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# Beyond heat stress: the heat shock factor family orchestrates multifaceted abiotic stress responses in perennial ryegrass

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## Abstract

Perennial ryegrass (*Lolium perenne* L.) is a vital cool-season forage and turfgrass species whose productivity is increasingly threatened by climate change-induced abiotic stresses. The Heat Shock Factor (*HSF*) family are key transcription factors known to mediate plant stress responses, yet their functional landscape in perennial ryegrass remains poorly characterized, especially beyond heat stress. In this study, genome-wide identification revealed 26 LpHSF genes in perennial ryegrass, harboring abundant stress-responsive *cis*-elements. Moving beyond a singular focus on thermotolerance, the study established a comprehensive spatio-temporal expression atlas of *LpHSF*s. The findings demonstrate that specific *LpHSF*s, particularly members of the *LpHSFA2* subclass, *LpHSFA3*, *LpHSFC1.3*, and *LpHSFC1.4*, are significantly upregulated under a broad spectrum of abiotic stresses, including salinity, alkalinity, and heavy metal exposure, indicating their roles as broad-spectrum stress regulators. Furthermore, this study uncovered distinct tissue-specific expression patterns, with Class A genes predominantly expressed in leaves and Classes B and C in roots and crowns, implicating their roles in organ development and stress adaptation. Notably, the expression of numerous *LpHSF*s during leaf senescence and their induction by hormones such as ABA and MeJA further highlights their functional diversity. This study provides the first integrated expression atlas of the *LpHSF* family, revealing their versatile roles in coordinating responses to diverse environmental and developmental signals. This work lays a crucial foundation for future functional studies and positions *LpHSF*s as prime targets for molecular breeding aimed at enhancing multi-stress resilience in perennial ryegrass.

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## Introduction

Perennial ryegrass (*Lolium perenne* L.) is a high-value cool-season species widely utilized as premium turfgrass and nutritious forage, prized for its high protein content, extended growing season, strong grazing tolerance, and excellent palatability. As the most extensively cultivated perennial forage grass in temperate regions, it plays a critical role in landscaping and ecological management[1,2]. However, climate change has led to increasingly frequent and severe abiotic stresses—including heat waves, drought, soil salinization, and heavy metal contamination. These stresses can significantly disrupt plant hormone homeostasis, while phytohormones like abscisic acid (ABA), auxin, jasmonic acid, etc, in turn, play critical regulatory roles in mediating plant responses and adaptation to such environmental challenges. The adverse effects markedly impair its growth, yield, and persistence, thereby threatening agricultural sustainability and food security[3,4]. In this context, uncovering the genetic basis of stress adaptation is essential for breeding resilient cultivars through molecular approaches[5,6].

Among key regulators of plant stress responses, the Heat Shock Factor (*HSF*) gene family stands out as a master transcription factor family involved in diverse abiotic and biotic stresses[7,8]. *HSF*s are characterized by conserved domains such as the N-terminal DNA-binding domain (DBD) and the oligomerization domain (HR-A/B), and are classified into subfamilies A, B, and C based on structural features and functional motifs—including activation domains (AHA) in class A and repressor domains in class B[9]. Functional studies across species have revealed their critical roles: in Arabidopsis, *AtHSFA1* acts as a master regulator of heat stress response (HSR)[10], while *AtHSFA2* overexpression improves tolerance to heat, salt, and osmotic stress[11]. In maize, *ZmHSF20* negatively regulates thermotolerance by modulating cell wall-related genes[12], whereas in lily, LlHSFC2 forms heteromeric complexes with HSFAs to enhance proteostasis under heat stress[13].

Although *HSF*s have been identified in several forage grasses—including 16 members in Italian ryegrass[14] and 25 in an earlier perennial ryegrass genome[15]—their functional characterization remains fragmentary, largely limited to heat stress. A comprehensive expression atlas capturing their roles across multiple stresses and developmental contexts is still lacking. This study systematically identified 26 *LpHSF*s from the updated perennial ryegrass genome, classifying them into 16 class A, six class B, and four class C members. A spatio-temporal expression atlas of *LpHSF*s was further established under diverse abiotic stresses (heat, salt, alkali, heavy metals) and hormone treatments, as well as across tissue types and senescence stages. Beyond confirming their canonical heat-responsive roles, broad-spectrum stress responsiveness and tissue-specific regulation were uncovered, highlighting the functional diversification of *LpHSF*s within the plant stress adaptation network. This work provides a foundational resource for elucidating HSF-mediated regulatory mechanisms and supports molecular breeding aimed at enhancing multi-stress resilience in perennial ryegrass and related species.

## Materials and methods

### Identification of the *LpHSF* gene family in perennial ryegrass

The protein sequences of HSF family members from *Arabidopsis thaliana*, *Zea mays*, and *Oryza sativa* were retrieved from the Plant

Transcription Factor Database (PlantTFDB; http://planttfdb.cbi.pku.edu.cn)[16]. HSF protein sequences from perennial ryegrass were acquired from its published genome[17] and used to construct a local protein database. Using BLASTP, proteins containing the HTH (helix-turn-helix) domain were identified by querying with the HSF sequences from *A. thaliana*, *Z. mays*, and *O. sativa*. Candidate genes were further screened using the hidden Markov model (HMM) profile of the HTH domain (PF00447) obtained from the PFAM database (http://pfam.xfam.org). Redundant sequences were filtered using the online tools HMMER (www.hmmer.org), and SMART (http://smart.embl-heidelberg.de). Non-redundant sequences and those predicted with high confidence were selected and designated as *LpHSF* genes. The coding sequences (CDS) of the *LpHSF* genes were extracted from the perennial ryegrass genome annotation files. Finally, the physicochemical properties of the LpHSF proteins, including molecular weight (MW) and theoretical isoelectric point (pI), were predicted using ProtParam (https://web.expasy.org/protparam).

