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Article

Genome-Wide Identification and Expression Analysis of HSP Gene Family in *Gracilaria vermiculophylla* under Heat Stress

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Abstract: Marine heatwaves driven by global warming are increasing in both frequency and intensity, posing a severe and persistent threat to intertidal red algae. Heat Shock Proteins (HSPs) are vital molecular chaperones that maintain protein homeostasis under environmental stress, but our understanding of HSP-mediated heat tolerance in marine macroalgae remains fragmentary. In this study, 54 HSP genes in *Gracilaria vermiculophylla* were identified and classified into five ancestral superfamilies (sHSP, HSP40, HSP60, HSP70, and HSP90) based on Hidden Markov Model research, phylogenetic analysis, and conserved motif distributions. Each family exhibited relatively conserved genetic structures and motif components, while distinct families showed functional diversity. Functional annotation and protein-protein interaction (PPI) analysis indicate that HSP70 occupies a central position in the chaperone network, with promoters enriched in light-responsive elements and motifs associated with ABA and MeJA signaling. Transcriptomic profiling under heat stress demonstrated a highly coordinated, family-specific response. sHSPs were strongly induced, with $\log_2$FoldChange values exceeding 10, while HSP70 and HSP90 were highly expressed and centrally connected in the chaperone network. Interestingly, the HSP40 and HSP60 superfamilies exhibited diverse expression patterns, including both significant up- and down-regulations, suggesting a hierarchical, network response to heat stress. In conclusion, this study reveals a stress-responsive chaperone network and also provides valuable insights for functional analyses and the genetic improvement of stress resilience in economic macroalgae. The result has important implications for protecting seaweed species diversity and community functions in coastal areas in the face of global warming.

Keywords: *Gracilaria vermiculophylla*; genome-wide analysis; heat shock protein family; heat stress; gene expression

1. Introduction

Marine heatwaves driven by global warming are increasing in both frequency and intensity, posing a severe and persistent threat to coastal ecosystems [1]. As primary producers inhabiting the intertidal zone, marine macroalgae are particularly vulnerable to rapid temperature fluctuations. Heat stress can disrupt cellular homeostasis, impair photosynthesis, and ultimately limit algal growth and development. To counteract this abiotic stress, algae have evolved many tolerance mechanisms [2–4]. Chaperone-mediated proteostasis is a core tolerance mechanism across various stress conditions [5,6]. Heat shock proteins (HSPs) are molecular chaperones widely

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distributed across organisms, from prokaryotes to eukaryotes [7,8]. These proteins are highly conserved structurally and participate in protein folding, stabilization, and repair during environmental stress [7,9]. HSPs are categorized into six families based on their molecular weight, sequence homology, and functional specialization, including small HSP (sHSP), HSP40, HSP60, HSP70, HSP90, and HSP100 [10,11].

Different HSP families perform distinct but coordinated functions in mediating responses to heat stress. sHSPs act as ATP-independent first responders by binding partially denatured proteins to prevent irreversible aggregation, subsequently transferring them to ATP-dependent chaperones such as HSP70 and HSP100 for refolding [12,13]. HSP40 (DnaJ) acts as an essential co-chaperone, with structural diversity that enables recognition of a wide range of misfolded proteins [14–17]. HSP70, which contains conserved nucleotide- and substrate-binding domains, forms the core of the proteostasis network by facilitating proper protein refolding in cooperation with HSP40 and nucleotide exchange factors (NEFs) [18–20]. Downstream, HSP90 further processes these proteins, particularly signaling proteins and transcription factors [21], while HSP60 provides a sequestered space for protein maturation [22,23]. HSP100 proteins are specialized in the disaggregation and recovery of stable protein aggregates [24]. These families form an integrated proteostasis network that protects cells from heat-induced damage to the proteome.

Extensive studies in land plants and algae have demonstrated that HSPs play crucial roles in heat stress resistance. Among them, HSP70 is a primary stress response protein, showing rapid and robust transcriptional induction under heat stress across diverse species [25]. In red algae such as *Neopyropia yezoensis*, membrane fluidization triggers the heat-induced expression of HSP70 genes, highlighting regulatory features that differ from those reported in land plants [26,27]. Functional studies further indicate that the regulation of HSP70 expression significantly affects growth and stress tolerance in microalgae, including *Phaeodactylum tricornutum* [28]. In addition to HSP70, other HSP families also exhibit stress-specific expression patterns. For example, sHSPs are preferentially induced under heat stress in red and green algae [29–31], while HSP40 responds to heat stress in dinoflagellates [32–34]. Overall, these studies underscore the importance of HSPs in algal heat stress resistance and suggest both conserved and lineage-specific regulatory features.

With the development of high-throughput sequencing, large amounts of algal genomic and RNA-omics data have been collated and released. Among the various stress-related proteins, the HSP family has emerged as a hotspot, and several HSP gene families have been identified in red algae, including *Pyropia haitanensis* [35], *Pyropia yezoensis* [36] and *Cyanidioschyzon merolae* [37]. However, these studies have largely focused on individual HSP genes or a single HSP family, and our understanding of comprehensive genome-wide characterization of HSP gene families in marine macroalgae, as well as the mechanisms underlying HSP-mediated heat tolerance, remains unclear. Red algae exhibit substantial diversity in morphology, habitat preference, and thermal exposure regimes, suggesting that HSP gene repertoires, family expansion patterns, and regulatory strategies may not be universally conserved [37]. Moreover, compared with land plants, red algae possess distinct evolutionary origins, cellular architectures, and life cycles [38]. Yet how HSP families are organized at the genomic level and coordinated in their regulation under heat stress across diverse red algal species remains largely unresolved.

Gracilaria vermiculophylla is a globally distributed red alga with significant ecological and economic value [39]. However, its growth in the intertidal zone is restricted by heat stress, and the composition and specific functions of the HSP gene family in this species remain poorly understood. In this study, we aim to elucidate the role of HSP genes in regulating red algae's response to heat stress and use the recently published high-quality genome of *G. vermiculophylla* to identify and analyze the HSP gene family, followed by detailed analyses of their structural features and expression dynamics under heat stress [40]. This study provides a basis for understanding the potential roles of HSPs in red algal thermal tolerance and establishes a molecular foundation for future studies on stress adaptation and genetic improvement of economically important macroalgae.

2. Materials and Methods

2.1. Identification and Physicochemical Analysis of HSP Genes

The genome and protein sequences of *G. vermiculophylla*, collected from Shandong Province (36.09°N, 120.47°E), were retrieved from NCBI (GCA_054346105.1) and Figshare (https://doi.org/10.6084/m9.figshare.28667702.v3, accessed on 9 January 2026) [40]. Identification of HSP family members in the *G. vermiculophylla* genome was performed using a Hidden Markov Model (HMM) search. The HMM profiles corresponding to the specific HSP domains, including HSP40 (PF00226), HSP60 (PF00118), HSP70 (PF00012), HSP90 (PF00183), and sHSP (PF00011), were downloaded from the Pfam database (http://pfam.xfam.org/, accessed on 9 January 2026). We employed HMMER 3.4 to screen the *G. vermiculophylla* proteome using the "--cut_tc" parameter [41]. To ensure reliability, all candidate sequences were further verified for the presence of completely conserved domains.

Redundant sequences and short fragments were manually removed. Finally, the physicochemical properties of the identified HSP proteins were analyzed. We calculated the number of amino acids (AA), molecular weight (MW), isoelectric point (pI), aliphatic index (AI), instability index (II), and Grand Average of Hydropathy (GRAVY) by using the "Protein Parameter Calc" tools within the TBtools software suite (v2.490) [42].

2.2. Phylogenetic Analysis and Chromosomal Location of HSP Genes

Multiple protein sequences of the identified HSPs were aligned using MAFFT (v7.525) with parameters "--localpair" and "--maxiterate 1000" [43]. Then, we used trimAl (v1.5.rev1) with the "automated1" option to remove poorly aligned regions and improve tree quality [44]. Trimmed sequences were used to construct the phylogenetic tree in IQ-TREE (v3.0.1) with the "MFP" option [45]. We applied the ultrafast bootstrap method with 1000 replicates to assess branch reliability. The final tree was visualized and managed using the iTOL online tool (https://itol.embl.de/, accessed on 11 January 2026) [46]. For the chromosomal locations of HSP genes, we extracted chromosomal locations and gene density information from the *G. vermiculophylla* genome annotation (GFF3) file and visualized them using Tbtools (v2.490) [42].

2.3. Domains and Conserved Motifs of HSPs

For the functional prediction of HSPs, we used the Conserved Domain Database (CDD) to identify functional domains and SignalP (v6.0) to predict the presence of signal peptides through InterPro (https://www.ebi.ac.uk/interpro/, accessed on 13 January 2026) [47]. All results were visualized with a custom R (v4.5.1) script. The conserved motifs in the protein sequences encoded by HSP genes were identified using the Multiple EM for Motif Elicitation (MEME) program (v5.5.9) with the following parameters: maximum number of motifs set to 10 and default settings for other parameters (https://meme-suite.org/meme/tools/meme, accessed on 13 January 2026) [48]. Predicted motifs were annotated through the Tomtom online search tool (https://meme-suite.org/meme/tools/tomtom, accessed on 13 January 2026) with the JASPAR2026_CORE_plants_non-redundant_database.

