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Genome-wide analysis of the HSF family in *Allium sativum* L. and AsHSFB1 overexpression in *Arabidopsis* under heat stress

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Abstract

The heat shock transcription factor (HSF) family is one of the most widely studied transcription factor families in plants; *HSFs* can participate in the response to various stressors, such as heat stress, high salt, and drought stress. Based on garlic transcriptome data, we screened and identified 22 garlic *HSF*s. The HSF proteins of garlic and *Arabidopsis* can be divided into three (A, B, C) subfamilies. The phylogenetic relationship, chromosome localization, sequence characteristics, conserved motifs, and promoter analysis of the HSF family were analyzed through bioinformatics methods. RT-qPCR analysis showed that the nine selected genes had different degrees of response to heat stress. In addition, we isolated and identified a class B *HSF* gene, *AsHSFB1*, from garlic variety 'Xusuan No.6'. Subsequently, the *AsHSFB1* gene was overexpressed in *Arabidopsis thaliana*. Under heat stress, the germination rate and growth of wild-type plants were better than that of transgenic plants. Moreover, after heat treatment, the contents of peroxidase, catalase, and chlorophyll a and b of transgenic plants were lower, but the contents of malondialdehyde (MDA) and leaf conductivity were higher. Nitroblue tetrazolium (NBT) staining showed that the stained area of transgenic plant leaves was larger than that of the wild type. Further studies showed that *AsHSFB1* overexpression inhibited the expression of related reverse resistance genes. These results indicate that *AsHSFB1* might play a negative regulatory role in garlic resistance under high stress. Altogether, these findings provide valuable data for revealing the function of *HSF* genes and lay a foundation for the subsequent selection of heat-resistant garlic varieties.

Keywords AsHSFB1, *Arabidopsis*, Garlic, Heat shock factor family, Heat stress

Introduction

Heat, high salinity, and drought are the main abiotic stressors affecting plant growth [1]. Recent studies have indicated that heat transcription factor genes (*HSFs*) are key transcription factors (TFs) that play regulatory roles in plants under abiotic temperature stress and induce the transcription of heat shock proteins (HSPs) by binding to the reverse repetitive sequence region of heat shock elements [2]. As molecular chaperones, HSPs can cooperate with other proteins to reduce damage to plants caused by heat stress. include the DNA binding domain (DBD), oligomerization domain (OD, or HR-A/B), nuclear localization signal (nuclear localization signal, NLS), and nuclear export signal (NES) [3]. Near the N-terminus of HSFs is a highly conserved DBD, whose hydrophobic core forms a helix-turn-helix (H2-T-H3) structure that is required for specific binding of the HSE conserved motifs [4]. The NLS consists of basic amino acids and is mostly contiguous to the C-terminal HR-A/B [4]. According to the

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differences in the OD domain, the HSF family is divided into three categories, A, B, and C, each of which has subgroups, namely HSFA (A1–A9), HSFB (B1–B5), and HSFC [5].

Studies have shown that most HSF family members play a role in stress resistance by regulating a series of heat shock proteins, molecular chaperones, reactive oxygen scavenging enzymes, and other functional protein genes [6]. Among them, *HSFAs* have the largest number of gene members, and *HSFAs* are the main regulatory factors inducing plant heat tolerance and are widely involved in plant responses to heat shock, oxidation, and salt stress, the functions of which have been studied the most [7]. Many *HSF* genes exist in plants, and HSFs have been shown to be involved in the response to abiotic stress, such as heat and salt stress [8]. The size of the *HSF* gene family varies significantly among plant species. At present, at least 21 *Arabidopsis* HSFs [9], 56 wheat *HSFs* [10], 25 rice *HSFs* [11], 64 *Brassica napus* HSFs [12], and 18 peach *HSFs* [4] have been isolated and identified. Overexpression of the wheat *TaHSF3 gene* improved transgenic *Arabidopsis* heat resistance; in addition, *TaHSF3* expression in wheat seedlings increased under high temperatures [13].

Garlic (*Allium sativum* L.) is an important vegetable crop with rich nutrients and medicinal functions [14]. HSFs not only contribute to stress response but also participate in plant growth and development [15, 16]. To the best of our knowledge, there are currently no reports of garlic HSF identification or function analysis. In this study, we aimed to evaluate the structure and expression profile of the *HSF* gene family in garlic. A total of 22 genes were identified and characterized as members of the HSF gene family in garlic; additionally, we performed bioinformatic analyses of phylogenetic relationships, conserved domains, and motifs. In addition, the heat stress tolerance of newly obtained transgenic *Arabidopsis thaliana* expressing *AsHSFB1* were analyzed.

Materials and methods

Plant materials and growth conditions

The garlic (*Allium sativum L*) cultivar 'Xusuan NO.6' (surensuan202202) was used as the experimental material. It was conserved at the rhizome germplasm resource center of the Xuzhou Institute of Agricultural Sciences in the Jiangsu Xuhuai area. The *Arabidopsis* was a Colombian wild type (Col-0). For the heat stress, after 20 d, garlic plants were placed in a growth chamber at 38 °C to induce heat stress. Treatment with ddH_2O was used as the control. Each replicate included 10 plants. The leaves of garlic plants were sampled at 0, 1, 4, 8 and 24 h after treatment. The samples were frozen in liquid nitrogen and stored at − 80 °C for RNA extraction.

Identification of *HSF* genes in garlic

The sequences of *HSFs* in garlic were extracted based on an annotated garlic genome database. A total of 22 HSF sequences from *A. thaliana* were downloaded from the TAIR website (http://www.arabidopsis.org/). We obtained HSF sequences of other species from the plant TF database (http://planttfdb.cbi.pku.edu.cn/index.php).

Phylogenetic and motif analyses of AsHSFs

A phylogenetic tree was constructed using MEGA 11.0 (bootstrap value of 1000) [17]. Circos software was used to map AsHSF chromosome positions [18]. The protein characteristics of AsHSFs were analyzed using the ExPASy program (Table 1). The structure of 22 *AsHSF* genes in the annotated garlic genome file was visualized with TBtools software. MEME (http://meme-suite.org/index.html) and TBtools were used to analyze and visualize the conserved motifs of all the identified *AsHSF* genes [19].

Cis-acting element analysis of AsHSFs

The complete coding sequences and transcriptome sequences of the 15 AsHSF genes obtained from garlic were blasted against the local NCBI genome (garlic). *Asa0G02991.1*, *Asa5G00955.1*, *Asa6G06026.1*, *Asa4G02336.1*, *Asa6G04911.1*, *Asa4G02337.1*, *Asa4G01727.1*, and *Asa6G03712.1* were from the A group. *Asa1G03975.1*, *Asa7G06422.1*, *Asa1G01216.1*, *Asa7G07097.1*, *Asa5G00486.1*, and *Asa2G05473.1* were from the B group, and Asa8G01031.1 was from the C group. We selected about 2000 bp upstream of the *AsHSF* genes' start codon for promoter analysis. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the promoter region of 15 *AsHSF* genes.

Generation of transgenic *Arabidopsis*

According to the design principle of homologous recombinant primers, two restriction enzymes (*BamH* I and *Hind* III) were selected to clone AsHSFB1-F and AsHSFB1-R, and the AsHSFB1 gene was amplified. Two restriction enzymes, *BamH* I and *Hind* III, were used for double enzyme digestion of the pCAMBIA-1305 vector to obtain a linearized vector. The AsHSFB1 gene was amplified by recombinant primers, and the fragment was reassembled into the pCAMBIA-1305 vector. The recombinant plasmid was introduced into Agrobacterium tumefaciens GV3101 using the electric shock transformation method, and the genetic transformation of Arabidopsis thaliana was performed using the flower dipping method [20]. We used T3 transgenic plants (three

Table 1 Primer sequences for RT-qPCR

Gene ID	Forward primer (5′→3′)	Reverse primer (5′→3′)
Asa0G02991.1	*AAGCAGCAGCAACAGTCAACAT*	*TCGGCATTTGTTCAAGCATCGT*
Asa5G00955.1	*TCACTTCCGCCGCCATTCCT*	*TGTTCAGTTGGTGGTCGCTTGT*
Asa6G06026.1	*GGCTCCACCTCAACCTCAGAAG*	*GCTGTCCATCAACGGCAGATAC*
Asa6G04911.1	*CGCCTCCGAATCCACCTCATAA*	*CCTTCGATCTGGTTGCCGTTCA*
Asa7G06422.1	*ATCCACCGCCGACACAAAGC*	*ACAGTTCAAGTTCCGCCACCTC*
Asa2G05473.1	*CAGCAGCAGCAGCAGTGACA*	*ACCGCCGTACTTGGACATAAGC*
Asa8G01031.1	*TTCGTCGTCGTTGATCCGTTCG*	*TTCTTCCTCGCCGCCTTCCTT*
Asa4G01727.1	*CGCCTCCGAATCCACCTCATAA*	*TCTCAACGTCCAAGCCTTCCAA*
Asa6G03712.1	*ACAGTCGGAGCAGAGCATACCT*	*CGATGTCCACGCATGAAGTCCT*
SAND	*GCGTCAACGAATGTTCCAATTACCA*	*TCTCTTCAGTCTCAACTTCATCAGCAT*
AtActin	*CAGTGTCTGGATTGGTGGTTCTATC*	*TCCCGTCATGGAAACGATGT*
AtHSFA1	*TCGTCAGCTCAATACTTATGGATTC*	*CACATGACATCCCAGATCCTTGC*
AtLEA	*CGTTCAGTTCAGCGTTTTAGC*	*GGAAGCGGCGGTGTTATG*
AtHSP70	*TTGTTGGACATTGACCTCTC*	*GGCAAACTTTTAATTTTTATCCG*
AtHSP98	*CGTTCGGTGATGTAGCGGTCTG*	*CGTTCGGTGATGTAGCGGTCTG*
AtRD22	*CCTAACGCGGCTCTTTCTT*	*TCTTCCGAACCAGCTTCCAC*
AtAPX1	*ACTACCCAACCGTGAGCGAAGA*	*TGCCATGCGAGTCGGACCAT*

transgenic lines AsHSFB1-OE1, AsHSFB2-OE2, and AsHSFB3-OE3) for further physiological and biochemical analysis.