## Phylogenetic analysis and classification of the *LpHSF* gene family

The HSF proteins of *Lolium perenne*, *Oryza sativa*, *Triticum aestivum*, *Zea mays*, and *Arabidopsis thaliana* were downloaded from the perennial ryegrass[17], rice[18], wheat[19], corn[20] genome annotation project, and TAIR11[21], respectively. The HSF protein sequences of *Lolium perenne*, *Oryza sativa*, *Triticum aestivum*, *Zea mays*, and *Arabidopsis thaliana* were used for multiple sequence alignment with Muscle[22]. The maximum-likelihood phylogenetic tree was constructed using IQ-tree[23] with the VT+F+R10 model identified as the best substitution model using ModelFinder[24]. Ultrafast bootstrap[25] with 1,000 replications was used to test the branch supports. The *LpHSF* genes were divided into different subgroups on the basis of the classification of perennial ryegrass, rice, wheat, corn, and Arabidopsis *HSF*.

## Gene structure, *cis*-acting analysis, and chromosomal locations

To visualize the exon-intron structure of *LpHSF* genes, the gene structure was displayed using TBtools[26], according to the perennial ryegrass genome annotation file[17]. The analysis of cis-acting elements of 2,000 bp upstream of the 26 *LpHSF* genes was performed by using the PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PlantPAN 4.0 (http://plantpan.itps.ncku.edu.tw/plantpan4/index.html), and then the illustrations were drawn using the TBtools[26].

The chromosomal locations of the *LpHSF* genes were obtained from the perennial ryegrass genome annotation files and mapped to the corresponding chromosomes using TBtools[26]. In order to beautify the chromosome distribution of *LpHSF* genes, the online tool MapGene2Chrome (MG2C, http://mg2c.iask.in/mg2c_v2.1)[27] was used to make it.

## Plant materials and growth conditions

The perennial ryegrass seeds (cv. 'Buena Vista') were germinated at Sichuan Agricultural University in a growth chamber for 10 d. Germination was carried out in petri dishes lined with filter paper and moistened with sterile water. The seedlings were then transplanted into a hydroponic box containing 1/2 Hoagland (pH = 5.8) for 4 weeks. The Hoagland's solution was changed every 3 d. The growth temperature was 25/22 °C (day/night) with and a 16/8 h light/dark photoperiod.

## Stress treatments and several hormone treatment

To assess the expression patterns in response to various treatments, one-month-old perennial ryegrass seedlings were subjected to drought stress (20% PEG6000), salt stress (250 mM NaCl), heat stress (38/33 °C, day/night), alkaline stress (200 mM, pH = 9.4), heavy metal stress (1.33 mM $CdCl_2$)[28,29], and phytohormones treatment[30] such as gibberellins (GA, 100 μM), auxin (IAA, 20 μM), abscisic acid (ABA, 50 μM), salicylic acid (SA, 100 μM), methyl jasmonate (MeJA, 20 μM), ethephon (ETH, 200 μM), N-(Phenylmethyl)-9H-purin-6-amine (6-BA, 25μM). After 0, 1, 4, 12, and 72 h of different treatments, samples were collected, rapidly frozen in liquid nitrogen, and stored at −80 °C before RNA extraction.

## RNA extraction, cDNA synthesis, and RT-qPCR analysis

Total RNA was isolated using the HiPure Plant RNA Mini Kit (Magen Biotech Co., Ltd., China), according to the manufacturer's protocol. MonScript™ RTIII All-in-One Mix with dsDNase (Monad Biotech Co., Ltd., China) was used for the synthesis of cDNA. RT-qPCR analyses were performed using the Tag SYBR® Green qPCR Premix x (BestEnzymes Biotech Co., Ltd., Canada), in accordance with the manufacturer's protocol; the reactions were run using the CXF Connect™ Real-Time System (Bio-Rad). *The EUKARYOTIC INITIATION FACTOR 4 ALPHA* (*EIF4A*) was selected as the internal reference gene. Relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method[31]. The primers used in this study are listed in Supplementary Table S1.

## Statistical analysis

All statistical analyses were conducted in JMP software (Version 10, SAS Institute Inc., USA). Results are reported as mean values ± SEM derived from three independent biological replicates. To determine the statistical significance of differences in *LpHSF*s transcript abundance among different organs and in response to various abiotic stresses and phytohormone treatments, Fisher's protected least significant difference (LSD) test was applied, with a probability level of 0.05 considered statistically significant.

# Results

## Identification and phylogenetic analysis of *HSF* family in perennial ryegrass based on a new genome version

Based on the newly released genome version of perennial ryegrass[17], a total of 26 *HSF* genes (*LpHSF*s) containing the characteristic HTH domain were identified and systematically classified into three classes (A, B, and C) with 16, six, and four members, respectively, and named accordingly from *LpHSFA1B* to *LpHSFC1.4* (Fig. 1a). The coding sequence lengths of these *LpHSF*s ranged from 369 bp (*A7A*) to 2,067 bp (*A1B*), while the molecular weights of the encoded proteins varied from 1,2581.2 Da (*A7A*) to 74,431.97 Da (*A1B*), with isoelectric points spanning 4.74 (*A2.5*) to 10.69 (*C1.1*).

To elucidate their evolutionary relationships, a phylogenetic tree was constructed using HSF protein sequences from *Lolium perenne*, *Oryza sativa*, *Triticum aestivum*, *Zea mays*, and *Arabidopsis thaliana*. The classification was further supported by distinct functional domains: Class A members possess an activation domain (AHA), Class B contains a repressor domain (RD), and Class C comprises the remaining members. Phylogenetic analysis revealed that *LpHSF*s cluster closely with other Gramineae homologs, indicating a common evolutionary origin. Notably, this study identifies 26 *LpHSF*