2.4. Cis-Acting Regulatory Elements of HSPs

The promoter sequences of each HSP gene (2 kb upstream of the transcription start site) were extracted from the *G. vermiculophylla* genome. These sequences were then submitted to the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 14 January 2026) to predict cis-acting regulatory elements [49]. The final distribution of these regulatory elements along the promoters was visualized using the TBtools software [42].

2.5. GO and KEGG Enrichment of HSPs

To investigate the biological functions of the identified HSP genes, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. The protein sequences of all HSPs were first obtained from the *G. vermiculophylla* genome. These sequences were then submitted to the OmicShare Tools online platform (https://www.omicshare.com/tools, accessed on 15 January 2026) for functional annotation and enrichment [50]. GO terms were classified into three categories: biological process, molecular function, and cellular component. For both GO and KEGG analyses, the entire set of protein-coding genes in the genome was used as the background. Significantly enriched terms and pathways were identified using a p-value < 0.05. The enrichment results were finally visualized using chord diagrams to illustrate the relationships between specific HSP genes and their functional categories.

2.6. Protein-Protein Interaction Network Construction

The protein sequences of identified HSPs were submitted to the STRING database (https://string-db.org/, accessed on 15 January 2026) to construct a protein-protein interaction network. Since the *G. vermiculophylla* genome was not in the core database, we mapped the sequences to the reference species *Gracilariopsis chorda*. We set the minimum required interaction score to 0.7 (high confidence) to ensure network reliability. The interaction information was then analyzed and visualized by Cytoscape software (v3.10.4) [51]. The top eight proteins with the highest degree values were identified as the network's central hub proteins.

2.7. Expression Analysis of HSP Genes

Publicly available RNA-seq datasets were retrieved from the Sequence Read Archive (SRA) database via BioProject PRJNA528173 (NCBI). These datasets were generated from *G. vermiculophylla* collected from Shandong Province (37.0°N, 122.0°E), the same species and geographic region as the genome assembly used in this study. The RNA-seq data were included solely to provide independent transcriptomic evidence for the expression patterns of identified HSP genes under the reported experimental conditions rather than to validate gene expression in the sequenced individual.

The RNA-seq datasets were generated from the same species collected from the same geographic population (Shandong Province) as the genome assembly used in this study. Algal thalli were cultured under 30 μmol photons m^{-2} s^{-1} with a 12 h/12 h (light/dark) photoperiod. Two temperature conditions were applied: 25 °C as the control (C) and 36 °C as the heat stress treatment (H). The heat treatment lasted for 6 h [52]. Raw sequencing data were filtered using fastp (v0.23.4) [53] and then mapped to the *G. vermiculophylla* reference genome through HISAT2 (2.2.1) [54]. Then, gene count data were quantified and quality controlled using featureCounts (v2.1.1) [55] and MultiQC (v1.0.dev0) [56]. To ensure data reliability, only genes with a Counts Per Million (CPM) value greater than 1 in at least three samples were retained for subsequent analyses.

The filtered gene counts were normalized using the Transcripts Per Million (TPM) method. Principal Component Analysis (PCA) and sample correlation analysis were conducted on the OmicShare Tools online platform (https://www.omicshare.com/tools, accessed on 23 January 2026) [50]. Differential expression analysis between the control and heat-stress groups was performed using the DESeq2 (v1.48.2) R package [57]. Differentially Expressed Genes (DEGs) were identified based on an adjusted *p*-value (padj) < 0.05 (corrected using the Benjamini-Hochberg method) and an absolute |log2(FoldChange)| > 1.

3. Results

3.1. Identification and Characterization of HSP Gene Family Members

A total of 54 HSP genes were identified in the *G. vermiculophylla* genome. Among them, 52 candidates were initially identified by Hidden Markov Model (HMM) search, and two additional chloroplast-encoded members from the Pfam database were identified through BLASTP (v2.12.0) and NCBI searches. These genes were classified into five major superfamilies: 8 sHSPs, 25 HSP40s, 12 HSP60s, 6 HSP70s, and 3 HSP90s. The HSP40 superfamily was the most abundant, followed by the HSP60 and sHSP superfamilies. Notably, the HSP40 gene family was further classified into three groups based on domain architecture. Four members (Gv1.03G03310.1, Gv1.04G06270.1, Gv1.08G04720.1, and Gv1.20G03830.1) were identified as Type I (DnaJ-A), which possess both Zinc-finger and C-terminal domains. One member (Gv1.20G01540.1) was classified as Type II (DnaJ-B), possessing only a C-terminal domain. The remaining 20 members were categorized as Type III (DnaJ-C).

The physicochemical properties of these 54 HSPs are summarized in Table 1. The protein lengths ranged from 114 to 945 amino acids, and the molecular weights ranged from 12.25 kDa to 102.29 kDa. The sHSP superfamily exhibited the smallest MW values (16.14 to 33.55 kDa), while the HSP70s and HSP90s contained the largest proteins. Notably, the MW value of Gv1.13G01870.1 and Gv1.21G00980.1 exceeds 90 kDa, suggesting their potential classification within the HSP110 subfamily of the HSP70 superfamily. The predicted pI values spanned from 4.60 to 11.51. HSP70s and HSP90s were acidic (pI < 7), whereas the HSP40s, and sHSPs superfamilies contained both acidic and highly basic members. The instability index ranged from 24.55 to 94.98, with almost half of the members exhibiting an index greater than 40, suggesting that these proteins are generally unstable *in vitro*, consistent with the nature of dynamic molecular chaperones. The aliphatic index values ranged from 51.86 to 112.31, indicating varying levels of thermal stability for these proteins. GRAVY values were mostly negative, ranging from −1.34 (Gv1.04G04810.1) to 0.12 (Gv1.21G01260.1). Only three members (A0A345U988, Gv1.05G04100.1, and Gv1.21G01260.1) had positive GRAVY values. The predominantly negative GRAVY values indicate that the majority of *G. vermiculophylla* HSPs are hydrophilic, which supports their high solubility in the cytoplasm or organelle matrices.

3.2. Phylogenetic Analysis and Distribution of the HSP Gene Family Members

To verify the classification of HSP family members in *G. vermiculophylla*, a Maximum Likelihood (ML) phylogenetic tree was constructed from protein sequences of all 54 members. The phylogenetic analysis showed that all 54 HSPs were clearly divided into five distinct superfamilies, including HSP40, HSP60, sHSP, HSP70, and HSP90 (Figure 1). Each superfamily clustered into a single independent clade with high bootstrap support values, particularly within the sHSP and HSP70 superfamilies. The HSP40 superfamily exhibited the highest internal diversity, forming

several smaller subgroups within its clade. This clear clustering pattern showed that members within the same subfamily were more closely related in sequence, suggesting a shared evolutionary relationship.

Table 1. Bioinformatic characteristics of HSPs in *G. vermiculophylla*.