Heat treatment and physiological determination of transgenic plants

Arabidopsis seeds of both wild-type (WT) and overexpressing (OE) strains were uniformly seeded on Murashige and Skoog (MS) solid medium. After 3 days of vernalization, WT and transgenic *Arabidopsis* seeds were exposed to heat stress at 46 °C for 30 min, and the seed germination rate was observed. The untreated seeds were cultured at 22 °C (light 16 h/ dark 8 h) for 7 days, and then transferred to a sterile soil. After 18 days, three transgenic plant lines and WT *Arabidopsis* plants were placed in growth chambers and subjected to heat stress. The treatment conditions were as follows: 38 °C heat shock treatment for 2 h, 22 °C growth recovery for 2 days, then 40 °C heat treatment for 3 days, and finally 22 °C growth recovery for 7 days. There were three replicates for each of the above processes. The leaves were collected at 0, 1, 4, and 8 h, and the leaves of the control group were immediately frozen in liquid nitrogen and stored at − 80 °C. Then, 0.1 g of leaf tissue was taken according to the manufacturer's instructions (Beijing Solarbio Technology Co., Ltd., China) and 1 mL of extract liquid was added for ice bath homogenization; the mixture was then centrifuged at 4℃ for 10 min at 10,000 g [21, 22]. After that, the supernatant was placed on ice for measurement. To calculate the POD content, first, the weight of the original leaf sample (W) was recorded. Then, the reagents in the kit were added into the EP tube in sequence and then mixed, after which 200 μL of the mixture was transferred to a 96-well plate. Then, the light absorption value (A) at 470 nm was recorded for 30 s (A1) and 1 min 30 s (A2), and the ΔA was calculated by subtracting A1 from A2. After that, the following formula was used: POD (U/g) = 9800 × ΔA ÷ W. To calculate the CAT content, 10 μL of the sample, as well as the working liquid provided in the kit, were added to a 96-well plate and mixed. The initial absorption value (A1) at 240 nm and the absorption value after 1 min (A2) were then determined, and the difference between them was calculated as follows: ΔA = A1 − A2. Finally, the following formula was used: CAT (U/g) = 764.5 × ΔA ÷ W. To calculate the MDA content, the determination tube and the blank tube were arranged according to the instructions in the kit, and then 200 μL of the mixture's supernatant was absorbed into the 96-well plate to determine the absorbance of each sample at 532 nm and 600 nm. The following was then calculated: $\Delta A532 = A532_{determination} - A532_{blank}$, $\Delta A600 = A600_{determination} - A600_{blank}$, ΔA = ΔA532 − ΔA600. Finally, the following formula was used to determine the MDA content: MDA (nmol/g) = 53.763 × ΔA ÷ W.

The accumulation of superoxide anion (O^{2-}) in Arabidopsis leaves was analyzed by NBT staining [23]. The collected fresh leaves were incubated overnight at 28°C in 0.05% (w/v) NBT staining buffer and decolorized with 75% ethanol. When all the chlorophyll had been removed, the final picture was taken. The stained leaves were then observed and photographed. The conductivity meter method was used to determine the relative

conductivity [24]. A 0.1 g leaf sample was placed into a test tube filled with 15 ml of deionized water. After 24 h, the initial conductivity (Cinitial) was measured with a conductivity meter, and then the leaves were boiled at 100 °C for 15 min, after which they were cooled to room temperature and their final conductivity (Cmax) was measured. Three biological replicates were used for all treatments. The relative electrical conductivity was calculated as follows: EL = Cinitial/Cmax × 100%. The content of chlorophyll was determined by the ethanol extraction colorimetric method [25]. Leaf chlorophyll was extracted with 95% ethanol in the presence of a small amount of quartz sand and calcium carbonate powder, and the entire extraction process was carried out under dark conditions. The absorbance values at 665, 649 and 470 nm were determined using a spectrophotometer with 95% ethanol as the control, and the chlorophyll a and b contents were calculated.

Quantitative real-time PCR analysis

Total RNA from garlic 'Xusuan No.6' leaves and T3 transgenic *Arabidopsis* thaliana leaves was isolated using a plant total RNA purification kit, and cDNA was synthesized using a FastQuant RT Kit (Tiangen Biotech, Beijing, China). RT-qPCR was performed following the instructions of the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China). The quantitative primers were designed using Primer Premier 6.0. *SAND* was used as the internal control for normalization [26]. All primers are listed in Table 2. The reaction process consisted of 40 cycles of pre-denaturation at 95 °C for 10 min, followed by denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 40 s. The PCR reaction system had a volume of 20 μL, with each PCR system containing 2.0 μL cDNA, 0.4 μL positive and negative quantitative primers, 10 μL SYBR Premix Ex Taq, and 7.2 μL ddH_2O.

Statistical analysis

Data in this paper were analyzed using the minimum significant difference (LSD) test in SPSS 17.0 statistical software. Statistical data are presented as the mean ± the standard error of the mean, and one-way ANOVA was performed to determine statistical significance. The statistical significance threshold was set to $P < 0.05$. Student's t-test method was used in $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$(***) and $P < 0.0001$(****) levels were used to analyze the significance of differences in 9 *AsHSF* genes data under heat stress. Three biological replicates were performed.

Table 2 Physicochemical analysis of *AsHSF* TFs

Gene ID	Length of CDS (bp)	Molecular weight (Da)	Predicted isoelectric point (PI)	Number of amino acids	Chromosome location	Subfamily
Asa5G00955.1	699	26,498.92	6.47	232	5	A1
Asa6G06026.1	1353	46,276.46	4.82	411	6	A1
Asa4G01727.1	834	32,479.51	4.88	277	4	A2
Asa4G02336.1	588	22,411.72	9.06	195	4	A2
Asa4G02337.1	924	35,301.92	5.35	307	4	A2
Asa6G04911.1	951	36,311.93	4.92	316	6	A2
Asa6G03712.1	1224	46,224.52	5.23	407	6	A3
Asa4G05278.1	1392	51,395.00	5.01	463	4	A5
Asa1G03975.1	1176	44,808.68	5.79	391	1	A4
Asa1G01216.1	357	13,538.24	4.84	118	1	B1
Asa7G07097.1	732	28,239.34	6.98	243	7	B1
Asa7G01431.1	438	16,165.60	5.77	145	7	B1
Asa0G02991.1	1353	50,937.21	4.91	450	-	B1
Asa5G00486.1	699	26,498.92	6.47	232	5	B2
Asa2G05473.1	875	31,448.31	6.22	292	-	B2
Asa8G02166.1	831	31,343.28	8.80	276	8	B3
Asa7G06422.1	531	20,572.27	9.41	176	7	B3
Asa0G03146.1	765	30,069.97	6.84	254	-	B4
Asa8G02160.1	987	36,423.65	9.01	324	8	B4
Asa6G01673.1	801	31,336.03	6.46	246	6	B4
Asa8G01031.1	705	26,884.36	5.63	234	8	C
Asa0G01036.1	876	34,108.92	6.03	291	-	C

Results

Genome-wide identification and chromosomal distribution of HSF genes in garlic

In order to analyze the HSF transcription factor family in garlic, we constructed a phylogenetic tree using the amino acid sequences of 21 proteins from *Arabidopsis thaliana* and 22 proteins from garlic. The 22 HSFs were divided into group A (A1, A2, A3, A4, A5, A6, A7, A8, A9), group B (B1, B2, B3, B4), and group C. In Fig. 1, these are represented by different colors. The HSFA, HSFB, and HSFC classes contained 9, 11, and 2 members, respectively. As shown in Fig. S1, the highest number of proteins was in group B, followed by groups A and C.