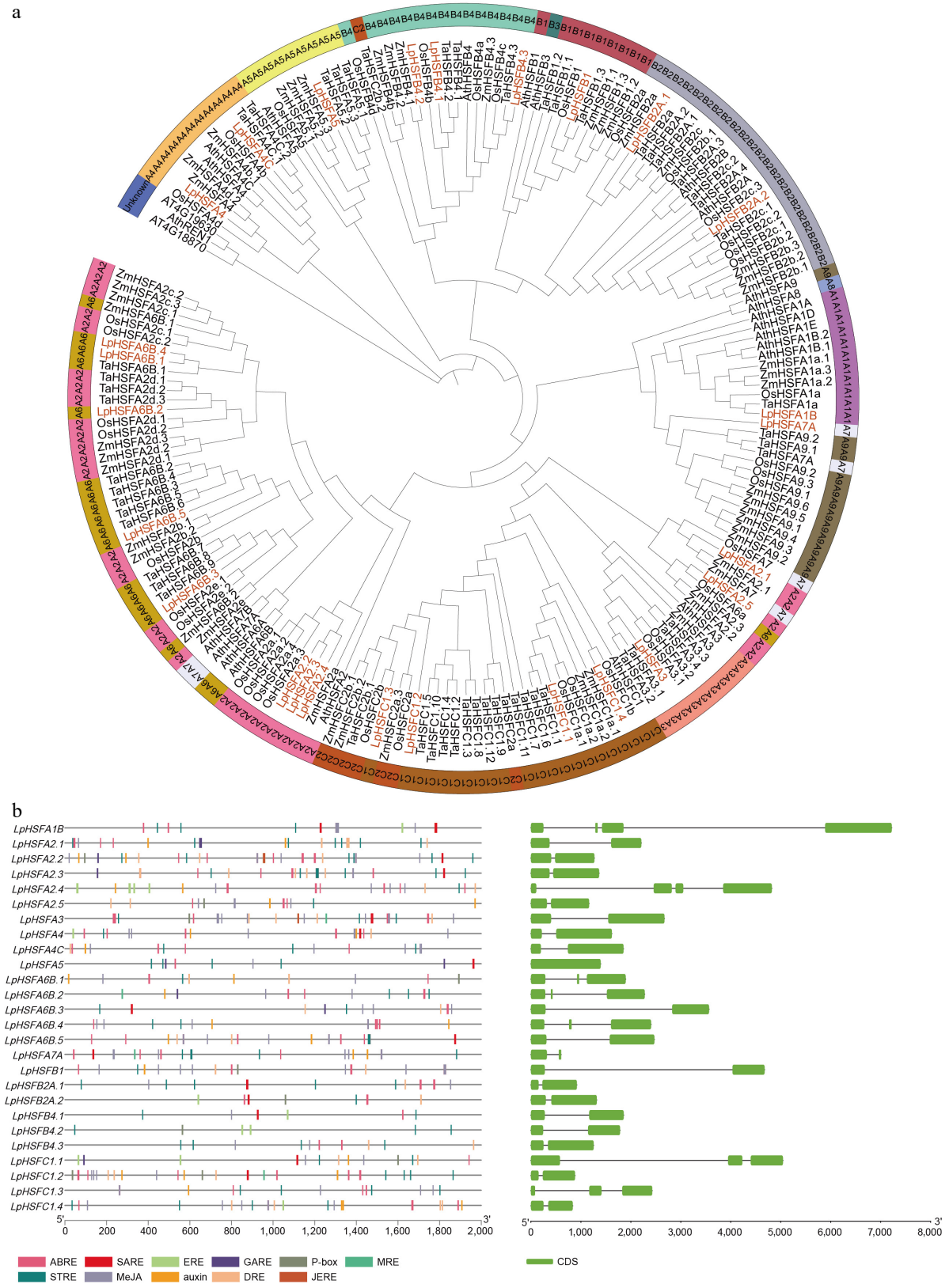


**Fig. 1** (a) Unrooted phylogenetic tree representing relationships among the HSF proteins of *Lolium perenne*, *Oryza sativa*, *Triticum aestivum*, *Zea mays*, and *Arabidopsis thaliana*. The genes in *Lolium perenne* are marked in orange, while those in *Oryza sativa*, *Triticum aestivum*, *Zea mays*, and *Arabidopsis thaliana* are marked in black. For the identification of subgroup A, the following color scheme was applied: A1 (mediumorchid), A2 (hotpink), A3 (lightsalmon), A4 (lightorange), A5 (khaki), A6 (goldenrod), A7 (aliceblue), A8 (skyblue), and A9 (olivedrab). In subgroup B, B1, B2, B3, and B4 were assigned the colors indianred, bluegray, slategray, and aquamarine, respectively. Within subgroup C, C1 was colored dark_goldenrod, and C2 was designated chocolate. Non-grouped members were uniformly colored slate_blue. (b) The *cis*-acting elements of the *LpHSF*s promoter region from perennial ryegrass, and gene structures of *LpHSF*s from perennial ryegrass in which the exons region is indicated by green boxes.

members, differing from the previous report[15] by the inclusion of additional members such as *A6B.4*, *A7A*, and *A2.4*, a discrepancy likely attributable to the improved genome assembly used here. Detailed information on all identified *LpHSF* genes is provided in Supplementary Table S2.

## Genomic distribution and putative functional analysis of *LpHSF* genes

Chromosomal localization analysis revealed that the 26 identified *LpHSF* genes are distributed across all seven chromosomes. As illustrated in Supplementary Fig. S1, chromosome 4 harbors the highest number, with eight genes (*A1B*, *A6B.3*, *A2.2*, *A2.3*, *A2.4*, *B4.2*, *A7A*, and *A6B.2*). Five genes (*A6B.4*, *A5*, *B4.3*, *B1*, and *B2A.2*) are located on chromosome 5, while four ones (*A2.1*, *C1.1*, *A4C*, and *C1.4*) reside on chromosome three. Chromosome 2 contains three *HSF*s (*B4.1*, *A6B.5*, and *B2A.1*), and chromosomes 1, 6, and 7 each contain only two genes.

To gain functional insights, the *cis*-elements in the promoters of these genes (*pLpHSF*s) were analyzed using PlantCARE. The results indicate that most *pLpHSF*s contain a variety of hormone-responsive and mRNA recognition elements (Fig. 1b; Supplementary Table S3), suggesting potential roles in abiotic stress responses and hormone signaling pathways. Furthermore, gene structure analysis showed diversity in intron-exon organization (Fig. 1b). Specifically, *A5* is intronless, *A1B* and *A2.4* contain three introns, five genes (*A6B.1*, *A6B.2*, *A6B.4*, *C1.1*, and *C1.3*) have two introns, and the remaining *LpHSF* genes possess a single intron.

## Expression profiling of *LpHSF* genes across tissues

The tissue-specific expression profiles of *LpHSF* genes were investigated to elucidate their potential roles in development and leaf senescence (Fig. 2). The analysis revealed that 23 out of 26 *LpHSF*s were highly expressed in leaves at different developmental stages. Strikingly, 15 A-type, three B-type, and two C-type *LpHSF*s displayed peak expression in the older, lower-positioned leaves ($4^{th}$ or $5^{th}$). Notably, six genes (*A2.4*, *A4*, *A4C*, *A6B.2*, *A6B.3*, *B2A.1*) exhibited leaf-specific expression.

