterized by a repressor domain (RD) consisting of tetrapeptide LFGV in the C-terminal region.

Heat shock transcription factor genes were first cloned in yeast in 1988 (Wiederecht et al., 1988). Unlike the small Hsf family in yeast and animals, plants hold complex and large Hsf gene families. 21, 25, 38, 40, 25, 32 Hsf genes have been found in Arabidopsis, rice, soybean, cotton, pepper and poplar, respectively (Nover et al., 2001; Chauhan et al., 2011; Li et al., 2014; Wang et al., 2014; Guo et al., 2015; Zhang et al., 2016). Among monocots and eudicots, the largest number of Hsf genes were found in wheat (56 members) (Xue et al., 2014).

Plant Hsfs do not only play a role in heat stress response, but also function both in response to other stressors and during organ development. In addition, structural characteristics and diverse expression patterns of Hsf family genes have revealed functional diversification (von Koskull-Döring et al., 2007). Our understanding of plant Hsf gene function comes predominantly from studies of tomato and Arabidopsis thaliana. In tomato, constitutively expressed HsfA1a functions as a master regulator for induced thermotolerance, and cannot be replaced by any other Hsfs (Mishra et al., 2002). However, in Arabidopsis, due to functional redundancy no comparable master regulator role could be identified for any of its four HsfA1 genes (Liu et al., 2011; Nishizawa-Yokoi et al., 2011; Guo et al., 2016). HsfA2 is structurally and functionally similar to HsfA1, but is only strongly accumulated in stressed plants. Its interaction with HsfA1 and B1 forms a functional complex, responsible for regulating core aspects of heat stress response and recovery (Nishizawa et al., 2006; Charn et al., 2007; Scharf et al., 2012; Guo et al., 2016). Additionally, HsfA2 is also involved as a key regulator in other environmental stresses such as osmotic and oxidative stress (Ogawa et al., 2007; Wang et al., 2017). Previous investigations showed that heat- and drought-induced expression of HsfA3 depends on transcription factor DREB2A in Arabidopsis, this indicates that HsfA3 plays a role in drought stress signaling (Sakuma et al., 2006; Schramm et al., 2008). Ectopic expression of HsfA3 has also been shown to enhance thermotolerance in Arabidopsis (Li et al., 2013). Group A4 Hsfs are involved in controlling reactive oxygen species homeostasis of plants, and group A5 Hsfs act as specific repressors of HsfA4 (Yamanouchi et al., 2002; Baniwal et al., 2007). Orthologous HsfA4a was reported to confer cadmium tolerance in wheat and rice (Shim et al., 2009). HsfA9 participates in embryo development and seed maturation in Arabidopsis and sunflower (Almoguera et al., 2002; Kotak et al., 2007); in addition, overexpression of seed-specific HaHSFA9 in tobacco confers tolerance to severe dehydration at vegetative stage (Prieto-Dapena et al., 2008).

Unlike class A Hsfs, class B Hsfs lack an AHA motif and show no independent function as transcriptional activators (von Koskull-Döring et al., 2007). However, in tomato, heat-induced HsfB1 acts as a coactivator with HsfA1a (Bharti et al., 2004). Moreover, Arabidopsis HsfB1 is inactive as a coactivator, although AtHsfB1 can act as a repressor of heat stress-inducible Hsfs (Czarnecka-Verner et al., 2000, 2004). To date, limited information is available for class C Hsfs. The results from tomato and Arabidopsis revealed striking species-specific deviations in the functional diversification of Hsf family members (von Koskull-Döring et al., 2007; Scharf et al., 2012; Guo et al., 2016). Overall, comprehensive characterization is still needed to investigate the functions of Hsfs in plant abiotic stress responses, and in particular in response to high CO$_2$ conditions.

Brassica napus (rapeseed) is one of the most important oil crops in the world. Given the recent publication of the B. napus genome (Chalhoub et al., 2014), rapeseed is becoming an important crop model system (Zhu et al., 2016). While rapeseed plants are sensitive to water deficit and high temperature during all stages of growth and development, the typical stress-related Hsf transcription factors have not yet

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been characterized in *B. napus*. To investigate the potential roles of rapeseed *Hsf* s in abiotic stress responses, the present study identifies 64 genes encoding BnaHsf proteins in the *B. napus* genome and analyzes their phylogenetic relationships, gene and domain structures, putative *cis*-acting elements, and expression patterns across different tissues and under heat, drought and high CO₂ stresses. The results of this study will help to provide a foundation for further functional studies of BnaHsf genes, and improve our understanding of the functional diversification of plant *Hsf* genes under various environmental stresses.