According to the garlic genome sequencing data, the chromosome distribution of 22 *HSF* genes was analyzed. A total of 18 HSF genes were located on 6 garlic chromosomes, and the location of 3 HSFs was unknown (Fig. S2). *Asa4G02337.1* and *As4G02336.1* on chromosome 4 and *Asa8G02166.1* and *Asa8G2160.1* on chromosome 8 are located in roughly the same positions, indicating that there may be tandem duplication between these genes. The distribution of the 18 HSF genes on chromosomes was uneven, among which the number of genes on chromosomes 4 and 6 was the largest, with four. Chromosomes 7 and 8 contained three genes, and chromosomes 1 and 5 contained only two genes.

Gene structure, conserved motif and physical properties of HSFs

ExPASy was used to analyze the isoelectric points and other fundamental physical properties (Table 2). The amino acid lengths of the AsHSF proteins ranged from 118 (Asa1G01216.1) to 463 (Asa4G05278.1) amino acids. The theoretical isoelectric point and molecular weight varied from 4.82 (Asa6G06026.1) to 9.41 (Asa7G06422.1) and from 13,538.24 (Asa1G01216.1) to 50,937.21 (Asa0G02991.1), respectively. These proteins ranged from basic to acidic.

The visualization of the protein structure of 22 garlic AsHSF proteins are shown in Fig. 2A, and the results show that 22 AsHSF proteins had the conserved domain of HSF. As shown in Fig. 2B, there were differences in

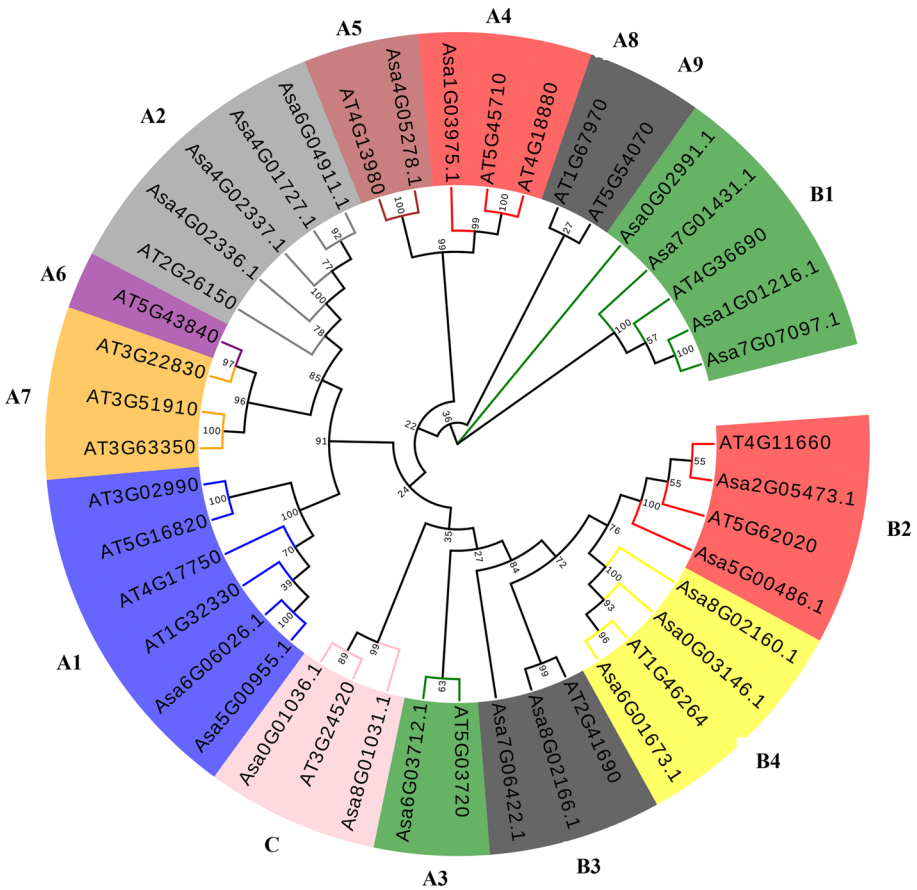

Fig. 1 Phylogenetic tree of heat shock transcription factors (HSFs) of *Arabidopsis thaliana* and garlic. Different colors and letters represent various subgroups

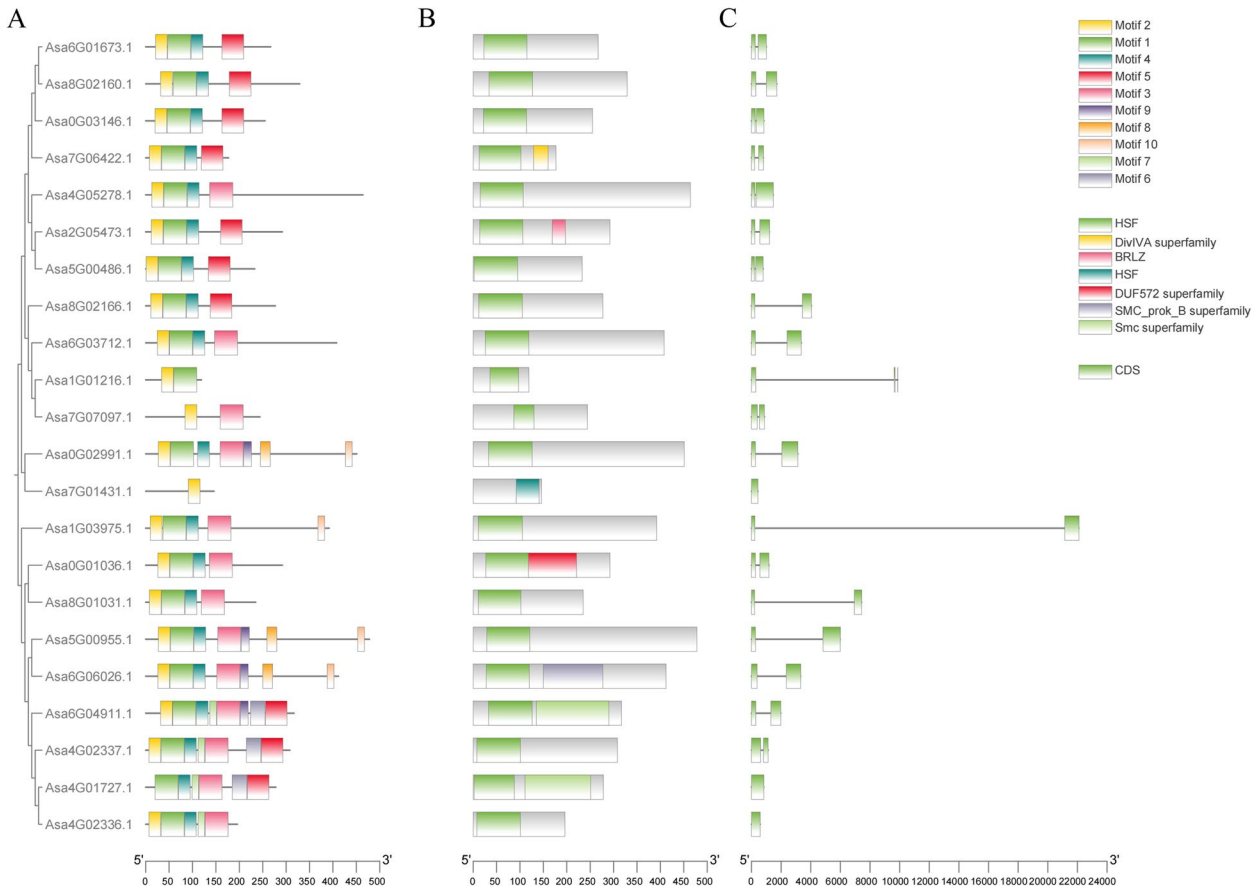

Fig. 2 Phylogenetic relationship, gene structure analysis, and motif distribution of garlic AsHSF genes. **A** Amino acid motifs in the AsHSF proteins are represented by colored boxes. The black lines indicate relative protein lengths. **B** Conserved domain structures of 22 *AsHSF* genes. **C** Exons and introns are indicated by rectangles and gray lines, respectively

motif type and location among members of the same subfamily, but there were no significant differences between members of different subfamilies, indicating the diversity of motif sequences in the garlic HSF subfamily. The structural organization details revealed that only 2 of the 22 AsHSF genes contained no introns in the A2 subfamily, while the rest of the HSF genes had more than two exons (Fig. 2C).

Analysis of *cis*-acting regulatory elements in *AsHSF* gene promoters

To understand the role of *AsHSF* genes in plant growth and development and their ability to respond to stress, PlantCARE was used to predict the cis-acting elements of the *AsHSF* gene promoter regions. Figure 3 shows the 15 *HSF* genes selected for analysis. Apart from basic TATA-box and CAAT-box, *AsHSFs* also contained many hormone responses, stress responses, and growth-related elements. The most common elements were light responsiveness (Box 4) (in 93.3% of the promoters) and anaerobic induction elements (ARE) (in 86.6% of the promoters). In addition, *AsHSF* gene promoters also contained ethylene-responsive (ERE), drought inducible (MBS), and gibberellin-responsiveness (TATC-box) elements. The different *cis*-elements in the promoter regions of these *AsHSFs* imply that *AsHSFs* may function in plant development and stress responses.