In roots, 21 *LpHSF*s showed relatively high expression, with all B- and C-type members included. The C-type genes *C1.1*, *C1.3*, and *C1.4* were particularly prominent, showing their highest transcript abundance in root tissue.

Within the crown tissue, 13 *LpHSF*s were highly expressed. Among these, *A6B.4*, *B4.1*, *B4.2*, and *B4.3* achieved their maximum expression levels in the crown, with *B4.1* and *B4.2* exhibiting expression approximately 3.5-fold and 3-fold higher, respectively, than in their next highest-expressing tissue (root).

Only ten *LpHSF*s were highly expressed in the leaf sheath, and for all of them, the sheath was not their primary site of expression.

## Expression profiles of *LpHSF* response to phytohormones

Although the presence of abundant hormone-responsive *cis*-regulatory elements in *pLpHSF*s suggested their potential regulatory roles, expression profiling of all 26 *LpHSF*s was conducted under phytohormone treatments using RT-qPCR. As shown in Fig. 3, the expression of most *LpHSF*s was unstable under control conditions.

In response to ABA, half of the subfamily A members exhibited significant expression changes, including seven upregulated genes (*A2.1*, *A2.4*, *A2.5*, *A3*, *A6B.4*, *A6B.5*, *A7A*) and one downregulated gene (*A1B*). In contrast, only three subfamily B members (*B4.1*, *B2A.1*, *B2A.2*) were repressed, and three subfamily C members (*C1.2*, *C1.3*, *C1.4*) were upregulated.

Under MeJA treatment, 11 out of 16 subfamily A members responded, with six being upregulated (*A2.1*, *A2.3*, *A2.4*, *A4C*, *A6B.1*, *A6B.4*) and five downregulated (*A1B*, *A2.2*, *A2.5*, *A6B.5*, *A7A*). In subfamily B, *B2A.2* was induced, and *B2A.1* was suppressed. Among subfamily C members, *C1.1* and *C1.2* were upregulated, while *C1.3* was downregulated.

Following IAA treatment, *A2.3*, *A2.4*, and *A6B.2* were upregulated in subfamily A, whereas *A1B*, *A3*, *A5*, and *A6B.4* were downregulated. In subfamilies B and C, only *B2A.2* and *B4.2* were induced.

Under GA treatment, ten subfamily A members (*A1B*, *A2.1*, *A2.2*, *A2.4*, *A3*, *A5*, *A6B.1*, *A6B.2*, *A6B.3*, *A6B.4*) were upregulated, while *A4*, *B2A.1*, *B2A.2*, *B4.1*, *B4.2*, *C1.3*, and *C1.4* were downregulated.

In response to 6-BA, nine subfamily A members (*A2.1*, *A2.2*, *A2.4*, *A2.5*, *A4*, *A4C*, *A6B.1*, *A6B.3*), four subfamily B members (*B4.1*, *B4.2*, *B4.3*), and one subfamily C member (*C1.4*) were upregulated, whereas *A1B*, *A3*, and *B2A.2* were downregulated.

Under ETH treatment, only *B4.2* and *B4.3* were upregulated, while 12 subfamily A members (*A1B*, *A2.1*, *A2.2*, *A2.4*, *A2.5*, *A4*, *A5*, *A6B.2*, *A6B.3*, *A6B.4*, *A6B.5*, *A7A*) and one subfamily C member (*C1.1*) were downregulated.

After SA treatment, two subfamily A members (*A4C*, *A6B.4*) and two subfamily B members (*B4.1*, *B4.2*) were upregulated, while nine subfamily A members (*A1B*, *A2.2*, *A2.5*, *A3*, *A4*, *A5*, *A6B.3*, *A6B.5*, *A7A*) and three subfamily C members (*C1.1*, *C1.2*, *C1.3*) were downregulated.

Notably, *A6B.2* showed marked downregulation across multiple treatments. Within class B, all genes except *B1* exhibited no hormone responsiveness. Additionally, *A2.3*, *A4C*, *C1.1*, and *C1.3* were specifically induced by MeJA.

## Comprehensive expression profiling of *LpHSF* genes under abiotic stresses

The expression patterns of all 26 *LpHSF* genes were comprehensively profiled under various abiotic stresses, including heat, salt, cadmium (Cd), drought, and alkali treatments (Fig. 4). The qRT-PCR analysis under heat stress across four time points corroborated the transcriptomic data, establishing the reliability of the dataset. Beyond heat stress, the *LpHSF* family exhibited extensive transcriptional responsiveness to all other tested conditions.

Under salt stress, 15 *LpHSF*s were upregulated, comprising nine from subfamily A (*A1B*, *A2.1*, *A2.2*, *A3*, *A6B.1-A6B.5*), three from B (*B2A.1*, *B2A.2*, *B4.1*), and three from C (*C1.2–C1.4*). In contrast, five genes (*A2.3*, *A4*, *A4C*, *B4.3*, *C1.1*) were downregulated. Notable induction was observed for *A2.1*, *A3*, *B2A.2*, *C1.3*, and *C1.4*, which exhibited upregulation ranging from 3- to 200-fold, whereas *A4* and *B4.3* were the most significantly repressed.

Exposure to heavy metal (Cd) stress resulted in the upregulation of 13 *LpHSF*s, including *A2.1*, *A2.2*, *A2.5*, *A3*, and *A6B.5* from subfamily A; *B1*, *B2A.2*, *B4.1*, and *B4.2* from subfamily B; and *C1.2*, *C1.3*, and *C1.4* from subfamily C. Conversely, ten genes were downregulated, including *A1B*, *A2.3*, *A4*, *A4C*, *A5*, *A6B.1-A6B.3*, *B4.3*, and *C1.1*. Marked induction was observed for *A3*, *C1.3*, and *C1.4*, with expression peaks elevated by 6- to 100-fold, while *A4* was the most suppressed.