HSP Gene Family	No.	Gene ID	A.A	MW (Kda)	PI	II	AI	GRAVY	Duplication Type
HSP 40	1	Gv1.03G01560.1	643	72.96	9.34	57.06	65.66	−0.93	Singleton
	2	Gv1.03G03310.1	410	45.72	6.25	40.01	67.66	−0.58	Dispersed
	3	Gv1.04G04810.1	518	60.12	5.67	58.32	52.66	−1.34	Singleton
	4	Gv1.04G06270.1	461	49.22	9.05	48.86	65.88	−0.46	Dispersed
	5	Gv1.05G01890.1	136	14.91	6.88	37.84	77.50	−0.26	Dispersed
	6	Gv1.05G03140.1	645	71.69	5.44	46.77	75.19	−0.40	Singleton
	7	Gv1.08G00710.1	306	35.29	9.52	37.27	94.15	−0.45	Singleton
	8	Gv1.08G04720.1	364	40.30	5.78	24.55	71.73	−0.57	Dispersed
	9	Gv1.10G01000.1	506	55.57	5.94	42.04	79.76	−0.55	Dispersed
	10	Gv1.10G01350.1	560	62.31	9.15	41.36	72.75	−0.61	Dispersed
	11	Gv1.10G02490.1	616	69.76	9.67	45.15	61.01	−0.77	Dispersed
	12	Gv1.11G02200.1	114	12.25	11.51	25.92	77.19	−0.15	Singleton
	13	Gv1.12G02340.1	198	22.29	9.57	55.29	70.20	−0.61	Singleton
	14	Gv1.13G00730.1	280	30.42	9.90	57.5	53.79	−0.62	Dispersed
	15	Gv1.14G02770.1	123	14.02	9.72	94.98	79.26	−0.29	Singleton
	16	Gv1.17G00350.1	301	33.14	9.61	49.09	58.94	−0.99	Dispersed
	17	Gv1.18G01340.1	685	77.90	5.84	48.00	83.02	−0.38	Singleton
	18	Gv1.18G02330.1	503	56.35	6.38	48.01	85.63	−0.34	Singleton
	19	Gv1.18G02550.1	376	42.65	10.62	63.65	51.86	−0.91	Singleton
	20	Gv1.19G03000.1	285	33.55	5.45	32.26	60.46	−0.96	Singleton
	21	Gv1.20G00750.1	354	40.40	5.09	41.96	76.67	−0.50	Dispersed
	22	Gv1.20G01540.1	334	36.62	9.41	36.34	62.25	−0.63	Dispersed
	23	Gv1.20G03830.1	442	47.42	9.75	38.20	62.40	−0.56	Dispersed
	24	Gv1.21G01770.1	181	20.06	7.08	41.09	81.44	−0.19	Dispersed
	25	Gv1.21G01970.1	136	14.89	6.88	34.41	77.50	−0.26	Dispersed
HSP 60	26	Gv1.05G03300.1	538	57.33	5.92	25.28	97.25	−0.06	Dispersed
	27	Gv1.05G04100.1	549	58.83	5.90	34.07	101.31	0.07	Dispersed
	28	Gv1.08G02720.1	537	58.94	5.90	34.74	92.23	−0.21	Dispersed
	29	Gv1.08G03170.1	593	63.28	5.07	35.22	94.54	−0.15	Dispersed
	30	Gv1.09G03580.1	574	60.92	5.38	26.49	96.71	−0.06	Dispersed
	31	Gv1.10G04300.1	529	57.64	7.18	32.50	103.61	−0.06	Dispersed
	32	Gv1.12G03020.1	573	62.13	5.77	28.08	94.33	−0.11	Dispersed
	33	Gv1.18G03050.1	544	58.66	5.77	38.61	102.78	−0.04	Dispersed
	34	Gv1.20G00570.1	543	59.94	6.05	43.30	101.09	−0.14	Dispersed
	35	Gv1.21G01260.1	558	59.29	6.25	36.87	108.01	0.12	Dispersed
	36	Gv1.22G00010.1	382	41.79	5.79	38.65	103.38	−0.01	Dispersed
	37	A0A345U988	528	57.25	5.62	31.70	112.31	0.02	\
HSP 70	38	Gv1.03G08340.1	663	72.70	4.89	37.80	83.39	−0.43	Dispersed
	39	Gv1.13G01870.1	945	102.29	5.61	39.17	82.94	−0.41	Dispersed
	40	Gv1.13G02550.1	664	72.31	5.20	40.00	79.92	−0.44	Dispersed
	41	Gv1.18G02360.1	669	71.70	5.41	32.48	84.45	−0.34	Dispersed
	42	Gv1.21G00980.1	878	95.07	5.03	36.61	83.03	−0.33	Dispersed
	43	A0A345TTP3	621	68.22	5.05	38.06	92.90	−0.44	\
HSP 90	44	Gv1.06G01910.1	846	96.08	4.60	34.12	73.43	−0.76	Dispersed
	45	Gv1.09G01340.1	752	84.86	4.93	42.49	79.34	−0.49	Dispersed
	46	Gv1.18G01030.1	749	85.27	4.75	39.37	83.86	−0.60	Dispersed
sHSP	47	Gv1.04G02900.1	143	16.14	9.21	48.96	85.87	−0.42	Tandem
	48	Gv1.04G02910.1	146	16.47	7.76	43.52	72.05	−0.56	Tandem
	49	Gv1.06G04130.1	165	18.81	5.39	35.92	70.24	−0.91	Dispersed
	50	Gv1.08G01980.1	211	23.50	6.36	45.51	80.90	−0.39	Dispersed
	51	Gv1.13G00430.1	165	18.84	5.76	32.13	77.33	−0.76	Dispersed
	52	Gv1.17G03130.1	160	18.02	7.23	57.15	95.00	−0.35	Dispersed
	53	Gv1.17G03150.1	146	16.57	9.46	45.13	78.30	−0.32	Dispersed
	54	Gv1.20G00630.1	169	18.96	6.66	55.19	80.30	−0.50	Dispersed

[MW (Molecular Weight in Kilo Daltons), A.A (number of amino acids), pI (Isoelectric point), AI (Aliphatic Index), II (Instability Index) and GRAVY (Grand Average of Hydropathy)].

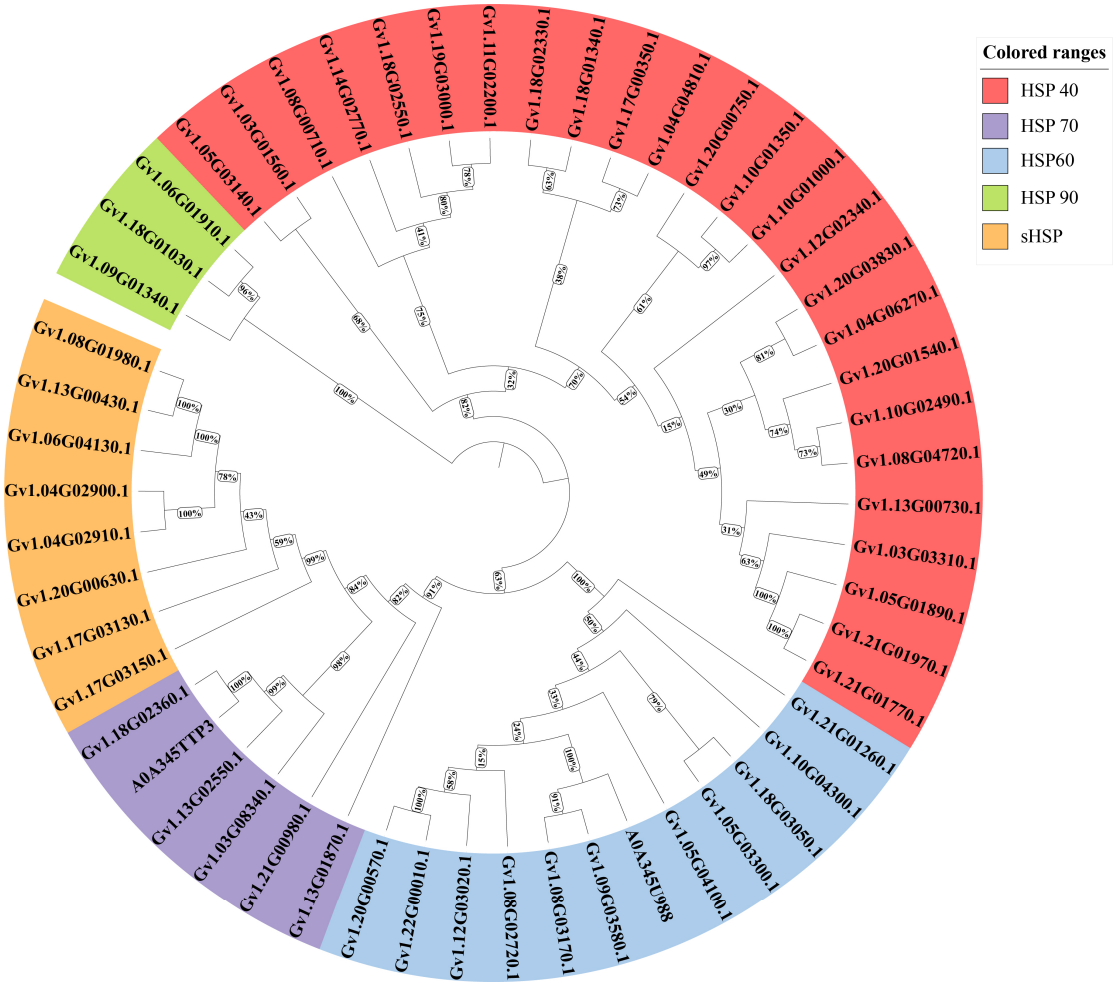


Figure 1. Phylogenetic tree of HSP families in *G. vermiculophylla*. The colors represent different heat shock proteins: red, HSP40; purple, HSP60; blue, HSP70; green, HSP90; orange, sHSP.

The chromosome locations of HSPs were visualized using TBtools (Figure 2). Among the 22 chromosomes, 52 HSP genes were unevenly distributed across 17 chromosomes and showed significant variation per chromosome (Supplementary Table S1). Chr8, Chr18, and Chr 20 contained a higher density of HSP genes compared to other chromosomes. Interestingly, several gene clusters were identified on specific chromosomes, including the sHSP gene pair Gv1.04G02900.1/Gv1.04G02910.1 on Chr4 and Gv1.17G03130.1/Gv1.17G03150.1 on Chr17, as well as the Gv1.18G02330.1/Gv1.18G02360.1 on Chr18 (Figure 2).