Interaction networks of AsHSFs and related proteins

We used STRING software to analyze the relationship between AsHSFs and related proteins and constructed a protein interaction network [27]. As shown in Fig. 4, a total of 22 HSF proteins were associated with each other. The interaction score between HSFA2 (Asa7G01431.1) and the HSP101 protein was the highest (0.987). HSP101 is a cytoplasmic heat shock protein required for high-temperature adaptation, indicating that the Asa7G01431.1 gene may regulate heat stress processes. All 22 HSFs had protein interactions with HSP90.1, HSF70, and HSF101, suggesting that the activation of downstream HSP expression by HSF may be

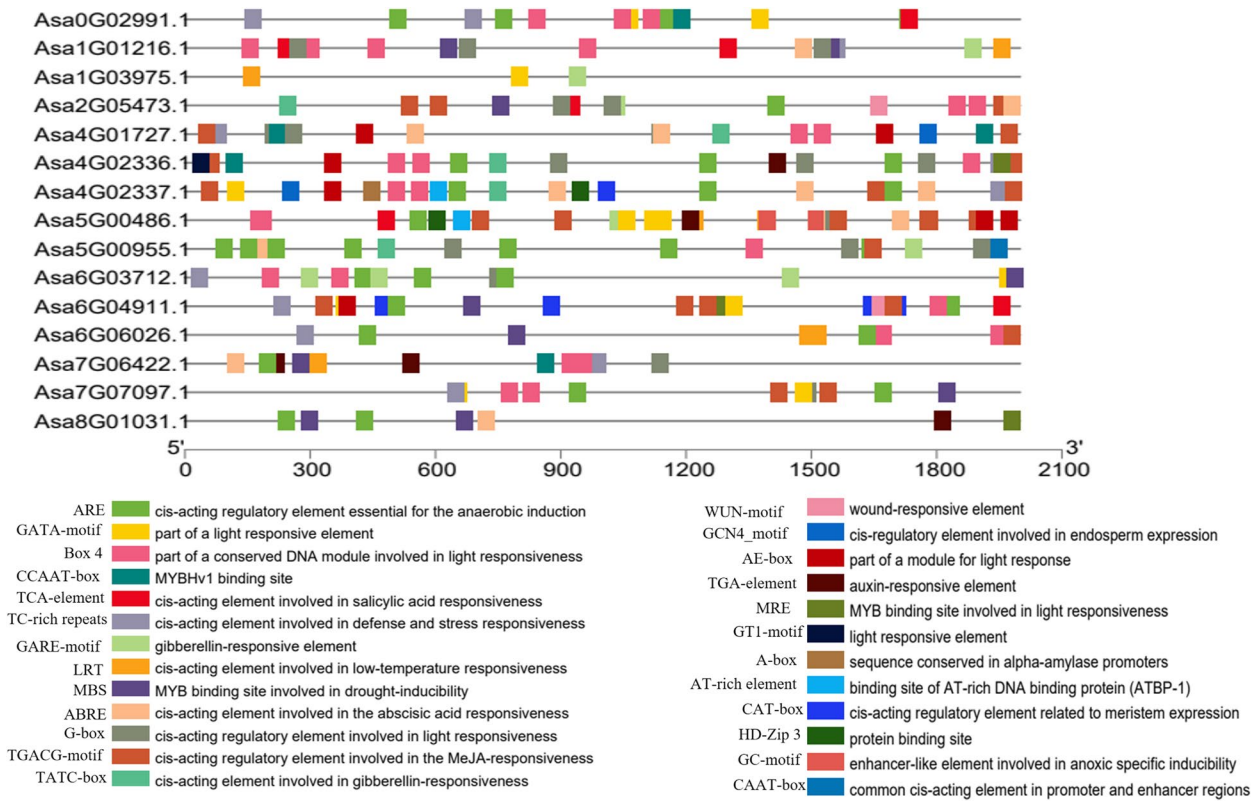

Fig. 3 Cis-elements in the promoters of 15 *AsHSF* genes

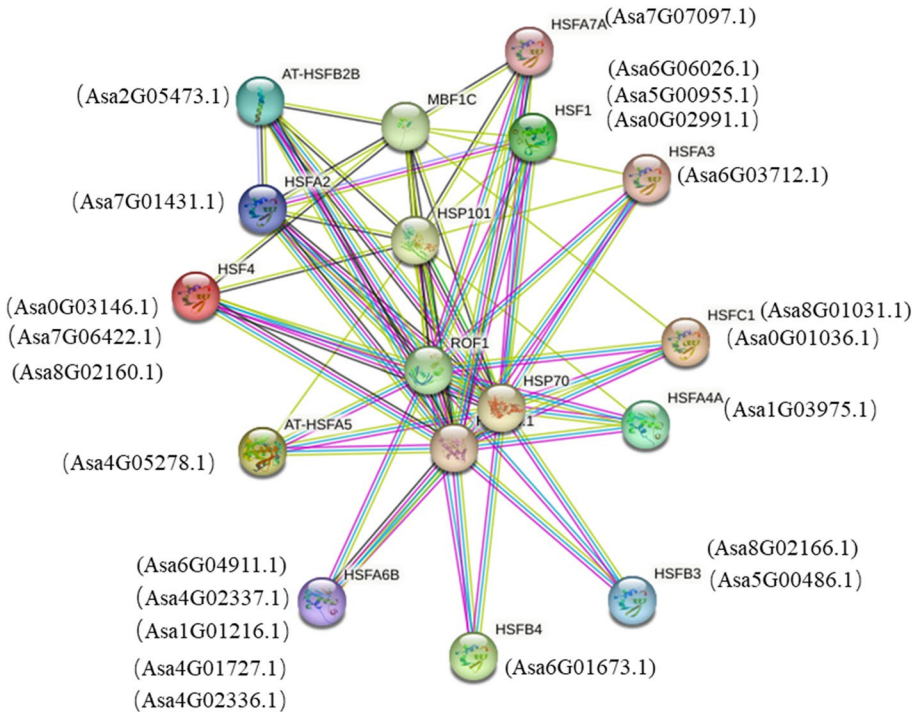

Fig. 4 Functional interaction networks of 22 AsHSFs. The thickness of the lines between genes represents the association strength

an important molecular mechanism for improving plant heat tolerance.

Expression analysis of garlic *AsHSF* genes under heat stress

Considerable evidence has shown that *HSFs* are involved in plant resistance to heat stress, salt, and drought stress. The expression profiles of 9 *AsHSFs* (*Asa0G02991.1*, *Asa5G00955.1*, *Asa6G06026.1*, *Asa6G04911.1*, *Asa4G01727.1*, *Asa6G03712.1*, *Asa2G05473.1*, *Asa7G06422.1*, *Asa8G01031.1*) in different developmental stages and heat temperature stress time points (0, 1, 4, 8, and 24 h) were analyzed by RT-qPCR. As shown in Fig. 5, all 9 TF genes showed different levels of response to heat stress. After heat stress, the relative expression levels of *Asa0G02991.1*, *Asa6G06026.1*, *Asa6G04911.1*, and *Asa6g03712.1* showed a trend of increasing first