**MATERIALS AND METHODS**

**Identification of Hsf Genes in Brassica napus**

The gene sequence of *B. napus* were downloaded from the genome database1, to gather the probable candidate *B. napus* Hsf amino acid sequences, the Hsf-type DBD domain (Pfam: PF00447) was submitted as a query in a protein BLAST search of the *B. napus* genome database. Hsf gene sequences from *Arabidopsis* (Nover et al., 2001) were retrieved from the TAIR database (Lamesch et al., 2012) and used as queries to perform repetitive BLAST searches against the Phytozome database v9.1 (Goodstein et al., 2012). BLAST searches were also performed against the NCBI nucleic-acid sequence data repositories. All protein sequences derived from the BLAST searches were examined using domain-analysis programs. Molecular weight, isoelectric point, functional domains, and amino acid signal peptides of BnaHsfs were calculated using the ExPASy online servers2.

**Multiple Sequence Alignments and Phylogenetic Analysis**

Multiple sequence alignment of Hsf proteins from *B. napus* were performed using the program ClustalX 1.83 (Thompson et al., 1997). The phylogenetic tree was constructed using the neighbor-joining (NJ) method by MEGA 6 program (Tamura et al., 1997). The phylogenetic tree was constructed using the *Phylogenetic Analysis Using Parsimony (PhyML)* software (Guindon and Gascuel, 2003), the bootstrap value was set at 1000 replications to assess tree reliability.

**Domain and Gene Structure Analysis**

The MEME program3 (Bailey et al., 2009) was used for identification of conserved motifs, with the following parameters: number of repetitions: any; maximum number of motifs:15; and the optimum motif widths: 6–200 amino acid residues. Exon/intron organization of the *Hsf* genes in *B. napus* was illustrated using Gene Structure Display Server program (GSDS4) (Hu et al., 2015) by alignment of the cDNAs with their corresponding genomic DNA sequences.

1http://www.genoscope.cns.fr/brassicane/s/
2http://cn.expasy.org/tools
3http://meme-suite.org/tools/meme
4http://gds.cbi.pku.edu.cn

**Regulatory Cis-Element Analysis**

Prediction of putative *cis*-elements was performed using Signal Scan Search against the plant *cis*-acting regulatory DNA elements (PLACE) database (Higo et al., 1999). A 2-kb sequence upstream of ATG initiation codon of BnaHsf genes was selected as the promoter region for this analysis.

**Plant Materials and Growth Conditions**

Rapeseed seeds were germinated on a filter paper, and then transplanted to soil pots growing in the greenhouse at Oil crops research institute (Wuhan, China) with conditions of a temperature of 22°C, LED sodium lamp and a humidity of about 50–70% for 6 weeks. The plants were then transferred to growth chamber programmed under specific environmental conditions for 2–3 days before stress treatment. The conditions in growth chamber were set as follows: temperature of 25°C and humidity of 50% in 16 h light; temperature of 22°C and humidity of 60% in 8 h dark.

**Stress Treatments**

The high CO₂ stress was performed in a growth chamber (AR-41L2, United States) in which CO₂ gas can be accurately and stably controlled in the range of 100–300 ppm. The conditions of growth chamber were set as follows: CO₂ concentration of 1000 ppm, light intensity of 100 umol/m²/s, temperature of 25°C and 60% relative humidity. Leaf samples were collected at 1, 3, and 6 h during the treatment.

The heat and drought stress were performed in a common growth chamber. For heat stress, the chamber was set with temperature of 40°C and humidity of 60%. Leave samples under heat were collected at 1, 3, and 6 h during treatment. For drought stress, the chamber was set as follows: temperature of 25°C and humidity of 40% in 16 h light; temperature of 22°C and humidity of 50% in 8 h dark; withholding water for 7 days, leaf samples were collected at 1, 2, and 3 days during drought treatment.

All the collected leaf samples were immediately frozen in liquid nitrogen, and stored at −70°C for further analysis.

**RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis**

The RNA was isolated from leaf tissues using an RNA extraction kit (Takara, Dalian), according to the manufacturer’s instructions. The first-strand cDNA was synthesized by the Prime Script RT reagent Kit (Takara, Dalian). Real-time quantitative PCR was performed using 2 µl of cDNA in a 20 µl reaction volume with SYBR Premix Ex Taq (Takara) on a 7500-Fast real-time PCR System (Applied Biosystems). Gene-specific primers were designed (Supplementary Table 1). The rapeseed *TMA7* gene (BnaC05g11560D) was used as an internal control to normalize the expression level of the target gene, which has highly stable expression level in different tissues and under various growth conditions. Each treatment was repeated three times independently. The thermal cycler was set as follows: an initial incubation at 50°C for 2 min and 95°C for 5 min, followed by 40 cycle at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. The relative...
quantification of BnaHsfs transcription levels was determined by the methods described previously (Livak and Schmittgen, 2001).