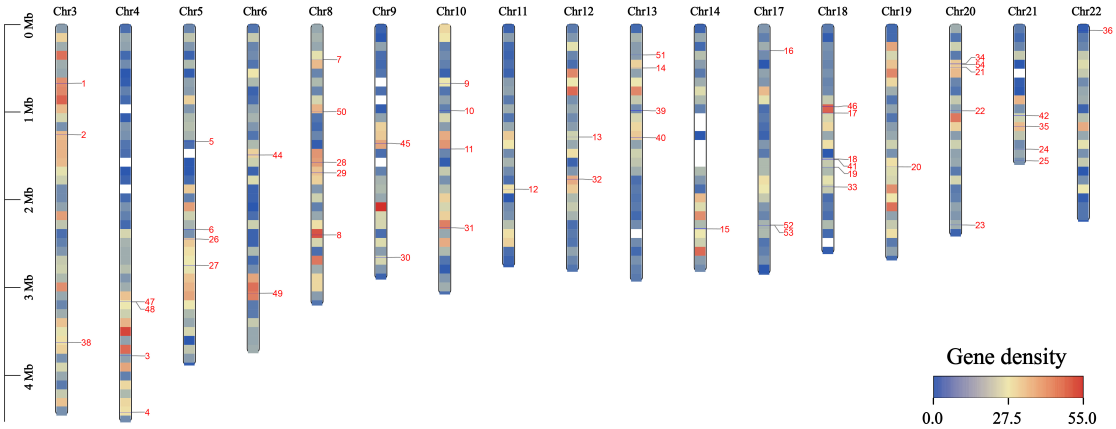


Figure 2. Distribution of the HSP gene family members on *G. vermiculophylla* chromosomes. HSP genes are labeled with gene numbers on the chromosome. The scale bar on the left indicates the length of the chromosome. Gene density is indicated by a color gradient from blue (low) to red (high).

To further investigate the basis of this distribution pattern, we analyzed duplication relationships among the HSP genes using MCScanX (v1.0.0) with an E-value cutoff of E-value of 1×10^{-10} (Table 1) [58]. Of the 52 HSP genes, 39 (75%) were classified as dispersed duplicates, consistent with their broad distribution across 17 chromosomes and suggesting that dispersed duplication may have contributed substantially to shaping the chromosomal distribution of the HSP gene family. In contrast, only one HSP gene pair (Gv1.04G02900.1/Gv1.04G02910.1) was identified as a tandem duplication, suggesting that although the HSP genes are widely distributed across the genome, tandem duplication was not the predominant duplication pattern associated with HSP gene family expansion in *G. vermiculophylla*.

3.3. Conserved Motifs of HSP Genes

To further explore the structural characteristics and functional conservation of the HSP family, we analyzed conserved motifs across all HSP family members using the MEME Suite. A total of 10 distinct motifs were identified for each superfamily, and their distribution patterns were highly consistent with the phylogenetic classification (Figure 3).

The HSP40 superfamily demonstrated significant diversity in its motif composition. It has been observed that the number and lengths of motifs in HSP40 ranged from 1 to 5 and 8 to 50, respectively. Among the different types, Type I (DnaJ-A) members and a small minority of Type III (DnaJ-C) had the highest number of conserved motifs. This structural variation is consistent with the earlier classification of HSP40 into three types based on their different domain architectures. In addition, motifs 1 and 2 were conserved in most HSP40 genes except Gv1.08G04720.1 and Gv1.11G02200.1 (Figure 3).

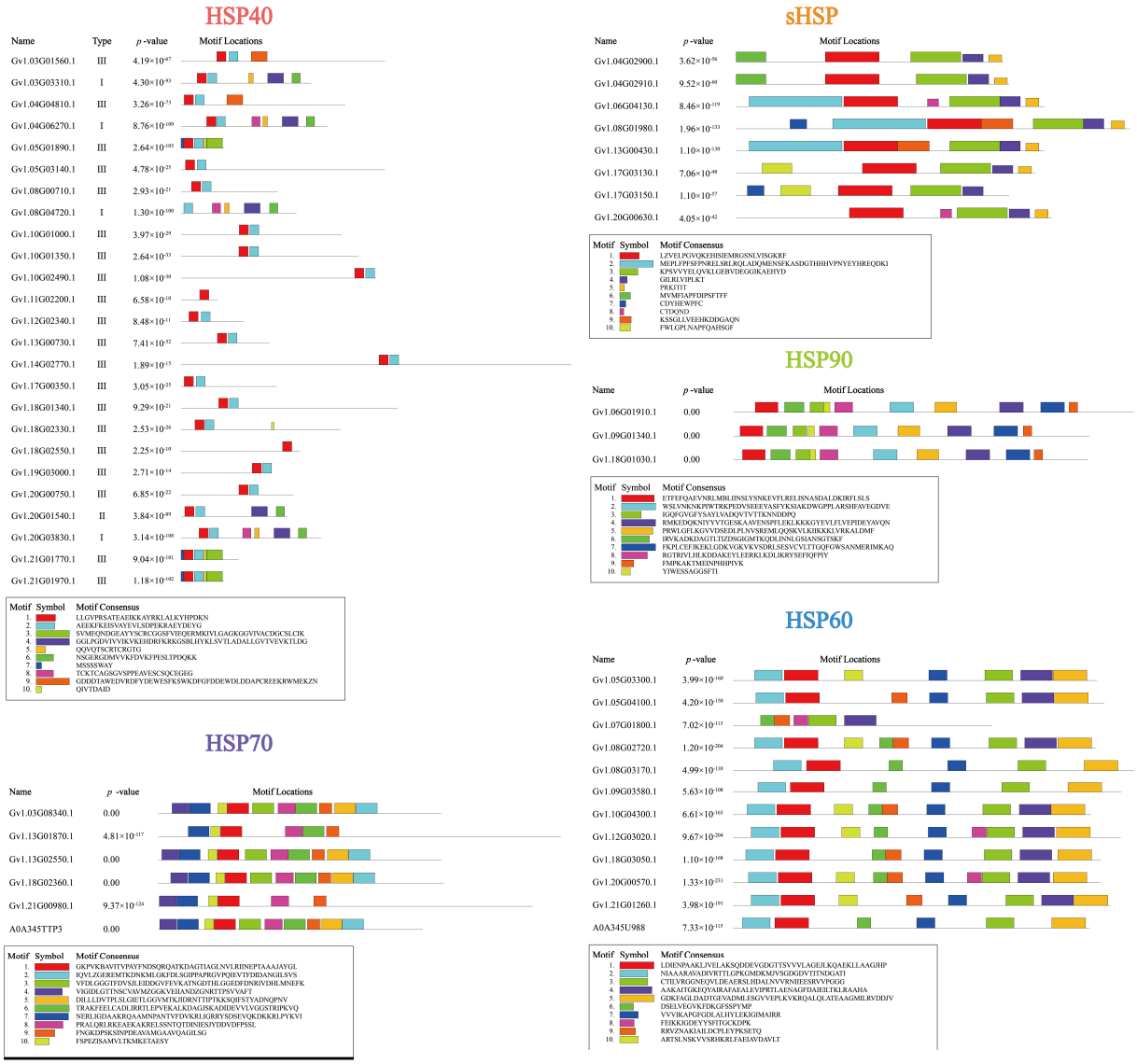


Figure 3. The conserved ten motifs of HSP40, HSP60, HSP70, HSP90 and sHSP in *G. vermiculohpylla*.

It has been observed that the number and lengths of motifs in sHSP ranged from 5 to 7 and 7 to 50, respectively. Motifs 1, 3, 4, and 5 were conserved across all sHSP genes, and the most conserved motif (7 motifs) is found in Gv1.08G01980.1. This superfamily showed moderate variation, with motif patterns generally following the phylogenetic tree's clustering (Figure 3).

In contrast, the HSP60 and HSP90 superfamilies also showed highly uniform motif distributions within their respective clades. The number and lengths of motifs in HSP60 ranged from 5 to 9 and from 21 to 50, respectively. In addition, all HSP90 genes contained 10 motifs, with lengths ranging from 13 to 50. This consistency suggests that these superfamilies have maintained stable structural frameworks throughout evolution to ensure efficient chaperone activity (Figure 3).

The HSP70 superfamily is a major group of molecular chaperones essential for protein folding and stress tolerance [36]. The HSP70 superfamily exhibited the most conserved motif arrangement in *G. vermiculophylla*. Among these members, two genes (Gv1.13G01870.1, Gv1.21G00980.1) possessed 6 motifs and four genes (Gv1.03G08340.1, Gv1.13G02550.1, Gv1.18G02360.1 and A0A345TTP3) possessed 10 motifs. The lengths of these motifs ranged between 21 and 50. Almost all members contained a complete set of ten motifs in a fixed order, reflecting a highly stable functional structure. Detailed annotation revealed that these motifs are associated with several key regulatory elements (Table 2). Specifically, Motifs 1, 7, and 9 were identified as DNA-binding One Zinc Finger elements, which typically participate in transcriptional regulation and environmental stress responses. Motifs 3, 5, and 8 were categorized as MYB-related motifs, which play significant roles in plant development and abiotic stress tolerance. Furthermore, Motif 4 was associated with Trihelix domains that are often involved in light-responsive and environmental adaptation processes. Motif 10 was linked to NAC elements, which are essential regulators of stress signaling and developmental pathways. Motif 6 was identified as a basic leucine zipper (bZIP) factor, a group frequently associated with hormone signaling and heat stress responses. Finally, Motif 2 was consistently observed across the entire family, although its specific functional role remains unknown, as it did not match any current database annotations (Table 2). These differences in motif arrangement across HSP superfamilies likely reflect their functional specialization in helping *G. vermiculophylla* adapt to diverse environmental challenges.

Table 2. Ten conserved motifs of HSP 70 proteins in *G. vermiculophylla*.