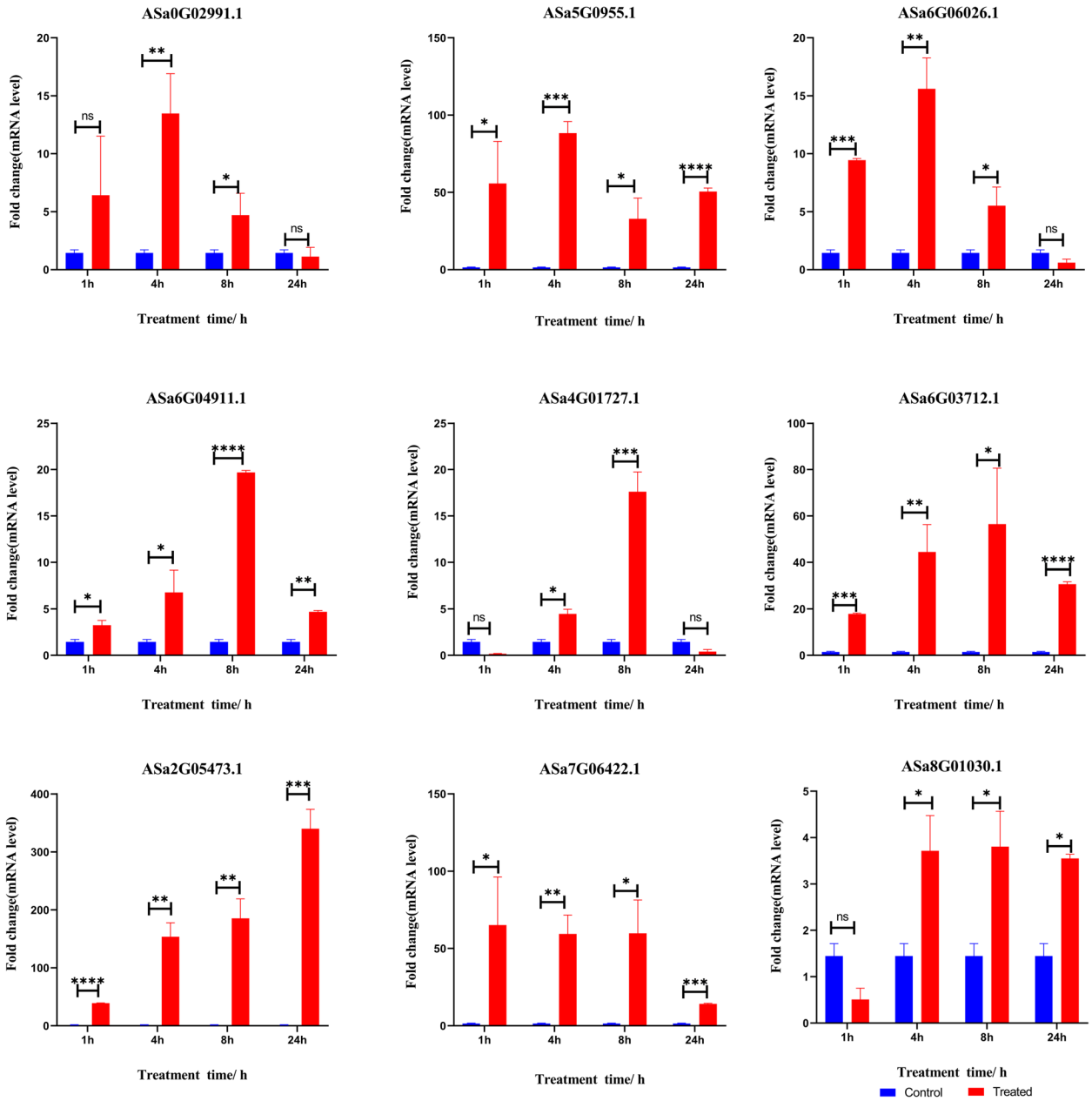

Fig. 5 Expression profiling of 9 *AsHSF* genes of garlic under heat stress. Data are expressed as the means ± standard deviation (SD) of three replicates. Error bars represent standard deviation among three independent replicates. The significance levels were based on $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****)

and then decreasing and reached the lowest value of relative expression levels 24 h after treatment. With the increase in treatment time, the relative expression levels of Asa2G05473.1 and Asa8G01031.1 showed an increasing trend; after 1 h of treatment, the expression level increased rapidly and remained at a high level.

Studies have shown that plant tissue-specific expression plays a role in its growth and development. In the leaf blade, roots, and bulb of garlic, 9 *AsHSF* genes showed different expression levels, and some *AsHSF* genes of the same subfamily often had similar expression patterns (Fig. 6). For example, the expression levels of the *Asa2G05473.1* and Asa*7G06422.1* genes from the B subfamily were significantly higher in the bulb than in the leaf blade and roots, which could be used to analyze the tissue-specific expression patterns of other genes from the same subfamily in garlic. *Asa5G00955.1*, *Asa6G04911.1*, and *Asa6G03712.1* had the lowest expression level in the leaf blade, followed by the roots, and the highest expression level in the bulb. The relative expression levels of the *Asa6G04911.1* and *Asa8G01031.1* genes in the roots were significantly higher than those in the leaf blade and bulb.

Screening and identification of plants overexpressing the *AsHSFB1* gene

Transgenic *Arabidopsis thaliana* overexpressing *AsHSFB1* under the control of CaMV35S promoter was constructed (Fig. 7A). Three independent transgenic lines were obtained, as confirmed by genomic DNA PCR and qRT-PCR analyses. Using the genomic DNA of WT *Arabidopsis thaliana* and hygromycin-resistant screened plants as templates, PCR detection was performed on homozygous strains of the T3 generation using primers 35S-F and AsHSFB1-R (Fig. 7B). There was no target band in the WT, but in the three transgenic lines, a band

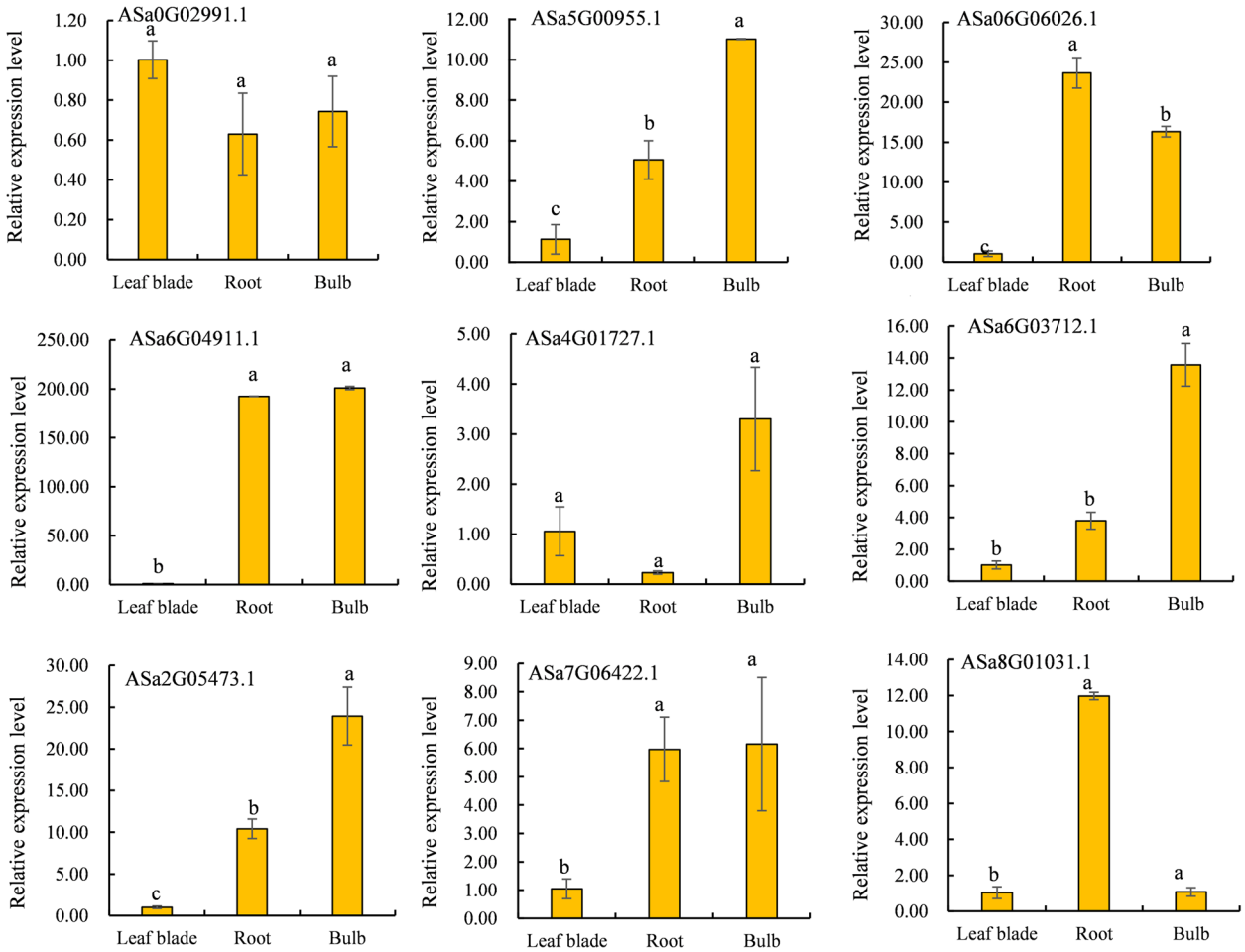

Fig. 6 Expression profiling of 9 *AsHSF* genes of garlic in different tissues. Data are expressed as the means ± standard deviation (SD) of three replicates. Bars represent mean standard deviation (SD). The letters above the bars indicate significant differences between the gene expression level ($P < 0.05$)