**RESULTS**

**Genome-Wide Identification of Hsf Genes in Brassica napus**

To identify Hsf genes in *B. napus*, candidate genes were selected by using the conserved Hsf domain (PF00447) from the Pfam database to query the *B. napus* genome. Meanwhile, the amino acid sequences of 21 AtHsfs from *Arabidopsis* were used to protein BLAST the *B. napus* genome. A total of 64 BnaHsfs (BnaHsf01-BnaHsf64) were identified as members of the Hsf gene family in *B. napus*, through simultaneous consideration of the conservation of the DBD domain, the coiled-coil structure from the SMART database, and the CD-search of the NCBI database (Table 1).

Fifty-four BnaHsf genes were distributed unevenly among the 19 chromosomes of *B. napus* from A01 to A10 and C01 to C09; however, 10 members (including BnaHsf02) were located on unanchored scaffolds that could not be mapped to a specific chromosome. Most BnaHsf genes were localized to chromosome A03 and C03 (8 and 7 Hsf genes), while there was only one BnaHsf on chromosome A04 and A06 (Table 1). The deduced proteins of the BnaHsf ranges from 226 amino acids (aa; BnaHsf56) to 517 aa (BnaHsf19) in length, with predicted isoelectric points (pl) varying from 4.61 (BnaHsf07) to 9.16 (BnaHsf57 and BnaHsf60) and molecular weight (MW) from 26.42 kDa (BnaHsf56) to 58.24 kDa (BnaHsf19) (Table 1).

**Phylogenetic Analysis and Multiple Sequence Alignment of BnaHsf Proteins**

Among the best-studied 21 *Arabidopsis* Hsfs, 15 members belong to group A and 5 members belong to Group B, and only one Hsf has been classified as part of group C (Nover et al., 2001). To explore the classification and the evolutionary characteristics of the BnaHsf genes, an unrooted phylogenetic tree was generated using protein sequences of BnaHsfs (Figure 1 and Supplementary Datasheet 1).

According to this phylogenetic analysis, the BnaHsf genes were grouped into three classes, A (BnaHsf01-BnaHsf43), B (BnaHsf44-BnaHsf60), and C (BnaHsf61-BnaHsf64), as in *Arabidopsis*. Class A was the largest and consisted of 43 members from eight subclasses (A1–A8). In *Arabidopsis*, class A is further subdivided into 9 subclasses, A1–A9, with A9 (At5g4070) appearing as a single branch of the AtHsfs molecular phylogeny. However, no orthologous HsfA9 genes were found in B. napus, indicating that Hsf genes in this subgroup were lost. Class B consisted of 17 members from four subclasses (B1–B4), and class C was the smallest, containing only 4 members (Figure 1 and Table 1).

Multiple sequence alignment analysis of BnaHsfs proteins showed that a typical *B. napus* Hsf protein contains 5 conserved domains, including of DBD, OD, NLS, NES, and AHA domains in order from N-terminal to C-terminal. Among these, the highly structured DBD and OD domains are the most conserved sections in each BnaHsf. The OD domain (HR-A/B region) served as an important basis for classification of BnaHsfs. *B. napus* class B Hsf proteins, like other plant Hsf proteins, are compact and lack an insertion between the HR-A and HR-B regions (Figure 2), while an insertion of 21 aa in length was found in BnaHsfA and an insertion of 7 aa in length was found in BnaHsfC between the HR-A and HR-B regions. Thus, class A members are less conserved than class B and C members.

**Structure and Motif Analysis of BnaHsf Proteins**

To study the structural diversity of BnaHsf genes, the exon/intron organization of individual BnaHsf genes was analyzed by comparing cDNA sequences with the corresponding genomic DNA sequence. The detailed gene structures are shown in Figure 3A. The number of introns ranges from zero to five in BnaHsf genes. Five introns were found in BnaHsf31, while none were found in the BnaHsf63/64 gene pair. Most BnaHsfs contained one or two introns (Figure 3A).

Conserved motif analysis was conducted by using MEME, and 15 motifs were detected in BnaHsf proteins (Figure 3B and Supplementary Figure 1). The DBD and OD domains, composed of motif 4, motif 1 and motif 2, were the most conserved and were found in almost all 64 BnaHsf members, while motif 1 was absent in BnaHsf43. The NLS motif (motif 9) and NES motif (motif 15) were found in most class A and B members but not in class C BnaHsf proteins. Motif 3 is the insertion between the HR-A and HR-B regions that was found only in class A and class C members. The AHA motif (motif 6) was found in most class A members but not in classes B and C. In general, the structure of Hsf proteins was conserved throughout the BnaHsf gene family.

**Tempo-Spatial Expression Profiles of Hsf Genes in Brassica napus**

To examine spatial and temporal expression profiles of BnaHsfs across different tissues and organs, an expression pattern map was drawn based on RNA-seq data (Supplementary Table 2) from twelve rapeseed tissues (leaf, root, stem, sepal, silique, pericarp, bud, stamen, ovule, new pistil, mature pistil and wilting pistil).