Motif	Motif Sequence	Annotation
Motif 1	GKPVKBAVITVPAYFNDSQRQATKDAGTIAGLNVLRIINEPTAAAJAYGL	DNA-binding One Zinc Finger
Motif 2	IQVLZGEREMTKDNKMLGKFDLSGIPPAPRGVPQIEVTFDIDANGILSVS	NA
Motif 3	VFDLGGGTFDVSJLEIDDGVFEVKATNGDTHLGGEDFDNRIVDHLMNEFK	MYB-related
Motif 4	VIGIDLGTTNSCVAVMZGGKVEIIANDZGNRTTPSVVAFT	Trihelix
Motif 5	DILLLDVTPLSLGIETLGGVMTKJIDRNTTIPTKKSQIFSTYADNQPNV	MYB-related
Motif 6	TRAKFEELCADLIRRTLEPVEKALKDAGJSKADIDEVVLVGGSTRIPKVQ	Basic leucine zipper factors
Motif 7	NERLIGDAAKRQAAMNPANTVFDVKRLIGRRYSDSEVQKDKKRLPYKVI	DNA-binding One Zinc Finger
Motif 8	PRALQRLRKEAEKAKRELSSNTQTDINIESJYDDVDFPSSL	MYB-related
Motif 9	FNGKDPSKSINPDEAVAMGAAVQAGILSG	DNA-binding One Zinc Finger
Motif 10	FSPEZISAMVLTKMKETAESY	NAC

3.4. Functional Annotation and Enrichment Analysis of the HSPs

To elucidate the potential biological functions and metabolic pathways of the identified HSP genes in *G. vermiculophylla*, GO and KEGG enrichment analyses were performed on the 50 nuclear-encoded HSP genes (Figure 4), excluding two chloroplast-encoded genes due to insufficient genomic annotation.

The KEGG pathway analysis revealed the metabolic roles of the HSP family, with 36 members from various superfamilies assigned to specific pathways (Figure 4a). The results showed a significant enrichment of genes in the "Protein processing in endoplasmic reticulum" category. All members of the sHSP superfamily, as well as the majority of HSP40, HSP70, and HSP90 members, were enriched in this pathway. In contrast, the HSP60 superfamily exhibited antiviral defense functions, including the "Phagosome" and "RNA degradation" pathways. This suggests that while the other superfamilies focus primarily on protein processing, the HSP60 superfamily plays specialized roles in intracellular degradation and in regulating RNA stability. Notably, one HSP70 gene (Gv1.18G02360.1) was also enriched in the "RNA degradation" pathway, suggesting a potential synergy between these superfamilies in maintaining RNA stability. Furthermore, the HSP40 superfamily demonstrated functional diversity: Gv1.18G02330.1 was involved in "Porphyrin metabolism" and Gv1.12G02340.1 in the "Sulfur relay system". In addition, one HSP90 member (Gv1.18G01030.1) was enriched in the "Two-component system", suggesting a potential role in environmental signal transduction.

The GO analysis further classified the HSP genes (18 genes unannotated) into three primary categories: biological process, molecular function, and cellular component (Figure 4b). Like KEGG, the HSP40 superfamily exhibited remarkable functional diversity, including "Hsp70 protein binding," "Ribosome binding," and "Zinc ion binding," among others. Notably, the HSP40 superfamily did not show enrichment in "ATP binding" or "ATP-dependent protein folding chaperone" functions, which distinguished it from the other superfamilies (Figure 4b). This diversity suggests that HSP40s act as specialized adapters that recruit various client proteins to the chaperone machinery. In contrast, the other superfamilies showed more specialized enrichment patterns. The sHSP superfamily was predominantly enriched in "Response to heat," suggesting its important role in defense against heat stress. Both HSP60 and HSP90 superfamilies exhibited a comprehensive set of functions related to energy-driven protein stabilization, including "ATP hydrolysis activity," "Unfolded protein binding," "Protein folding," "ATP binding," and "ATP-dependent protein folding chaperone". The HSP70 superfamily showed a more focused enrichment, specifically in "ATP binding" and "ATP-dependent protein folding chaperone". These results suggest that while HSP60 and HSP90 handle a wider range of misfolded protein interactions, HSP70 members serve as core engines of ATP-dependent folding processes.

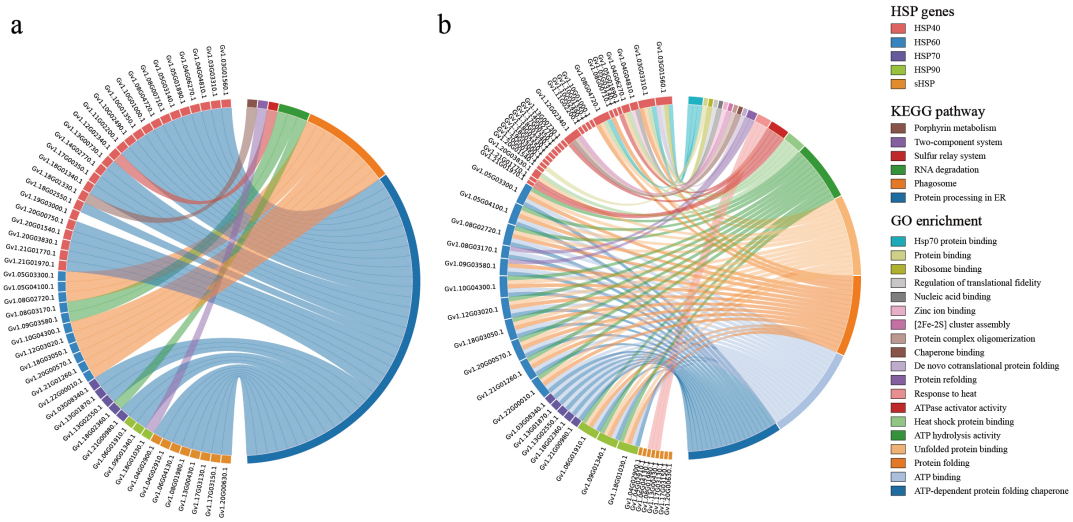


Figure 4. GO and KEGG enrichment analysis of HSP gene family members in *G. vermiculophylla*. (**a**) Chord diagram of KEGG pathway enrichment. (**b**) Chord diagram of GO term enrichment.

3.5. Protein-Protein Interaction Network and Hub Gene Identification

To investigate the potential functional relationships among HSP members, a protein-protein interaction (PPI) network was constructed based on the 52 nuclear-encoded HSPs using the interaction information from *Gracilariopsis chorda*. Setting the minimum required interaction score to 0.7 resulted in 32 HSP genes being mapped to the predicted interaction network (Figure 5). All genes included in the network exhibited interactions with at least one other HSP member, indicating potential functional associations among different HSPs in *G. vermiculophylla*. Network topology analysis showed that HSP70 members occupied central positions in the predicted PPI network. All identified HSP70 genes exhibited high connectivity, with each interacting with more than 20 other proteins (Supplementary Table S2). Among them, Gv1.18G02360.1 (HSP70) showed the highest connectivity, with a maximum degree of 40, suggesting that it may represent a potential hub in the HSP interaction network. In addition to HSP70 members, one HSP40 gene (Gv1.09G01340.1) and two HSP90 genes (Gv1.18G01030.1 and Gv1.06G01910.1) also showed relatively high connectivity, with interaction degrees exceeding 20. These connectivity patterns suggest potential functional associations among HSP70, HSP40, and HSP90 members in the predicted interaction network. Overall, the PPI analysis identified HSP70, HSP40, and HSP90 members as highly connected nodes, with HSP70 members occupying particularly central positions in the predicted network.