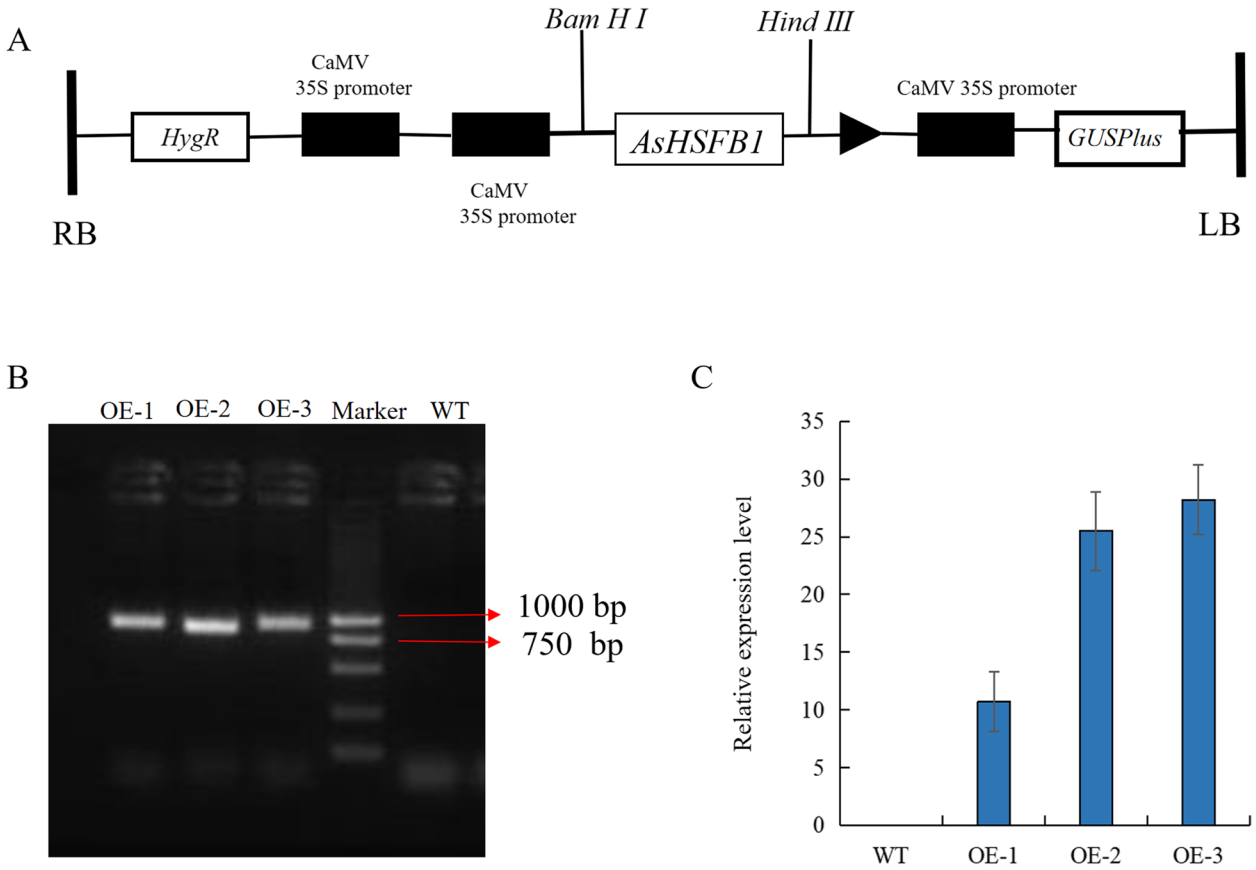

Fig. 7 PCR and RT-PCR detection of AsHSFB1-overexpressing plants. **A** Schematic diagram of the overexpression construct. **B** PCR was used to determine whether the *AsHSFB1* gene was transferred into *Arabidopsis thaliana*. Marker, WT, and OE represent the DNA marker (DL2000), wild type, and *AsHSFB1* overexpression lines, respectively. **C** *AsHSFB1* expression in transgenic plants was detected by qRT-PCR

of the same size as the target fragment was amplified, which was verified by sequencing to be consistent with the target gene sequence, indicating that the target gene was integrated into the *Arabidopsis* genome. The expression levels of *AsHSFB1* in different transgenic strains showed significant differences (Fig. 7C). For example, the expression level of OE-2 was significantly higher than that of the other two strains, indicating certain differences in the expression levels among different transgenic strains.

Effect of *AsHSFB1* overexpression on plant growth in *Arabidopsis* plants under heat stress

To further clarify the role of *AsHSFB1* in heat stress, WT and transgenic Arabidopsis thaliana seeds were placed at a high temperature of 46°C for 30 min after 3 days of vernalization. As shown in Figs. 8A and B, after 3 d of heat treatment, 100% of the WT seeds germinated, while the germination rate of OE1, OE2, and OE3 of transgenic strains was lower than that of the WT, and the germination rate of OE3 was the lowest at 80% (Fig. 8C). To further verify the effect of *AsHSFB1* overexpression on the heat tolerance of plants, WT and OE *Arabidopsis* were seeded on MS medium, heat treated at 44°C for 70 min at 5 days after seeding, and resumed growth at 22°C for 7 days. Under normal conditions, there was no significant difference in the growth of the WT and OE plants. After heat treatment, both WT and transgenic plants were wilted to varying degrees, and the leaves of WT *Arabidopsis* seedlings were yellowish green in large areas, but the transgenic *Arabidopsis* seedlings were completely bleached and died (Figs. 8 D and E). At the same time, the plants transferred to the culture medium for 18 days were treated with heat shock at 38°C for 2 h, resumed growth at 22°C for 2 d, heat treated at 40°C for 3 d, and finally resumed growth at 22°C for 7 d. The WT strains were still green, and the transgenic strains were wilted to varying degrees. The OE3 strain seriously withered (Fig. 8F). Based on the observed phenotypic results, O^{2-} accumulation in the leaves of transgenic and WT *Arabidopsis* plants was detected, and the amount of O^{2-} accumulated in transgenic Arabidopsis plants was higher than that in

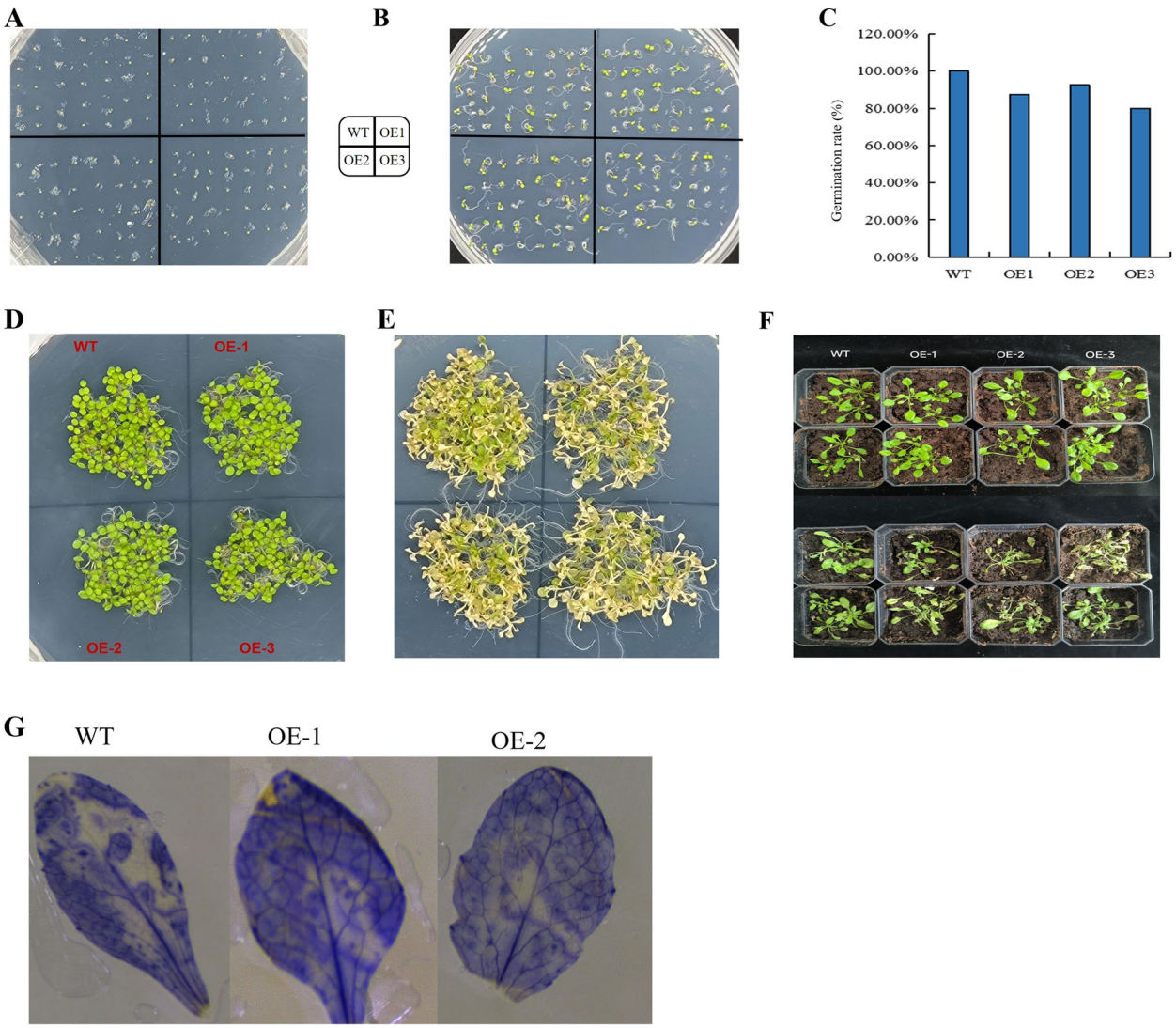

Fig. 8 Effect of *AsHSFB1* overexpression on seed germination rate and heat resistance of transgenic plants. **A** Wild-type (WT) and transgenic *Arabidopsis thaliana* after 3 days of vernalization treated at 46 °C for 30 min. Photos taken before HS treatment. **B** Photos taken after 3 d at 22 °C. **C** Comparison of germination rates among WT and transgenic plants after heat treatment. **D** *Arabidopsis* grown for about 7 days. **E** *Arabidopsis* grown for about 7 days, heat treated at 44 °C for 5 h, and resumed growth at 22 °C for 3 days. **F** *Arabidopsis* grown for about 18 days, heat shock treatment at 38 °C for 10 h, and recovery at 22 °C for 5 days. **G** The accumulation of O^{2-} in WT and transgenic *Arabidopsis* plants (OE-1and OE-2)

WT *Arabidopsis* plants. The number of blue dots on the leaves of WT *Arabidopsis* plants was lower than that of transgenic *Arabidopsis* plants (Fig. 8G).