Under drought conditions, 15 genes were upregulated, including nine A-subfamily members (*A2.1*, *A2.2*, *A2.4*, *A2.5*, *A3*, *A6B.1*, *A6B.4*, *A6B.5*, *A7A*), three from the B-subfamily (*B2A.1*, *B2A.2*, *B4.1*), and three from the C-subfamily (*C1.1*, *C1.2*, *C1.4*). Eight genes were downregulated, including *A2.3*, *A4*, *A4C*, *A5*, *A6B.2*, *A6B.3*, *B1*, and *B4.3*. The expression of *A2.1* and *A3* was steadily induced, while *C1.4* showed the most dramatic upregulation, and *A2.3* was the most strongly repressed.

A distinct expression profile emerged under alkali stress, which triggered the most widespread upregulation, affecting 18 *LpHSF*s.

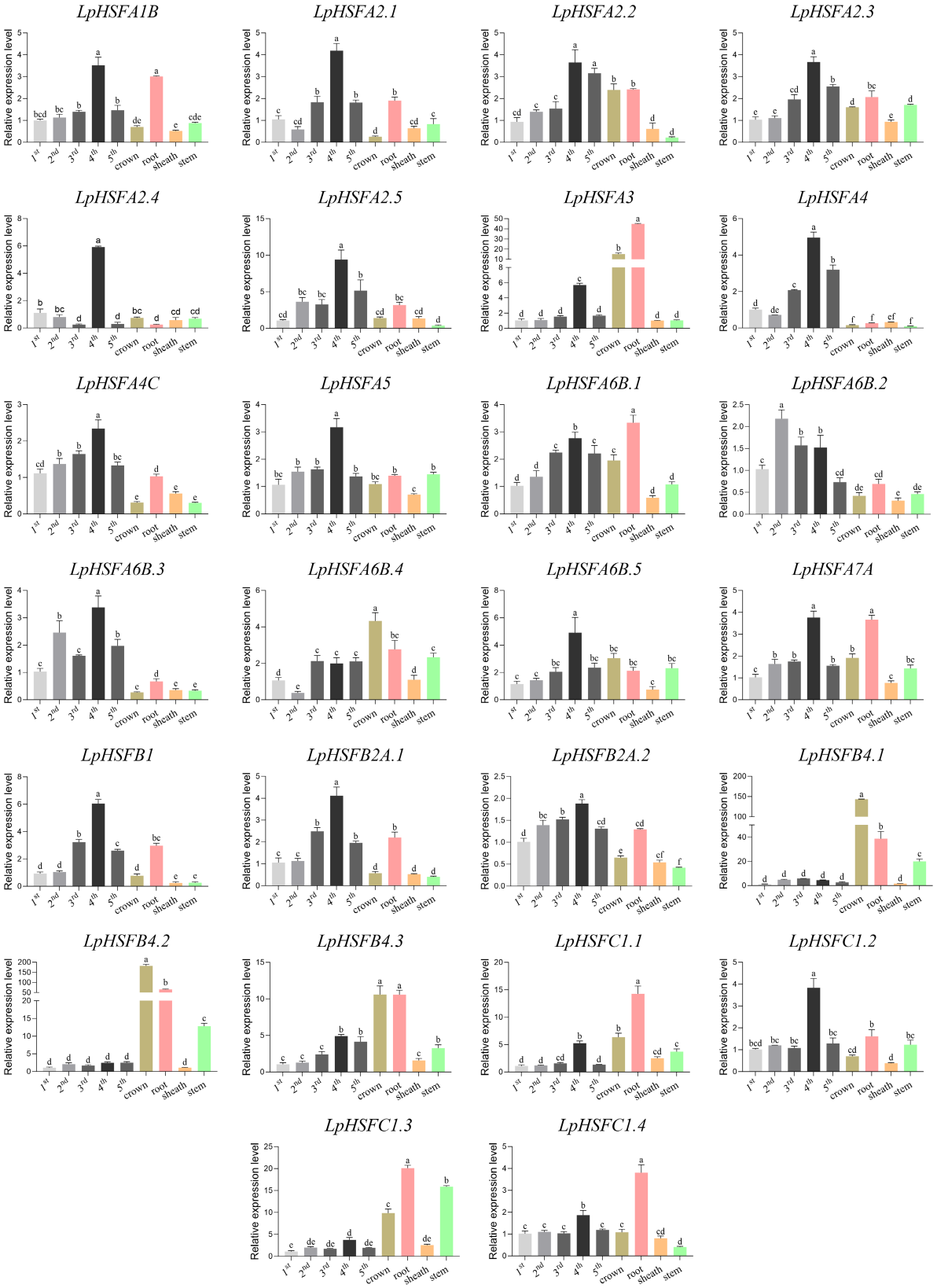


**Fig. 2** Expression patterns of *LpHSF* in different tissues. The statistical significance was determined using Fisher's protected least significant difference (LSD) test ($p < 0.05$).

These included 12 A-subfamily members (*A1B*, *A2.1*, *A2.2*, *A2.3*, *A2.5*, *A3*, *A5*, *A6B.1*, *A6B.3*, *A6B.4*, *A6B.5*, *A7A*), two from B-subfamily (*B1*, *B2A.2*), and all four C-subfamily members. Only five genes (*A4*, *A6B.2*, *B4.1*, *B4.2*, *B4.3*) were downregulated. The induction patterns of *A2.1*, *A3*, and *C1.4* under alkali stress were notably consistent with those observed under drought.