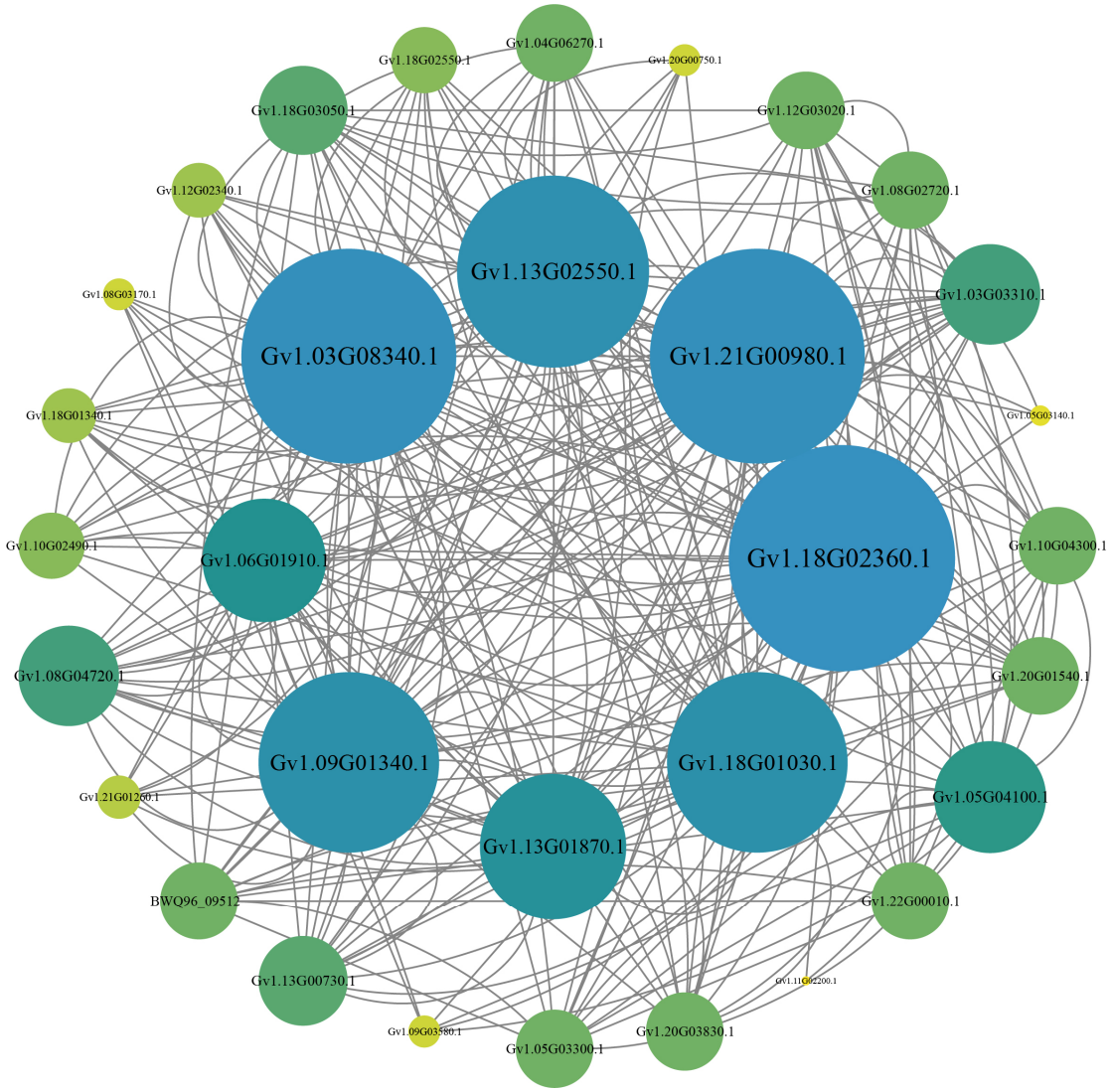


Figure 5. Protein-protein interactions of HSP genes in *G. vermiculophylla*. The network was constructed with a minimum required interaction score of 0.7 (high confidence), and each line represents an interaction. Both the size and color intensity of the nodes are mapped to the degree of centrality.

3.6. Expression Analysis of HSP Genes under Heat Stress

To investigate the transcriptional responses of *G. vermiculophylla* to heat stress, we retrieved and reanalyzed publicly available RNA-seq data comprising three control samples (C1, C2, C3) and three heat-stress samples (H1, H2, H3). More than 86% of the clean reads from all six samples were successfully aligned to the *G. vermiculophylla* genome. Quantification using featureCounts showed that the number of assigned reads in the control group ranged from 30 to 33 million, whereas that in the heat-stress group ranged from 27 to 32 million. MultiQC analysis further indicated that the proportion of reads assigned by featureCounts ranged from 57% to 64%. These results showed comparable sequencing depth and read assignment rates between the control and heat-stress groups. PCA and correlation heatmap analyses further demonstrated strong within-group sample correlations, supporting the consistency among biological replicates (Supplementary Figure S1). Of the identified HSP family, 50 HSP genes remained after CPM filtering and were used for differential expression analysis.

The majority of these 50 HSP genes were significantly induced by heat stress ($|\log_2(\text{FoldChange})| > 1$), although the magnitude and direction of the response varied across superfamilies (Supplementary Table S3). The sHSP genes exhibited the most dramatic transcriptomic induction (Figure 6). Five sHSP members (Gv1.04G02900, Gv1.04G02910, Gv1.06G04130, Gv1.08G01980, and Gv1.13G00430) were massively up-regulated, with $\log_2$FoldChange values ranging from 10.30 to 12.65 (Supplementary Table S3). These transcriptomic results suggest that sHSPs may be highly responsive to heat stress in *G. vermiculophylla*. In the HSP90 superfamily, all three analyzed members were also significantly up-regulated (Figure 6). Notably, the hub gene Gv1.18G01030 showed a very high induction with a $\log_2$FoldChange of 7.78 (Supplementary Table S3). The HSP70 superfamily showed a highly consistent upward trend (Figure 6). All five analyzed members were significantly induced

(log_2FoldChange > 2). The key hub gene identified in the PPI network, Gv1.18G02360, exhibited a strong induction (log_2FoldChange = 4.71). Another member, Gv1.21G00980, reached a log_2FoldChange of 6.10 (Supplementary Table S3). The sustained high expression of these hub genes is consistent with their potential roles in the cellular protein protection network. The HSP40 superfamily exhibited the most diverse expression patterns, reflecting its functional complexity. 11 HSP40 genes were significantly upregulated, with Gv1.20G01540 showing the greatest induction (log_2FoldChange = 6.28). However, 5 HSP40 genes (Gv1.05G03140, Gv1.18G02550, Gv1.19G03000, Gv1.21G01770 and Gv1.21G01970) were significantly downregulated. Similarly, the HSP60 superfamily also exhibited diverse expression patterns. Gv1.08G03170 was significantly induced (log_2FoldChange = 5.31), and two members (Gv1.12G03020 and Gv1.20G00570) were downregulated. In addition to heat treatment, we also analyzed the expression patterns of these HSP genes under high-light stress (Supplementary Table S4) [59]. However, none of the members exhibited significant differential expression under this condition.

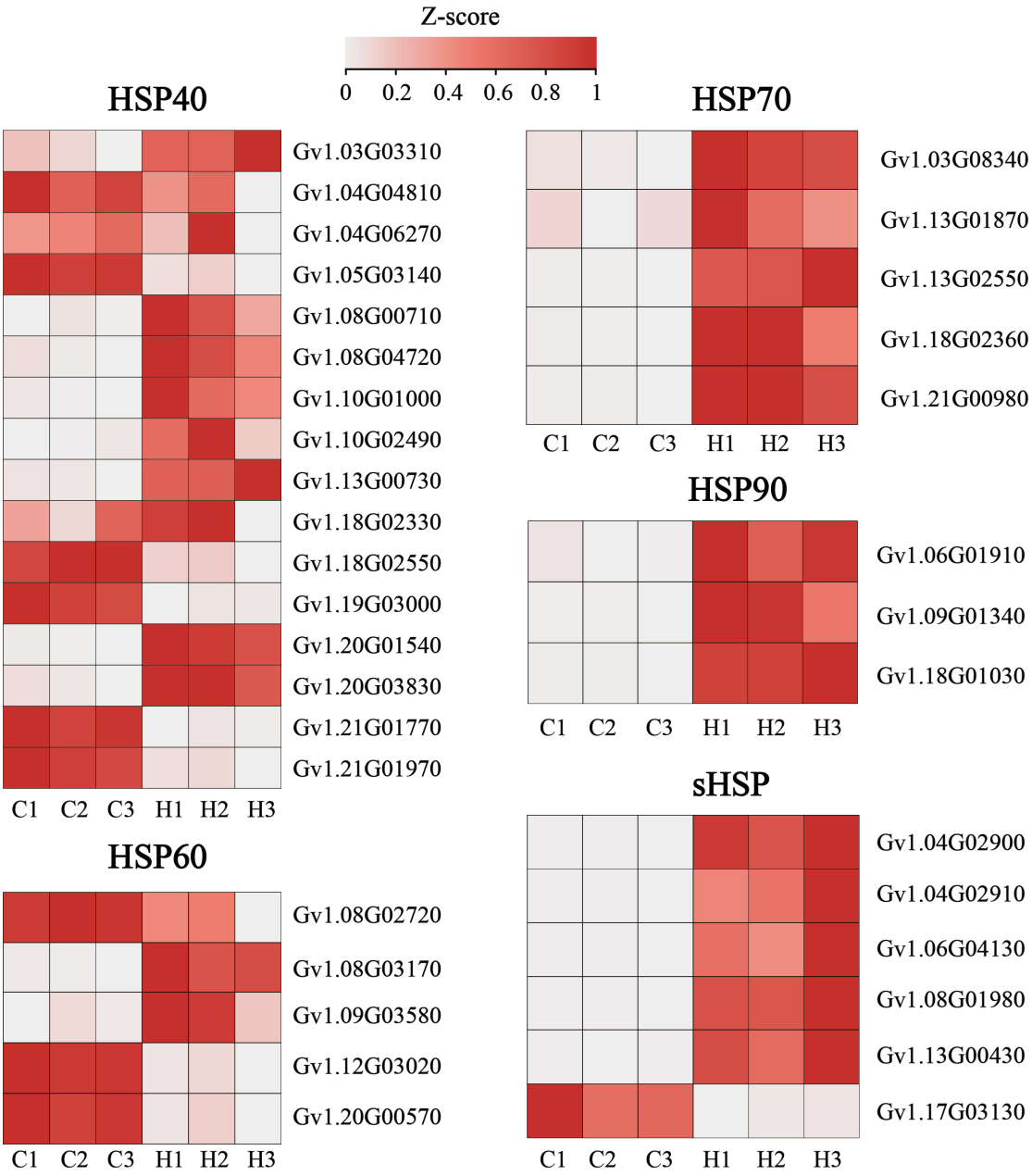


Figure 6. Heat map showing the expression profiles of HSP40, HSP60, HSP70, HSP90, and sHSP genes in *G. vermiculophylla* based on publicly available RNA-seq datasets. Expression values were normalized using the Z-score transformation of TPM values.