Physiological and biochemical changes following heat stress

When plants are stressed, the antioxidant system is one of the main ways for plants to remove toxic reactive oxygen species (ROS) from their bodies [28]. As shown in Fig. 9, under normal growth conditions, the superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) activities of transgenic *Arabidopsis* leaves were not significantly different from those of the WT. However, under heat stress, POD and CAT protective enzyme activities were visibly lower in the OE lines than in the WT. MDA is negatively correlated with cell membrane stability and can be used as an index to evaluate cell membrane damage [28]. Here, the MDA content of WT plants after heat stress was significantly lower than that of transgenic plants.

Heat stress significantly affects the physiological process of photosynthesis and eventually leads to an increase or decrease in chlorophyll content [29]. Under normal growth conditions, there was no significant difference in

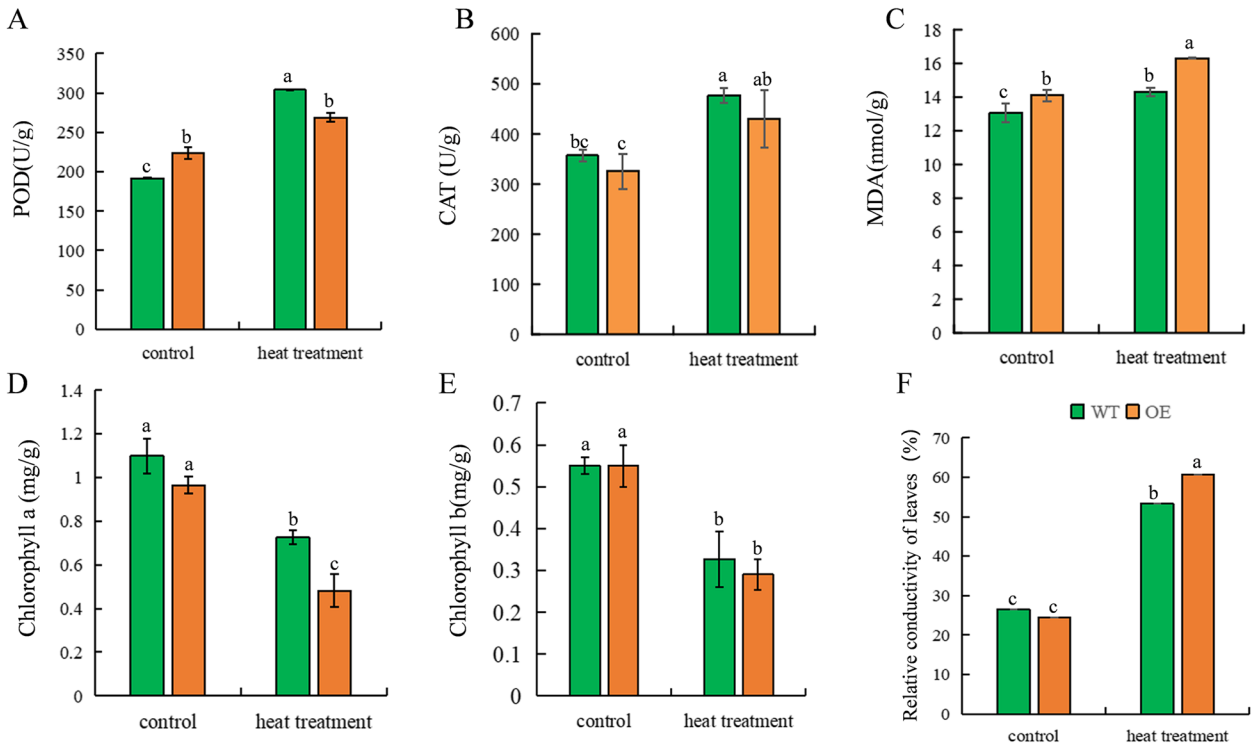

Fig. 9 Analysis of physiological traits in transgenic and WT *Arabidopsis thaliana* plants profiles under heat stress treatment. **A** Activity of POD enzyme in transgenic and WT *Arabidopsis thaliana* plants measured after heat stress **B** Activity of CAT enzyme in two transgenic and WT *Arabidopsis thaliana* plants measured after heat stress **C** MDA contents in transgenic and WT *Arabidopsis thaliana* plants measured after heat stress. **D**, **E** Chlorophyll a or chlorophyll b content in two transgenic and WT *Arabidopsis thaliana* plants measured after heat stress. **F** Relative electrical conductivity (REC) in transgenic and WT *Arabidopsis thaliana* plants measured after heat stress

chlorophyll a or chlorophyll b content. After heat stress, the chlorophyll a content of the WT leaves was significantly higher than that of transgenic leaves, and the chlorophyll a content was 1.5 times that of WT leaves. The chlorophyll b content was 1.12 times that of the WT. The leaf relative electrical conductivity (REC) can characterize the damage to plant cell membranes [30]. In contrast, the REC was higher in the OE lines than in the WT. The phenotypic results indicate that *AsHSFB1* may negatively regulate the heat tolerance of *Arabidopsis*.

Effects of *AsHSFB1* overexpression on the expression of other resistance-related genes

To determine whether *AsHSFB1* induced the expression of heat stress-responsive genes and how they are related, the transcriptional expression levels of resistance-related genes (*AtHSFA1*, *AtLEA*, *AtAPX1*, *AtHSP70*, *AtHSP98*, *AtRD22*) were detected by RT-qPCR (Fig. 10). Under normal growth conditions, some heat-related genes were induced, with low expression at a normal temperature but sharply increased expression at a high temperature, for example, *AtHSFA1*, *AtHSP70,* and *AtHSP98*. The relative expression levels of the *AtHSFA1* and *AtAPX1* genes increased rapidly after 1 and 4 h of heat stress. The expression levels of *AtHSFA1* and *AtAPX1* genes in transgenic *Arabidopsis thaliana* were significantly higher than those in the WT, but the expression levels of *AtHSFA1* and *AtLEA* genes in transgenic *Arabidopsis thaliana* were significantly decreased after 8 h of treatment. The expression levels of heat shock protein genes *AtHSP70* and *ATHSP98* in transgenic *Arabidopsis thaliana* were significantly lower than those of the WT in each period of heat stress, which may be due to the inhibitory effect of *AsHSFB1* on downstream gene expression, indicating that the *AsHSFB1* gene may negatively regulate heat resistance in *Arabidopsis thaliana*.

Discussion

Garlic is an important bulb vegetable crop that is nutrient rich. Heat stress is the main adverse environmental factor affecting the growth and development of garlic, reducing its yield and quality [31]. When plants are subjected to heat stress, the physiological and biochemical states of plants change to different degrees to improve the survival rate [32]. Therefore, it is of great significance to study the heat resistance of garlic. The main function of TFs is