We found that BnaHsf genes were differently expressed among the subclasses in 14 tissues and at different developmental stages (Figure 4). BnaHsfA6 subclass (BnaHsf29-BnaHsf34) exhibited root specific expression but was hardly detected in other tissues. Subclass A7 (BnaHsf35-BnaHsf40) was also root specific but BnaHsf35 and BnaHsf37 were also detected in reproductive organs such as petal, stamen and pistil. The BnaHsf A2, A3, A4C and A5 subclasses were the most abundant BnaHsf genes and were constitutively expressed among all tissues, as were BnaHsf23 and -24 in subclass A4A, BnaHsf46 and -47 in subclass B1, and BnaHsf48 and -49 in subclass B2A. Subclass B3 (BnaHsf55-56) showed a higher transcription level in root, sepal and bud tissues.
TABLE 1 | Summary information of the BnaHsf family genes in Brassica napus.

| Gene name   | Subfamily | Gene ID       | Size (aa) | pI   | MW (kDa) | Chromosome location          |
|-------------|-----------|---------------|-----------|------|----------|-----------------------------|
| BnaHsf01    | A1A       | BnaA01g08640D | 430       | 5.06 | 47.82    | chrA01:4144293..4145692     |
| BnaHsf02    | A1B       | BnaA02g02370D | 442       | 4.84 | 49.00    | chrA02:1454367..1456043      |
| BnaHsf04    | A1D       | BnaA08g09680D | 439       | 4.74 | 48.81    | chrC02:3678716..3680294      |
| BnaHsf05    | A1E       | BnaA09g17670D | 487       | 4.92 | 53.69    | chrA10:1299786..13000101     |
| BnaHsf06    | B1        | BnaC09g41040D | 441       | 4.86 | 48.71    | chrC09:43210122..43212302    |
| BnaHsf07    | A2        | BnaA03g22890D | 473       | 5.35 | 41.74    | chrA03:10918958..10920504    |
| BnaHsf08    | A3        | BnaA05g32280D | 306       | 8.58 | 34.64    | chrA05:22119903..22121886    |
| BnaHsf09    | A4A       | BnaA01g09690D | 517       | 5.81 | 50.33    | chrA01:4758848..4761452      |

(Continued)
BnaHsf55 and -56 in subclass B4 were specifically expressed in ovule tissues. In contrast, class BnaHsfC was inactive in ovule tissues.

Expression Patterns of BnaHsf Genes under Abiotic Stresses

To determine the potential role BnaHsf play in plant responses to different environmental stresses, the expression levels of BnaHsf genes under high temperature, drought and high CO₂ stresses were analyzed using RNA-seq data (Supplementary Table 3). The mRNA for these transcriptomic analyses were extracted from the leaves of rapeseed plants both in normal growth conditions and after 3 h of heat treatment, 3 days of drought treatment and 3 h of high CO₂ treatment. The results showed that BnaHsf genes were very sensitive to heat and drought stress (Figure 5). Members of subclasses A2, A4C, A5, and class B (except for the non-expressed subclasses B3 and B4) showed relatively higher basic transcription levels in leaf tissue under normal growth conditions. Among the subclasses with high basic expression levels, BnaHsf15 and -16, in subclass A2, were dramatically upregulated (>25-fold), becoming the predominant transcripts after 3 days of exposure to drought stress, but were suppressed after 3 h of heat treatment.

B1 members, except for BnaHsf45, were strongly induced by drought stress, and BnaHsf46 and -47 were strongly induced by heat. All B2 members were significantly upregulated under drought conditions, but were only slightly induced by 3 h of heat treatment. As observed in the subclasses with low basic expression, a moderate induction was seen in the A1E subclass after exposure to drought and heat. Members of subclasses A3 and A4A were strongly induced by both drought and heat stress. Strikingly, the highest induction (>350-fold for BnaHsf36~38) by drought was observed in A7A subclass, although expression of members of this subclass was hardly detectable under normal conditions. Heat treatment also resulted in a marked induction in A7A members. Members in class C were only upregulated by drought treatment. In the case of individual member genes, BnaHsf07 (in A1D) and BnaHsf40 (in A7B) were induced by drought stress, and BnaHsf43 in subclass A8 was strongly induced by heat stress.