3.7. Structural Domains and Regulatory Elements of the HSP70s

The HSP70 superfamily in *G. vermiculophylla* exhibits complex structural arrangements and diverse regulatory potentials that support its important role in stress response. We performed a detailed analysis of the cis-

acting regulatory elements and domains of the identified nuclear HSP70 genes to understand their functional specialization (Figure 7).

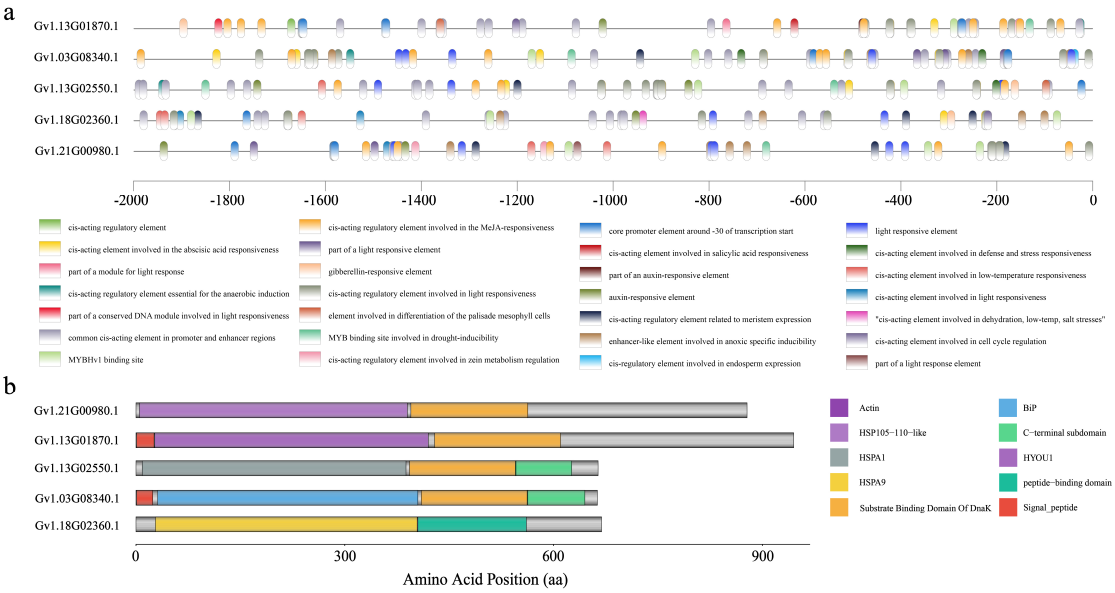


Figure 7. Analysis of the cis-acting regulatory elements and domains of HSP70 genes. (**a**) Predicted distribution of cis-acting regulatory elements in the promoter regions of HSP70 genes. (**b**) Domain analysis of HSP70 genes.

The promoter analysis identified a rich landscape of regulatory elements involved in hormone signaling and abiotic stress responses (Figure 7a). A high density of abscisic acid (ABA)-responsive elements was found across all HSP70 promoters, with Gv1.03G08340.1 and Gv1.13G02550.1 containing numerous binding sites. Elements involved in methyl jasmonate (MeJA)-responsiveness were also widely distributed, particularly in Gv1.13G01870.1 and Gv1.22G03620.1. Other hormone-related elements were detected in several members, including gibberellin-responsive and auxin-responsive motifs. In addition to hormone regulation, several stress-related elements were identified within the HSP70 promoters. These include MYB-binding sites involved in drought-induced expression and specific low-temperature-responsive elements found in Gv1.13G02550.1, Gv1.18G02360.1, and Gv1.21G00980.1. We also detected anaerobic induction elements and enhancer-like elements involved in anoxic-specific inducibility across most members. Furthermore, multiple light-responsive elements were present in every HSP70 promoter, highlighting the potential sensitivity of this superfamily to the marine light environment. Other regulatory features, such as MYBHv1-binding sites and motifs related to meristem expression, were also identified. These diverse regulatory features explain the versatile expression patterns of HSP70 genes and their ability to protect *G. vermiculophylla* from a wide range of environmental stress.

Domain analysis revealed that while all members share the core HSP70 framework, they possess distinct specialized domains (Figure 7b). At the N-terminus, Gv1.21G00980.1 contains an HSP105-110-like domain, Gv1.13G01870.1 is characterized by a HYOU1 domain, and Gv1.13G02550.1 possesses an HSPA1 domain. Gv1.03G08340.1 was identified as a BiP (binding immunoglobulin protein) homolog, while Gv1.18G02360.1 contains an HSPA9 domain. The substrate-binding properties of these proteins are also highly conserved. Substrate Binding Domains of DnaK were identified in Gv1.21G00980.1, Gv1.13G01870.1, Gv1.22G03620.1, and Gv1.03G08340.1. Meanwhile, Gv1.18G02360.1 features a specialized peptide-binding domain. At the C-terminus, both Gv1.13G02550.1 and Gv1.03G08340.1 contain a C-terminal subdomain, which is often involved in co-chaperone interactions. Additionally, SignalP analysis identified signal peptides at the N-terminus of Gv1.13G01870.1 and Gv1.22G03620.1, suggesting these specific chaperones are targeted to the secretory pathway or the endoplasmic reticulum.

4. Discussion

In marine environments, intertidal seaweeds are continuously exposed to heatwaves due to global warming. Given that they inhabit the demanding habitat of the intertidal zone, intertidal macroalgae have developed a set of protective mechanisms for survival [60]. HSPs are essential molecular chaperones in these protective mechanisms and have been extensively studied in higher plants, including Populus [61], tea plant [62], and various Poaceae species [9]. However, despite the significance of HSPs, systematic research on this gene family in red macroalgae

remains limited due to the lack of high-quality reference genomes. *G. vermiculophylla* is one of the most important economic agarophytes among red macroalgae and serves as a key species in coastal ecosystems [63]. This algal species showed a wide range of temperature (5–34 °C) tolerance [64]. Therefore, understanding the tolerance mechanisms of *G. vermiculophylla* under heat stress is necessary to clarify its resilience in dynamic coastal habitats. Here, we comprehensively analyzed the structural characteristics, functions, and expression patterns under heat stress of the HSP gene family.

In this study, we identified 54 HSP genes in *G. vermiculophylla*, comprising 52 nuclear-encoded and 2 chloroplast-encoded genes. This distribution indicates that the HSP system in this red alga is primarily regulated by the nuclear genome, while the chloroplast genome retains only a few members. In eukaryotes, the HSP superfamily differentiated early in evolution and has exhibited strong conservation within individual families, with each family retaining its characteristic core structural domains [9,65,66]. In this study, five HSP gene superfamilies were identified, and their high bootstrap values further confirm this evolutionary pattern. The identified family includes 8 sHSPs, 25 HSP40s, 12 HSP60s, 6 HSP70s, and 3 HSP90s. Notably, HSP40 is the largest subfamily, accounting for nearly 50% of the total HSP genes. This pattern is consistent with findings in rice (54%) and cotton (62%), suggesting that HSP40 represents the largest proportion of HSP genes, and may be evolutionarily conserved and associated with stress responses across species [11,67]. Notably, the structure of the HSP40 superfamily exhibits remarkable diversity, which enables responses to various types of stress. In contrast to the expansion of HSP genes in land plants via whole-genome duplication (WGD) [11,67], no gene duplication events were detected in *G. vermiculophylla*. Furthermore, the *G. vermiculophylla* genome contains a high proportion of transposable elements (TEs). This suggests that these HSP genes are likely shaped by the retention of ancestral genes, with potential contributions from transposable elements.

The physicochemical properties of several HSP superfamilies differ significantly, reflecting their diverse functional requirements. However, nearly all identified HSPs are hydrophilic, a common feature in eukaryotes that helps prevent chaperone aggregation under environmental stress [68]. The MW distribution in *G. vermiculophylla* follows patterns observed in other photosynthetic eukaryotes [9,69]. For instance, the sHSP superfamily consists of the smallest proteins, consistent with findings in *Arabidopsis* [70] and *P. yezoensis* [30]. Notably, two members (Gv1.13G01870.1 and Gv1.21G00980.1) exceed 90 kDa. These larger proteins are likely members of the HSP110/SSE subfamily [71]. In higher plants, HSP110 members act as nucleotide exchange factors for HSP70 [72]. Their presence in *G. vermiculophylla* suggests a conserved and sophisticated chaperone system. The broad range of PI further indicates functional specialization among the subfamilies. Most HSP70s and HSP90s in *G. vermiculophylla* are acidic, which may support their cytoplasmic localization and promote interactions with a diverse range of substrate proteins [73,74]. Despite the presence of acidic members in the HSP40 families, their basic characteristics are more prominent. This suggests that these proteins may be localized to different cellular compartments and interact with specific acidic substrates [15]. Notably, the pI distribution pattern of the HSP family in *G. vermiculophylla* is consistent with those of both animals and plants, suggesting that the HSP family may share conserved biochemical properties across eukaryotes [7,75]. Furthermore, high II values in several HSPs indicate that they are unstable *in vitro*. In the sHSP superfamily, 75% of the members exhibited a high instability index. A similar pattern was observed across other red algal lineages, including Cyanidiophyceae, Florideophyceae, Compsopogonophyceae, Porphyridiophyceae, Bangiophyceae, Rhodellophyceae, and Stylonematophyceae, where more than 76% of sHSP members showed high instability indices [37]. This conserved pattern suggests that the relatively high predicted instability of sHSPs may represent a common feature of red algal sHSP proteins. Similar patterns of high instability have been reported for sHSPs in both animals and plants [76,77]. This structural plasticity may induce conformational rearrangements and expose substrate-binding sites under heat stress, enabling the sHSPs to rapidly bind and refold various misfolded proteins [78,79]. As an indicator of thermal stability, higher AI values in HSP60s suggest these members may maintain stable oligomeric structures and perform chaperone functions in intertidal zone [80].