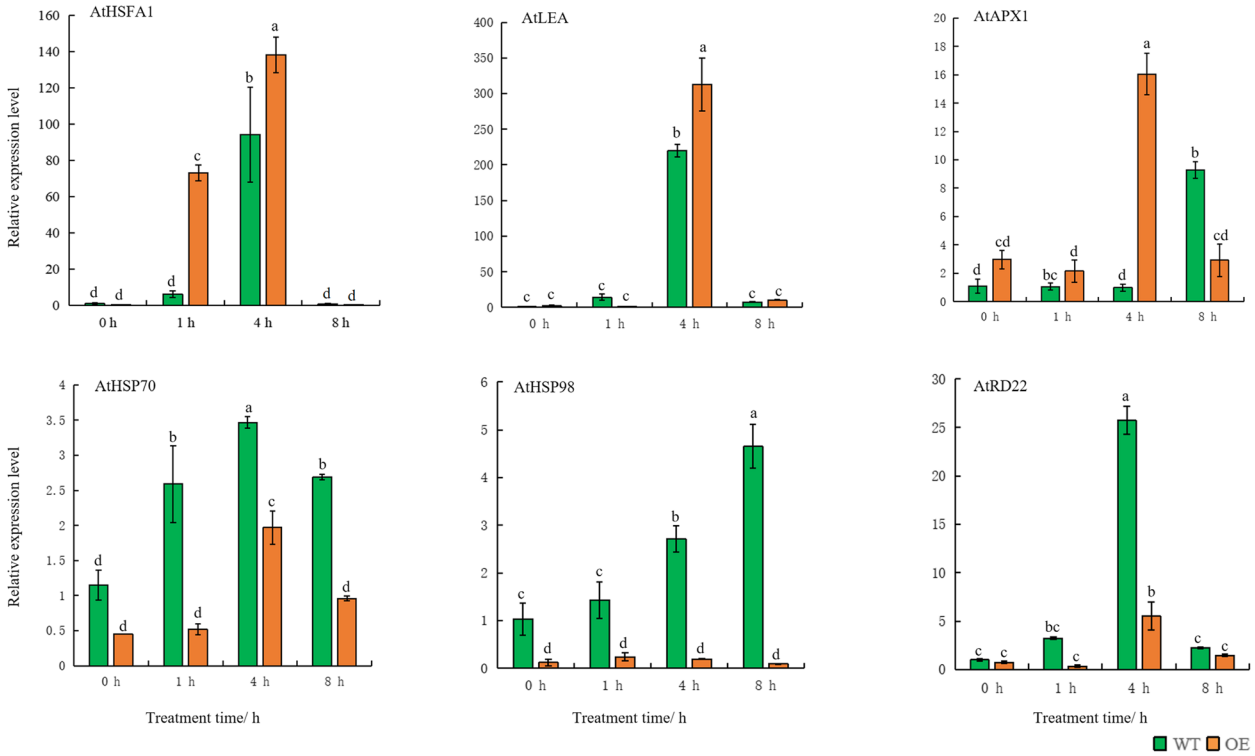

Fig. 10 Expression patterns of stress-responsive genes in wild-type and transgenic *Arabidopsis thaliana* under normal conditions and after heat stress treatment. Data are expressed as the means ± standard deviation (SD) of three replicates. Bars represent mean standard deviation (SD). The letters above the bars indicate significant differences between the gene expression level ($P < 0.05$)

to activate or inhibit gene transcription, which plays an important role in regulating plant growth and development and response to the environment [33]. *HSFs* are a class of transcriptional regulatory genes that can activate heat shock gene expression under heat stress and play a key role in plant response to heat stress [34].

At present, there is little research on *HSF* genes in garlic. The whole genome and transcriptome of garlic were sequenced, as garlic *HSF* genes family identification provides an advantageous platform. In this study, 22 *HSF* factors were identified, and phylogenetic analysis was performed with 21 genes of *Arabidopsis thaliana*, which were divided into subfamilies A, B, and C, containing 9, 11, and 2 *HSF* genes, respectively. The number of genes contained in each group differed, indicating that the *HSF* gene family of Chinese cabbage was not evenly distributed and that there was wide diversity among members. The 22 *HSF* genes of Chinese cabbage are widely present on 6 chromosomes, indicating that *HSF* genes have a wide range of common ancestors in the garlic genome. MEME analysis showed that the conserved domains of TFs in the same subfamily were similar, suggesting that these conserved motifs are related to the regulatory functions of different subfamilies. RT-qPCR showed that heat stress significantly induced the expression of garlic *HSF* genes, indicating that these genes are widely involved in the heat shock response of garlic leaves to high temperatures at the seedling stage.

Modern breeding methods can combine molecular biology with traditional breeding methods to improve plant varieties and increase breeding efficiency. Overexpressing white clover *TrHSFB2a* in transgenic *Arabidopsis* negatively regulated drought, heat, and salt stress tolerance [35]. We cloned the *AsHSFB1* gene and analyzed the influence of its overexpression on the heat resistance of *Arabidopsis.* Under heat stress, the germination rate and growth state of the WT were better than that of the transgenic lines. Heat stress destroy the normal metabolism of antioxidant enzyme activity. As important enzymes in the antioxidant enzyme system, POD, CAT, and MDA activities are important indicators of plant adaptability under stress [28]. After heat stress, the photosynthetic pigments of plant leaves are decomposed and deactivated, and the permeability of the cell membrane increases, resulting in a decrease in chlorophyll content and an increase in REC [36, 37]. In this study, the POD and CAT content in WT plants were higher than those of the OE lines. In contrast, the measurement of MDA content for the plasma membrane damage index showed that the MDA level of WT *Arabidopsis*

was much lower than that of transgenic *Arabidopsis*, representing higher lipid damage in overexpressing lines. In addition, the chlorophyll content of transgenic *Arabidopsis thaliana* was significantly lower than that of the WT, but the relative conductivity was higher than that of the WT. The regulation of stress-related gene expression is an important mechanism for plants to cope with abiotic stress [38]. As TFs, *HSFs* can affect the heat resistance of plants by regulating the expression of downstream heat-resistance genes, the most important of which are *HSP* genes [39]. In this study, *AsHSFB1* overexpression in transgenic *Arabidopsis thaliana* resulted in a weakening of the positive response of heat tolerance and other resistance genes to heat stress. Therefore, *AsHSFB1* is likely to regulate the heat tolerance of *Arabidopsis thaliana* by affecting the expression of these key factors. These findings provide evidence that *AsHSFB1* negatively regulates stress tolerance, at least in *Arabidopsis.*

Class B *HSFs* are considered transcriptional suppressors, and it is generally believed that the LFGV-conserved structure of class B is the key region for the inhibition of class A members [3]. Class B *HSFs* in *Arabidopsis* included five members, *HSFB1*, *HSFB2A*, and *HSFB4* [40]. Ikeda et al. found that the survival rate of *HSFB1-1*/*HSFB2-1* double mutant *Arabidopsis* seedlings subjected to heat stress was only 50% of that of the WT, which indicates that *HSFB1* and *HSFB2b* genes are necessary for plants to acquire heat resistance [41]. Zhang et al. showed that Class B *HSFs* have a positive effect on heat resistance in wheat. Overexpression of wheat *TaHSF3* in *Arabidopsis thaliana* enhanced the heat resistance of transgenic *Arabidopsis thaliana* [13]. Wang et al. found that *CvHSF30-2* improves heat resistance in Clematis by increasing HSP expression, which is negatively regulated by *CvHSFB2a* [42]. Therefore, Class B *HSFs* appear to play a complex and important role in regulating the thermal response of plants. In our study, *AsHSFB1* appears to be a negative transcriptional coactivator of some HSFA and HSP members. Therefore, future studies on the interaction of *AsHSFB1* with Class A *HSFs* and HSP may help us better understand the function of *HSFB* transcription factors in the plant response to heat stress.

In summary, we performed an evolutionary analysis of the HSF family in garlic to obtain comprehensive information on this family, including its protein features and phylogenetic relationships. Furthermore, the motifs and domains of the proteins, gene structures, promoter cis-elements, and heat treatment expression patterns of HSF genes were analyzed in garlic. In this work, an *HSF* gene, *AsHSFB1*, was first isolated from garlic and overexpressed in *Arabidopsis thaliana*. The growth state of WT Arabidopsis thaliana under heat stress was better than that of transgenic Arabidopsis thaliana. However, WT *Arabidopsis* maintained a high chlorophyll content and significantly increased antioxidant enzyme (CAT, POD) activity. The results indicated that *AsHSFB1* overexpression may negatively regulate the heat resistance of transgenic *Arabidopsis thaliana*. However, knowledge on the regenerative genetic transformation system of garlic is still limited, and most of the research on garlic focuses on its cultivation, breeding, physiology, and biochemistry. At present, the *AsHSFB1* gene has only been preliminarily verified in *Arabidopsis thaliana*, though attempts have been made to establish a garlic genetic transformation system to transfer the *AsHSFB1* gene into garlic. Unlike *Arabidopsis thaliana* and several other crop plants, the thermal response mechanism of horticultural plants, including garlic, is rarely investigated, so further exploration of the function of this gene will be the focus of our future work.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-11002-w.

Supplementary Material 1.

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Authors' contributions
YQQ and FJD initiated and designed the research. YQQ, YF, LCY, and ZYQ performed the experiments. LXJ, ZBW, GJ, LXJ, and LMQ analyzed the data. ZYQ contributed reagents, materials, and analysis tools. YQQ wrote the paper. FJD, YF, LCY, YY, and GJ revised the paper. All authors reviewed the manuscript.

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Data availability
The data used in this study used are from garlic genome sequencing data. The reference genome can be found here: https://doi.org/10.6084/m9.figshare.12570947. The accession numbers of the Arabidopsis thaliana proteins were retrieved from The Arabidopsis Information Resource (https://www.arabidopsis.org/). In this study, garlic variety "Xusuan No. 6" was taken as the research object. This variety was kindly provided by Dr. Yang Feng (Xuzhou Institute of Agricultural Sciences in the Jiangsu Xuhuai area).

Declarations

Ethics approval and consent to participate
Not applicable. The sampling of plant material was performed in compliance with institutional guidelines. The research conducted in this study required neither approval from an ethics committee, nor did it involve any human or animal subjects. The use of the garlic cultivar 'Xusuan NO.6' plants in this experiment was authorized by the Jiangsu Provincial Department of Agriculture and Rural Affairs.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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