Unlike drought and heat stress, high CO₂ treatment did not cause a significant effect on the transcription level of most of the BnaHsf family genes (Figure 5). However, three member genes, BnaHsf18 (in A3), BnaHsf21 (in A4A) and BnaHsf43 (in A8), were clearly induced (3 to 9-fold) by exposure to high CO₂ conditions. However, the expression of members of subclass A2 members (BnaHsf15/16) and of subclass B2 was largely suppressed by high CO₂ treatment.

qRT-PCR Expression Analysis of Selected BnaHsf Genes under Abiotic Stresses

Twelve BnaHsf genes from three main classes were selected for examination of their function under three stress conditions using quantitative Real-Time PCR (qRT-PCR). These genes included BnaHsf04 from subclass A1, BnaHsf15 and -16 from subclass A2, BnaHsf17 and -18 from subclass A3, BnaHsf20 and BnaHsf23 from subclass A4, BnaHsf42 from subclass A8, BnaHsf45 from subclass B1, BnaHsf46 from subclass B2 and BnaHsf61 from class C. qRT-PCR was carried out using rapeseed plants exposed to heat (0, 1, 3, and 6 h), drought (0, 1, 2, and 3 days), and high CO₂ (0, 1, 3, and 6 h).

The results of the qRT-PCR analyses were consistent with the expression patterns of selected BnaHsfs from RNA-seq data, and provided more details under progressive stresses (Figure 6 and Supplementary Table 4). The RNA-seq data showed that transcription levels of BnaHsf15-16 were decreased after exposure to 3 h heat (Figure 5), while in qRT-PCR results they were upregulated after 1 h of heat, and their expression then dropped to lower than basic levels (Figure 6). However, the strong induction of BnaHsf15-16 began with a marked reduction in transcription after 1 day of treatment under drought stress. In contrast to BnaHsf15-16, the expression levels of BnaHsf17-18 and BnaHsf43 were progressively induced by all prolonged drought, heat, and high CO₂ stress conditions. A similar pattern was found in BnaHsf43 under drought and heat conditions. For BnaHsf21-22, the induction in their transcription was weakened as heat stress processed, while under progressive drought, the induction by stress showed an opposite enhancement (Figure 6).

### Table 3

| Gene name   | Subfamily | Gene ID              | Size (aa) | pl    | MW (kDa) | Chromosome location   |
|-------------|-----------|----------------------|-----------|-------|----------|-----------------------|
| BnaHsf53    |           | BnaA09g21510D        | 361       | 4.66  | 38.21    | chrA09:14189356..14190030 |
| BnaHsf54    |           | BnaC09g52680D        | 361       | 4.66  | 38.21    | chrC09_random:2042369..2043875  |
| BnaHsf55    | B3        | BnaA03g19560D        | 239       | 5.17  | 27.92    | chrA03:9269373..9270563   |
| BnaHsf56    |           | BnaC03g23450D        | 226       | 5.88  | 26.42    | chrC03:13066673..13069836   |
| BnaHsf57    | B4        | BnaA08g04110D        | 335       | 9.16  | 38.15    | chrA08:3451960..3453065  |
| BnaHsf58    |           | BnaC08g04780D        | 335       | 9.16  | 38.33    | chrC08:5463460..5464644   |
| BnaHsf59    |           | BnaA10g55400D        | 329       | 8.71  | 37.39    | chrA10:3141954..3142758  |
| BnaHsf60    |           | BnaC03g03010D        | 330       | 7.85  | 37.67    | chrA03:29056023..29056970  |
| BnaHsf61    | C         | BnaA07g05580D        | 314       | 5.87  | 35.79    | chrA07:5882469..5883727  |
| BnaHsf62    |           | BnaC07g07130D        | 314       | 5.87  | 35.91    | chrC07:11406343..11407422  |
| BnaHsf63    |           | BnaA03g07460D        | 306       | 6.57  | 34.90    | chrA03:18569380..18570300  |
| BnaHsf64    |           | BnaC03g43960D        | 315       | 6.06  | 35.71    | chrC03:29056023..29056970  |
Regulatory Cis-Element Analysis of BnaHsf Genes

To identify the presence of putative regulatory cis-acting elements enriched in BnHsf genes, the promoter sequences upstream of their CDS were extracted and searched against the PLACE database (Higo et al., 1999). Analysis showed that HSEs were the most abundant cis-elements in promoter regions of BnHsf genes, including perfect type and active HSE variants (Table 2). Many of the subclasses of the BnHsf gene family possess the two types of HSE, although these are not present in subclasses A3 and A6A. Subclass A6A contains only one member, BnHsf29, which may be a pseudogene since its transcription cannot be detected in any tissues of rapeseed plants. The two members (BnHsf17 and 18) of subclass A3 were found to have three other major types of stress-related cis-elements present in the BnHsf family, STRE, DRE/CRT, and MYCATRD22. The STRE element was first found to be stress responsive in yeast and can serve as a specific binding site for HsfA1a in Arabidopsis (Martinez-Pastor et al., 1996; Haralampidis et al., 2002; Guo et al., 2008). STRE was present in most subclasses of BnaHsf except A1A, A6A, and A7. DRE/CRT and MYCATRD22, two types of cis-elements responsive to drought stress, also appeared in most BnHsf subclasses. Two other stress related cis-elements, ABREOSRAB21 and LTRE, were found in some of the BnaHsf family members. In addition, three CO2-responsive elements (CCRE1/2/3) (Ohno et al., 2012; Tanaka et al., 2016) were