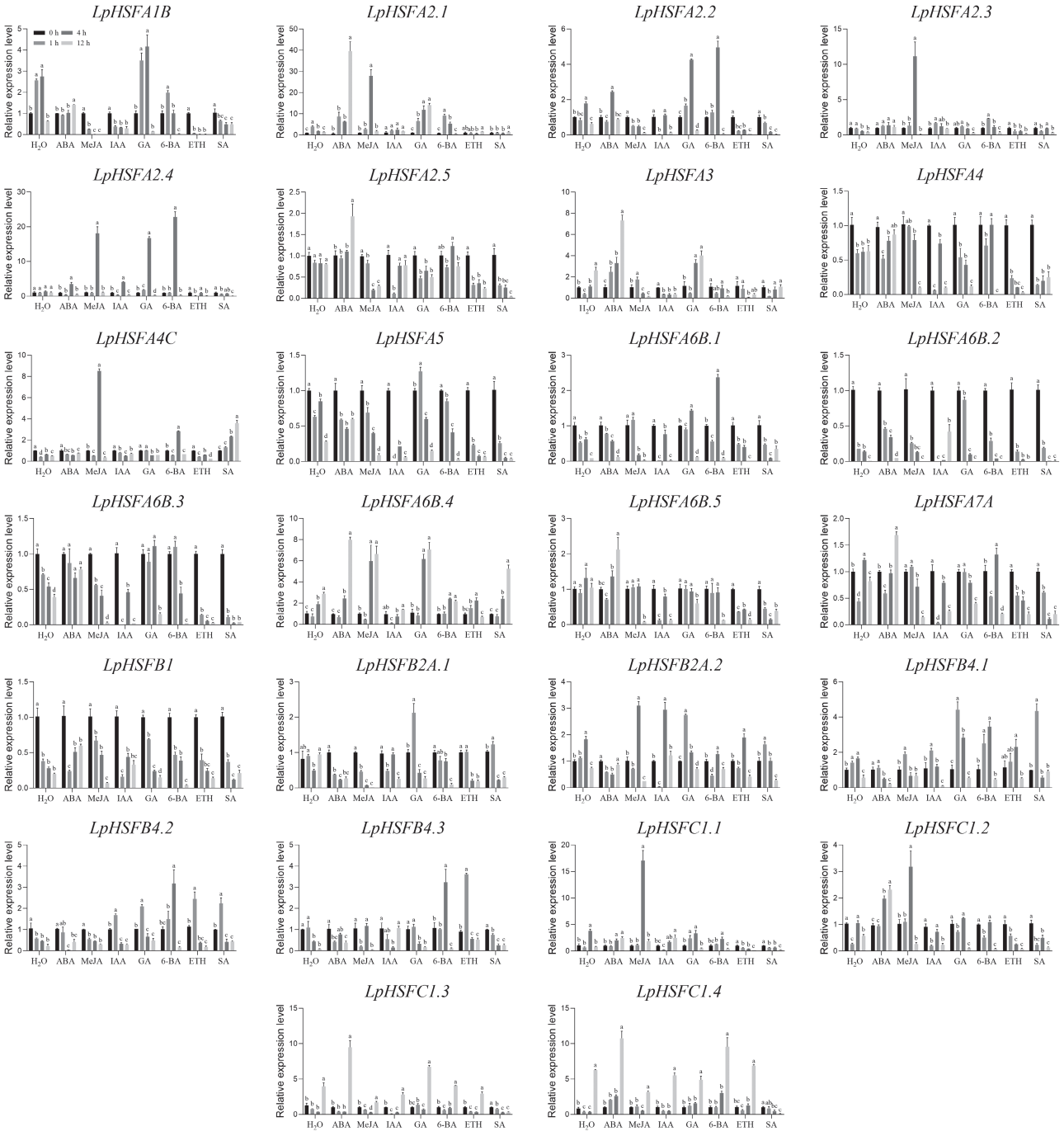


**Fig. 3** Expression profiles of 26 *LpHSF* genes in response to ABA, MeJA, IAA, GA, 6-BA, ETH, and SA treatments. The vertical bars indicate standard error. The statistical significance was determined using Fisher's protected least significant difference (LSD) test ($p < 0.05$).

# Discussion

Perennial ryegrass is a widely cultivated cool-season species valued both as forage and turfgrass. However, its productivity and survival are increasingly threatened by climate change-induced abiotic stresses, including extreme temperatures, salinity, alkalinity, and heavy metal toxicity. The heat shock transcription factor (*HSF*) family has been widely reported to play critical roles in plant responses to such stresses, positioning it as a key target for molecular breeding aimed at enhancing stress resilience[12,13,32]. Beyond their canonical role in thermotolerance, this study provides a comprehensive expression atlas of the *HSF* gene family in perennial ryegrass, revealing their functional diversity and tissue-specific regulation under multiple abiotic stresses, thereby underscoring their versatile roles in stress adaptation.

In this study, 26 *LpHSF* genes were identified from the perennial ryegrass genome. Promoter analysis revealed an abundance of hormone-responsive cis-elements, including those associated with abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid, auxin, and gibberellin. This composition is consistent with findings in *Dianthus caryophyllus*[33], *Phaseolus vulgaris*[34], and *Triticum aestivum*[35], suggesting an evolutionarily conserved role of *HSFs* in hormonal and abiotic stress signaling across plant species. The genome-wide identification and expression profiling further demonstrated that