Functional annotation and PPI analysis indicate that HSP proteins form a sophisticated chaperone network to respond to various environmental stresses. In this stress-responsive chaperone network, HSP70 represents a central component and is widely distributed across diverse cellular compartments, including the cytoplasm, nucleus, endoplasmic reticulum (BiP), mitochondria, and chloroplasts [81]. It is well established that HSP70 cooperates with other cellular chaperone systems to maintain protein homeostasis. For instance, HSP70 interacts with the J-domain of HSP40 to stimulate ATP hydrolysis [82]. Similarly, HSP100 proteins bind to the ATPase domain of HSP70 via their unique M-domain to coordinate protein disaggregation [83]. HSP70 also forms a stabilizing complex with HSP90 through the co-chaperone Hop/Sti1, thereby facilitating functional collaboration between the two chaperone systems [73]. In addition, HSP70 displaces sHSPs from sHSP-substrate complexes to initiate ATP-dependent protein refolding [84]. In mitochondria, mtHSP70 associates with the HSP60/HSP10

chaperonin complex and promotes HSP60 assembly [85]. In this study, the predicted PPI network showed that all HSP70 members exhibited strong interactions with other chaperone components, suggesting their potential central role in the chaperone network. The presence of specialized domains such as BiP, HYOU1, and HSPA9 also supports their predicted localization in specific compartments. In plants, the promoter regions of HSP70s are known to function as complex sensory hubs, enriched with various cis-acting elements associated with hormonal and environmental stresses [86,87]. The ubiquitous presence of putative light-responsive elements in the HSP70 promoters suggests that these genes may be subject to light-mediated regulation, consistent with reports of light-responsive cis-elements in HSP70 promoters of other species [88]. However, direct experimental evidence for light-responsive HSP70 expression is currently lacking in *G. vermiculophylla*. Similarly, the identification of putative low-temperature-responsive and MYB-related drought elements raises the possibility that HSP70 expression could be modulated by temperature and desiccation stress, as has been reported in other plant systems [89,90], though this remains to be experimentally verified in *G. vermiculophylla*.

The transcriptomic profiling of HSPs in *G. vermiculophylla* reveals a highly coordinated and superfamily-specific response to heat stress. The most striking result is the massive induction of the sHSP superfamily, with five members exhibiting $\log_2$FoldChange values exceeding 10. sHSPs are known to act as "first responders" under stress by rapidly binding with unfolded proteins and preventing irreversible aggregation in an ATP-independent manner [12,78]. This distinct level of upregulation, together with the consistent induction of sHSP genes under multiple stresses, including heat, oxidative, and copper stress, in *P. yezoensis* [30], suggests that sHSPs may play important roles in the early heat-stress response of *G. vermiculophylla*. In contrast to the explosive response of sHSPs, the HSP70 and HSP90 superfamilies exhibited a highly consistent and sustained upward trend. Similarly, in *Gracilaria lemaneiformis*, *hsp70-1* was strongly induced under heat stress, with significantly higher expression observed in the heat-tolerant "981" strain than in the wild type [91]. In addition, heat stress increased HSP70 abundance and significantly enriched HSP70-related pathways in *Fucus serratus* and *Saccharina japonica* [92,93]. Collectively, these findings suggest that HSP70 may play an important role in stress responses across macroalgae. The significant induction of key hub genes, such as Gv1.18G01030 (HSP90, $\log_2$FoldChange = 7.78) and Gv1.18G02360 (HSP70, $\log_2$FoldChange = 4.71), suggests that they may play important roles in the chaperone network. sHSPs bind misfolded proteins into stable complexes, which are subsequently processed by ATP-dependent HSP70 chaperones to facilitate protein solubilization and refolding [84]. Then, HSP90s further assist in the maturation of refolding proteins [94]. The HSP40 and HSP60 superfamilies demonstrated the most diverse expression patterns under heat stress. While many members were significantly induced to support the chaperone network, others were significantly downregulated, indicating that these families do not respond uniformly to heat stress and may have undergone functional divergence and specialization. In land plants, certain HSP40s or HSP60s are primarily involved in specific metabolic or developmental pathways rather than general stress defense [95,96]. The divergent responses of these two families in *G. vermiculophylla* likely indicate that some members focus on stress protection, whereas others, potentially involved in non-essential housekeeping functions, are downregulated to conserve energy during the stress response. Together with our findings, these comparative observations suggest that the superfamily-specific and coordinated HSP response observed in *G. vermiculophylla* may represent an adaptive response to the extreme and fluctuating heat conditions of the intertidal zone, potentially contributing to the species' resilience to the increasing frequency and intensity of marine heatwaves under climate change. It should be noted that the HSP expression patterns identified in this study were derived from publicly available RNA-seq datasets generated under a specific heat-stress condition (36 °C, 6 h), which exceeds the previously reported heat tolerance range of *G. vermiculophylla* (5–30 °C), suggesting that this treatment represents an acute, extreme heat exposure rather than routine intertidal temperature variation. Such short-term temperature spikes can occur during marine heatwave events or during low-tide emersion under intense solar radiation, when intertidal algae are directly exposed to ambient air temperatures that substantially exceed seawater temperatures. While these data provide valuable genome-wide insights into HSP responses under acute heat stress, the expression patterns identified through bioinformatic analyses alone have not been independently validated experimentally. Targeted validation, such as qRT-PCR, together with expression profiling under a broader range of ecologically relevant temperature and duration conditions, will be needed to confirm the expression patterns of HSP genes and further clarify their functional roles in the natural stress adaptation of *G. vermiculophylla*.

We acknowledge that differences in genetic background and experimental conditions may influence expression levels. However, consistent upregulation trends of HSP genes under heat stress across independent datasets support the robustness of our observations. Accordingly, our interpretation focuses on qualitative consistency rather than absolute expression values. Because the transcriptomic data were obtained from an independent study, the observed expression patterns should be regarded as preliminary evidence of HSP transcription under the reported conditions. Although the RNA-seq datasets originated from the same geographic

population as the genome assembly, differences in strain background and experimental conditions may have influenced gene expression. Future experiments using transcriptomic data generated from the sequenced strain under controlled stress treatments will further validate these findings.

5. Conclusions

This study reveals that *G. vermiculophylla* responds to heat stress by employing a conserved and sophisticated HSP-mediated molecular chaperone system. These HSPs form a highly integrated proteostasis network, with HSP70 occupying a central position. sHSPs are strongly induced and may act as rapid early responders, while HSP90 likely contributes to protein maturation. In contrast, the divergent expression patterns of HSP40 and HSP60 suggest functional specialization and potential hierarchical regulation within the chaperone system. In conclusion, this study characterized and analyzed the expression of the HSP gene family under heat stress, and the results provide a foundation for future research on stress biology of coastal seaweed species facing environmental changes, particularly the abnormal heatwave stress due to global warming.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/26093017 30229031/ALE-26060235-SI.zip. Figure S1: Principal Component Analysis (PCA) and sample correlation heatmap of *Gracilaria vermiculophylla* transcriptome. Table S1: Statistics of heat shock protein (HSP) genes in *Gracilaria vermiculophylla*. Table S2: Topological network parameters of HSP gene nodes in *Gracilaria vermiculophylla*. Table S3: Differential expression analysis of HSP genes between control and heat-stress treatment groups in *Gracilaria vermiculophylla*. Table S4: Differential expression analysis of HSP genes between high-light and low-light treatment groups in *Gracilaria vermiculophylla*.

Author Contributions

Conceptualization, methodology, software, formal analysis, investigation, writing—original draft preparation, visualization, J.X.; Data curation, visualization, K.D. and W.C.; Supervision, project administration, W.Z.; Validation, resources, writing—review and editing, J.J.; Writing—review and editing, funding acquisition, H.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

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Informed Consent Statement

Not applicable.

Data Availability Statement

All data are accessible within the manuscript and its supporting materials.

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Conflicts of Interest

The authors declare no conflicts of interest.

Use of AI and AI-Assisted Technologies

Artificial intelligence tools, including Generative Pre-trained Transformer (GPT), supported language editing and manuscript preparation; however, the research, interpretations, and conclusions presented in the manuscript are solely those of the authors. All authors have read and approved the final manuscript and take full responsibility for its content.

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