FIGURE 1 | Unrooted phylogenetic tree of rapeseed and Arabidopsis Hsf family. Amino acid sequences of Hsf proteins were analyzed using the neighbor-joining method with genetic distance calculated by MEGA6.0. The numbers at the nodes represent percentage bootstrap values based on 1,000 replications. The length of the branches is proportional to the expected number of amino acid substitutions per site.
**FIGURE 2** Multiple sequence alignment of the HR-A/B regions (OD domain) of rapeseed Hsf proteins.
observed in all BnHsf subclasses except A1A/B/D, A4A, A5, A6B, A7B, and B2A. The presence of these stress related cis-elements is likely responsible for the regulative expression patterns of BnHsf genes under drought, heat, and high CO₂ conditions. Moreover, some phytohormone responsive related cis-elements were enriched in promoter regions of BnHsf member genes, which may be involved in the stress acclimation response and development.

**DISCUSSION**

**High Number of Hsf Family Genes in Rapeseed Genome**

*Brassica napus* (rapeseed, genome AACC) is an amphidiploid species formed by recent interspecific hybridization between ancestors of *B. oleracea* (genome CC) and *B. rapa* (genome AA) (Chalhoub et al., 2014). In this study, we identified 64 Hsf genes...
in the genome of *B. napus*. Unlike yeast and animals, plants usually have many Hsf coding genes. There are 21 Hsf member genes in the model plant *Arabidopsis thaliana* (Nover et al., 2001), 25 members in rice (Chauhan et al., 2011), 56 Hsf genes in

**FIGURE 4** | The tempo-spatial transcription profiles of *BnaHsf* family genes in various tissues at different developmental stages. The different colors correspond to log2 transformed value.

**FIGURE 5** | Expression profiles of *BnaHsf* genes under drought, heat and high CO2 conditions. HT/CK, expression levels under heat vs. control; D/CK, expression levels under drought vs. control; HC/CK, expression levels under High CO2 vs. control. The different colors correspond to log2 transformed value.
wheat (Xue et al., 2014). To date, BnaHsfs represent the largest Hsf gene family in plant species of which Hsf member genes were analyzed. The diversification of plant Hsfs is presumably the result of gene- and whole-genome duplications (WGD) at different points in evolution, followed by gene loss (Chalhoub et al., 2014). In the case of rapeseed, the allopolyploid process, that followed from the fusion of genomes A and C, might also play a crucial role in the expansion of the BnaHsf gene family. In addition, the large size of the BnaHsf family may have been needed for adaptation of rapeseed to diverse climatic zones.

**Structural Analysis of BnaHsf Genes**

Similar to other plant Hsf families, the modular structure of rapeseed Hsf proteins is well conserved. While in comparison with that in Arabidopsis, there is no Hsf member in subclass A9 subclass in rapeseed. This differs from other eudicot plants, most of whom have sub class A9 Hsfs. This subclass is also lost in the Hsf family of B. napus (Huang et al., 2015). The DBD is characterized as a central domain for the Hsf protein: it specifically binds to HSEs in the target promoter region, and subsequently activates the transcription of associated heat-inducible genes. The DBD domain of plant Hsfs is encoded by two regions separated by an evolutionarily conserved intron, which was inserted immediately adjacent to the HTH DNA binding motif (Nover et al., 2001; Scharf et al., 2012). Most BnaHsf genes have this intron in their DBD domains; however, no intron was found in BnaHsf63 and -64 genes from class C, as shown by their gene structure (Figure 3A). As far as we know, the fact that this highly conserved intron in not present in the DBD domain of a plant Hsf is unique. Furthermore, this fact may indicate that BnaHsf63 and -64 have a novel regulation pattern relative to other Hsf genes.

**Diverse Transcriptional Patterns of BnaHsf Family Genes during Development and Abiotic Stresses**

The functional diversification of BnaHsf family genes was also found in the tempo-spatial expression profile of these genes during development and abiotic stress treatments. Among the tissues at different developmental stages, subclasses A1A, A2/3, A4C, A5, A8, and B2 were found to be constitutively expressed at relatively high levels in all the tissues examined. While almost all member genes from subclasses A6/7 and B3/4 were hardly detected in any tissue, BnaHsf35 from subclass A7A showed a high level of expression in root tissue. Subclasses B1 and B2A also showed high levels of expression in root tissue. Members of class C also showed increased expression in root tissue, but were not expressed in ovule tissue. These results suggest that these BnaHsf genes may be involved in root development. Under abiotic stress, many BnaHsf genes were upregulated in response to drought treatment. The number of drought induced BnaHsf genes was comparable to those induced by heat. This suggests that Hsf genes may also play an important role in the response and the acclimation to drought stress in rapeseed. Furthermore, the most inducible BnaHsf genes were upregulated by both drought and heat treatment, as shown by the combination of RNA-seq and qRT-PCR data. While BnaHsf43 of subclass A8 was only induced by heat, BnaHsf07 of subclass A1D and members of class C were predominantly upregulated by drought.