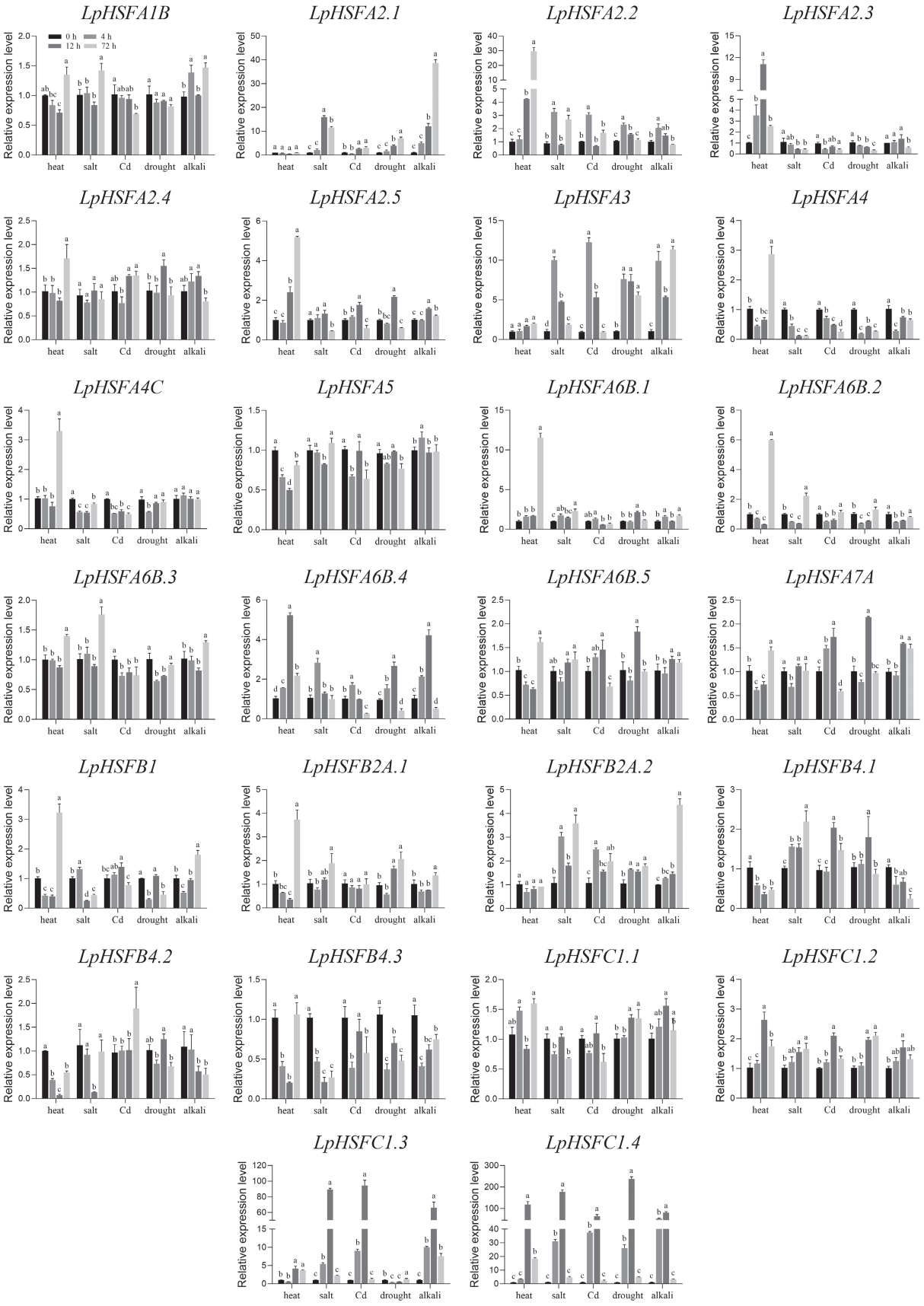


**Fig. 4** Expression profiles of 26 *LpHSF* genes under heat, salt, Cd, drought, and alkali stress. The vertical bars indicate standard error. The statistical significance was determined using Fisher's protected least significant difference (LSD) test ($p < 0.05$).

specific *LpHSF*s, particularly members of the *LpHSFA2* subclass, *LpHSFA3*, *LpHSFC1.3*, and *LpHSFC1.4*, exhibit broad-spectrum stress responsiveness beyond heat stress. For instance, *LpHSFA2.1* and *LpHSFC1.3* were significantly upregulated under salt stress, mirroring the function of *GmHSFB2b* in soybean, which acts as a positive regulator of salt tolerance[36]. Similarly, the induction of multiple *LpHSF*s under cadmium stress aligns with the role of *PvHSF16* in switchgrass as a positive regulator of cadmium tolerance[37]. Under heat stress, several Class A *LpHSF*s, including *LpHSFA2.2*, *LpHSFA2.3*, and *LpHSFA4*, were markedly upregulated, consistent with their established role as master regulators of the heat stress response. This functional parallel is observed in other species, such as *TaHSFA2h* in wheat[38] and *OsHSFA4d* in rice, which enhances thermotolerance by activating *HSP101*[39]. Thus, these heat-induced Class A *LpHSF*s likely serve as key positive regulators within the thermotolerance network of perennial ryegrass.

Class B *HSF*s have traditionally been regarded as transcriptional repressors[40], a view supported by their expression patterns under heat stress in this study. However, emerging evidence indicates functional divergence within this class. For example, *ZmHSF21* in maize acts as a positive regulator of cold tolerance[41], suggesting that Class B *HSF*s may exert both positive and negative regulatory effects depending on the species and stress type. Among Class C members, *LpHSFC1.4* responded to multiple stresses, including heat, salt, and alkali. In wheat, *TaHSFC2a* functions as a co-activator for Class A *HSF*s[38], whereas *OsHSFC1a* in rice acts by repressing a negative regulator[42]. This implies that stress-responsive Class C *HSF*s in ryegrass may operate either as transcriptional repressors or through the formation of heteromeric complexes with other *HSF*s. Collectively, the broad upregulation of *LpHSF*s under diverse stresses—such as salt, drought, alkali, and heavy metal exposure—aligns with recent studies in major crops including rice, maize, and wheat, underscoring the evolutionarily conserved role of *HSF*s as central regulators in plant abiotic stress networks.

Tissue-specific expression analysis revealed that most Class A *LpHSF*s were highly expressed in leaves, indicating a potential role in leaf development. In contrast, Class B and C members showed preferential expression in crowns and roots, suggesting specialized functions in below-ground organ development. Notably, many *LpHSF*s were also highly expressed in leaves across different developmental stages, implying a role in regulating leaf senescence. This observation is consistent with reports in rye (*Secale cereale* L.), where nearly half of the *HSF* members exhibit stage-specific expression[43]. In late-senescing barley genotypes, *HSF*s were significantly upregulated during late senescence stages, whereas no such enrichment was observed in early-senescing genotypes, indicating a positive correlation between *HSF* expression and leaf longevity[44]. Similarly, in bermudagrass, six *HSF*s were upregulated during dark-induced senescence, potentially facilitating the refolding of damaged proteins to maintain proteostasis[45]. In tall fescue, distinct functional specializations among *HSF* classes have been reported: Class A *HSF*s are rapidly induced under short-term heat stress and activate *HSP* expression to confer thermotolerance and delay heat-induced senescence; Class B members are also heat-induced but may participate in stress-senescence crosstalk through unclear mechanisms; and Class C *HSF*s are repressed by senescence, suggesting a role in modulating the antagonism between heat stress and senescence[46].