According to the combined transcriptional analysis, heat inducible BnaHsf genes could be divided into three groups. The first group consisted of BnaHsf15 and -16 (subclass A2), BnaHsf47 (subclass B1) and BnaHsf50 (subclass B2A), in which the expression of member genes exhibited an immediate and strong induction after 1 h of heat to a high level of 40~70-fold of that in non-stressed control, followed by a dramatic drop to the basic expression level after 3 and 6 h of heat treatment, even was slightly suppressed in subclass A2 after 3 and 6 h of heat. It may be that this group of genes governs early heat stress response. The second group contains BnaHsf21 and -22 of A4A, and BnaHsf46 of subclass B1. The transcription of the second group members showed also a fast and strong upregulation after 1 h of heat exposure. This upregulation of gene expression gradually declined after 3 and 6 h of heat, but still maintained at a high level relative to that in control. Genes from the second group might be involved in both early and late heat response. The last group greatly differed from the other two, comprised of BnaHsf17 and -18 of subclass A3 and BnaHsf43 of subclass A8. The genes from this third group were upregulated after 1 h of heat, and this induction was continuously enhanced as the stress treatment proceeded, finally peaking after 6 h of heat stress (46~550-fold vs. control). The members of this group likely have some function to facilitate acclimation to prolonged heat stress.

Drought induced BnaHsf genes seemed to have a single expression pattern. The genes continuously increased transcription during exposure to drought, and reached peak expression after the 3 days drought treatment. BnaHsf genes also played an important role in response to high CO2 treatment, as BnaHsf18 (A3), BnaHsf21 (A4A) and BnaHsf43 (A8) were strongly upregulated, while members of A2 and B2 subclasses were downregulated.

**Various Regulatory cis-Elements Enriched in the Promoters of BnaHsf Genes**

Regulatory element analysis revealed that there were many stress-related cis-acting elements enriched in the promoter regions of BnaHsf family genes. HSEs were found to be the dominant cis-elements (Table 2). Complex interactions may exist among BnaHsf genes, and these may come about via trans-acting regulation, as HSEs are marker binding sites for plant Hsf proteins. Previous work has supported this idea that HsfA1a/b target class B Hsf genes and are responsible for their induction during heat response in Arabidopsis (Lohmann et al., 2004; von Koskull-Döring et al., 2007), and that HsfA5 inhibits the activity of HsfA4 (Baniwal et al., 2007). Other abiotic stress-related cis-elements, including STR, DRE/CRT, MYCATRD22, ABRE, CCRE, and LTRF were also major regulatory elements found in BnaHsf genes. The presence of these stress-related elements seemed to be correlated to the expression response of BnaHsfs to heat, drought, and high CO2 treatments. For example, many drought related DRE/CRT and MYCATRD22 elements found upstream were associated with a marked induction of
TABLE 2 | Putative cis-elements enriched in the promoters of BnaHsf family genes.

| Sequence | BnaHsf subclass (members) | Element/stimulus annotation |
|----------|---------------------------|-----------------------------|
| NGAAPNNCTCN | A1A (Hsf02), A1B (Hsf03), A1D (Hsf08), A1E (Hsf10/11/14), A2 (Hsf15/16), A4A (Hsf19/21/23/24), A4C (Hsf25), A5 (Hsf28), A6B (Hsf31/33/34), A7A (Hsf35/38), A7B (Hsf39), A8 (Hsf42), B1 (Hsf44/46), B2A (Hsf50), B2B (Hsf51/54), B3 (Hsf55), C (Hsf63) | Perfect HSEs; heat stress elements |
| NGA/(N)(N)/ANT | A1D (Hsf09), A2 (Hsf16), A4A (Hsf19/22/23), A6B (Hsf31/34), A7A (Hsf36/37), A8 (Hsf41), B1 (Hsf46), B2A (Hsf48/50), B2B (Hsf51/54), B3 (Hsf59/60), C (Hsf61/62) | Active HSE variants |
| AGGGGG | A1B (Hsf03), A1D (Hsf08/09), A1E (Hsf13), A2 (Hsf16), A3 (Hsf17/18), A4A (Hsf19), A4C (Hsf26), A5 (Hsf28), B1 (Hsf44/45/47), B2A (Hsf50), B3 (Hsf55/56), B4 (Hsf57), C (Hsf64) | STRE; stress-responsive element |
| RYCGAC | A1A (Hsf02), A1D (Hsf08/09), A1E (Hsf11/13), A2 (Hsf15/16), A3 (Hsf17/18), A4A (Hsf19/20/22/23), A4C (Hsf26), A7A (Hsf35), A7B (Hsf40), B1 (Hsf44/45/47), B2A (Hsf50), B3 (Hsf56), B4 (Hsf57), C (Hsf64) | DRE/CRT; drought, cold |
| CACATG | A1A (Hsf02), A1B (Hsf05/06), A1D (Hsf07/09), A1E (Hsf14), A2 (Hsf15), A3 (Hsf17/18), A5 (Hsf27), A6B (Hsf34), A7A (Hsf36), A7B (Hsf40), A8 (Hsf41), B1 (Hsf45), B2B (Hsf53/54), B3 (Hsf56), B4 (Hsf59/60), C (Hsf61/62) | MYCATRD22; dehydration, ABA |
| ACCTTSSSC | A1B (Hsf03/04/06), A2 (Hsf16), A3 (Hsf17), A6A (Hsf29), A6B (Hsf34), A7B (Hsf39/40), B2B (Hsf53) | ABREOSRAB21; ABA-responsive elements |
| TGACGT | A1E (Hsf11), A2 (Hsf16), A3 (Hsf18), A4C (Hsf26), A6A (Hsf29), A7A (Hsf37), A8 (Hsf41/42), B1 (Hsf47), B2B (Hsf53), C (Hsf61/62/63) | CCRE1; CO2-responsive element |
| ACGCTCA | A1E (Hsf10/14), A2 (Hsf15/16), B1 (Hsf46), B2B (Hsf52), B3 (Hsf55), B4 (Hsf60), C (Hsf63/64) | CCRE2; CO2-responsive element |
| TGAACGC | A3 (Hsf18), A7A (Hsf36) | CCRE3; CO2-responsive element |
| CCGAAA | A1D (Hsf07), A2 (Hsf15), A6B (Hsf34), A7A (Hsf37), A7B (Hsf59), B1 (Hsf45/47), B2A (Hsf49), B2B (Hsf52/54), B4 (Hsf57) | LTRE; low temperature responsive element |
| TACACACA | A1B (Hsf03/06), A3 (Hsf17), A6A (Hsf29), A8 (Hsf43), B2A (Hsf50) | GARE1OSREP1; Gibberellin |
| GCCGCC | A1B (Hsf03/04), B3 (Hsf56), B4 (Hsf58), C (Hsf64) | GCCCORE; Jasmonic acid |
| AVTTCAAA | A1D (Hsf09), A1E (Hsf10/11), A4A (Hsf19/21), A4C (Hsf25/26), A5 (Hsf27/28), A6A (Hsf29), A6B (Hsf30/34), A7A (Hsf35/37), A8 (Hsf41/43), B1 (Hsf44/45), B2B (Hsf54), B3 (Hsf58), B4 (Hsf59), C (Hsf61/62) | ERELEE4; Ethylene; senescence |
BnHsf genes by drought stress. The two CCRE elements situated in promoter region also agreed with our observation of high induction levels of BnHsf18 under high CO₂ treatment. Unlike BnHsf15 and -16 of subclass A2, the heat highly inducible genes BnHsf17 and -18 of subclass A3 do not have functional HSE elements, but rather an STRE element was found upstream of the target genes. The STRE element was identified to be stress responsive, and serves as a direct binding site for HsfA1a besides HSE in Arabidopsis (Guo et al., 2008). Furthermore, the deletion of STRE from the promoter of the Arabidopsis Hsp90-1 gene decreased its promoter activity under heat stress conditions (Haralampidis et al., 2002). These findings suggest that STRE also plays a crucial role in transcriptional regulation under heat conditions, as do HSEs. Moreover, the different heat induced expression patterns of subclass A3 and subclass A2 BnHsf genes provides evidence for differential transcription regulation abilities of STRE and HSE element. Unexpectedly, rapeseed subclass A1A was not heat inducible, although HSE elements are found in the promoter. While HsfA1a serves as master regulator of thermotolerance in tomato (Mishra et al., 2002), it also functions actively in Arabidopsis under heat stress. These results may indicate differential gene regulation of rapeseed Hsf genes from those found in other plants, even those in the Brassicaceae.

CONCLUSION

Our genome-wide investigation of Hsf genes in B. napus reveals the largest plant Hsf gene family to date. With expression profile analysis, novel members of BnaHsf family were found to respond to high temperatures, as well as drought and high CO₂ stresses. Further characterization of these novel multifunctional BnaHsf genes will improve our understanding of the acclimation response of plants to multifactorial and combinational abiotic stresses, and may also provide useful genetic resources for further research on abiotic stress resistance in crops.

AUTHOR CONTRIBUTIONS

XZ and WH conceived and designed the research. XZ, CH, LZ, HL, JY, and ZH performed the experiments and bioinformatics. XZ and CH analyzed the data. XZ and CH wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01174/full?supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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