Previous studies have identified 16 *HSF* members in Italian ryegrass (*Lolium multiflorum* L.)[14]and 25 in perennial ryegrass[15]. While these studies primarily emphasized the role of *HSF*s in heat and drought responses, our time-course transcriptome analysis under heat stress revealed distinct temporal activation patterns among *LpHSF* members. Moreover, it was found that most *LpHSF*s are significantly induced by ABA and MeJA, highlighting their integration into hormone-mediated stress signaling pathways. Building on these findings, our promoter *cis*-element analysis elucidated the transcriptional regulatory basis of this response. The promoters were notably enriched with hormone-responsive motifs, particularly ABRE and MeJA elements. Strikingly, ABRE copy number exhibited a significant positive correlation with the level of heat-induced gene upregulation ($r = 0.45$, $p < 0.05$), functionally implicating this element in thermotolerance[47]. Similarly, the frequency of DRE elements correlated with transcriptional induction under both heat ($r = 0.49$, $p < 0.05$) and drought stress (Supplementary Table S4), suggesting its role as a signaling integrator[48]. Members such as *LpHSFA2.1* and *LpHSFA2.2*, which harbor multiple ABRE and DRE copies, showed pronounced induction under saline-alkali and heat stress (Fig. 5). This establishes a direct link between specific *cis*-regulatory architectures and the observed multi-stress expression profiles of key *LpHSF* genes.

In summary, the systematic expression profiling demonstrates that most *LpHSF* genes are transcriptionally regulated under diverse abiotic stresses and hormone treatments, reflecting the functional diversification of LpHSF proteins. This comprehensive analysis establishes a foundational resource for future investigations into the molecular mechanisms underlying stress adaptation in perennial ryegrass and supports the targeted selection of candidate genes for improving stress resilience through molecular breeding.

# Conclusions

This study provides the first comprehensive expression atlas of the *HSF* gene family in perennial ryegrass (Fig. 5), revealing its functional diversification beyond heat stress. The study demonstrates that specific *LpHSF*s, particularly from classes A and C, are transcriptionally regulated by a broad spectrum of abiotic stresses and hormonal treatments, and exhibit distinct tissue-specific expression patterns suggestive of roles in leaf and root development as well as senescence. These findings highlight the evolutionarily conserved role of *LpHSF*s as a central regulatory hub in stress adaptation and establish a crucial foundation for future functional studies aimed at molecular breeding for enhanced multi-stress resilience in perennial ryegrass.

## Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: Yu G; data collection: Li P, Zhou Y, Wang X; analysis and interpretation of results: Li P, Zhou Y, Wang X; draft manuscript preparation: Yu G, Li P; manuscript revision and review: Xie Z, Yu G, Zhang X; funding acquisition: Yu G, Zhang X. All authors reviewed the results and approved the final version of the manuscript.

## Data availability

All the LpHSF sequences were referenced in the NCBI genome (www.ncbi.nlm.nih.gov/datasets/genome/GCF_019359855.2/).

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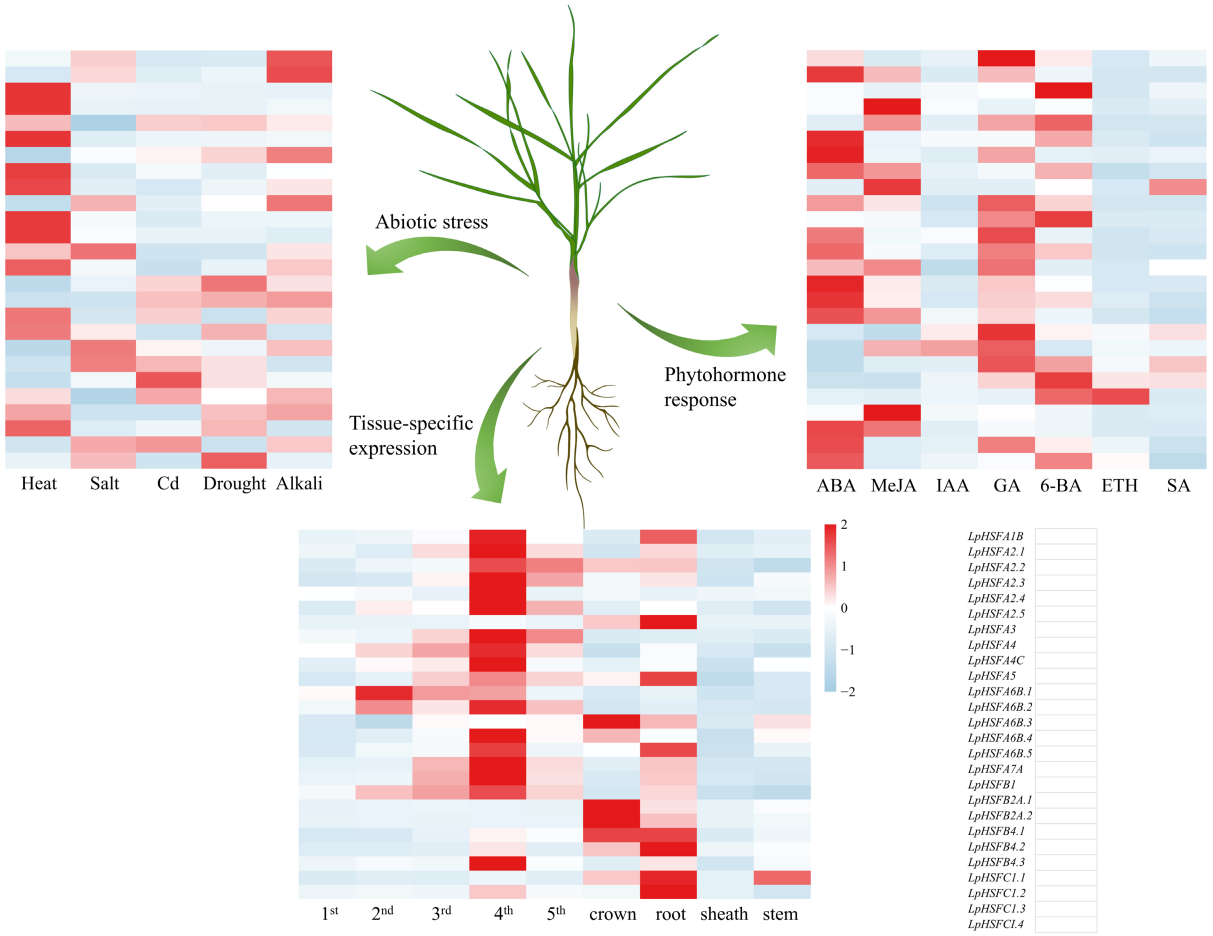


**Fig. 5** The expression profiles of the *LpHSF* gene family in perennial ryegrass under different abiotic stresses and hormone induction, and different tissues.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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