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Transcriptome alterations related to heat stress responses of wild and cultivated barley

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ABSTRACT

Heat stress, exacerbated by global warming, threatens food security by disrupting plant growth and productivity across many regions. The present study compared the transcriptome changes of heat-tolerant wild (*Hordeum vulgare* ssp. *spontaneum* L.) genotype and heat-sensitive cultivated *Hordeum* ('Mona' cultivar) barley subjected to control (24 ± 2 °C) and heat stress (40 ± 2 °C, 3 h) conditions via RNA sequencing with the Illumina Hiseq2500 platform. The wild barley genotype exhibited less impact from heat stress on growth and physiology than the 'Mona' cultivar. Heat stress led to 2141 differentially expressed genes (DEGs) in the heat-tolerant wild genotype and 1456 in the 'Mona' cultivar. Gene ontology enrichment analysis of the DEGs revealed that biological processes such as defense response to heat stress, proline and polyamine biosynthesis, and oxidative stress scavenging were predominantly involved in the thermo-tolerance of wild barley. Moreover, heat shock proteins, osmoprotectants, and catalytic activity were identified as the most critical molecular functions in response to high temperatures in wild barley. The significant alterations in the expression levels of candidate genes in response to heat stress highlight these genes' pivotal role in the thermo-tolerance of wild barley compared to the heat-sensitive 'Mona' cultivar. Comparing the evolved mechanisms in response to high temperatures between wild and cultivated barley helps identify the effective heat tolerance mechanisms in the thermo-tolerant wild genotype.

1. Introduction

Rising global temperatures due to significant climate change threaten agricultural production and, subsequently, food security (Hassan et al., 2021). Heat and drought stress, whether occurring individually or simultaneously, frequently impact many regions worldwide, significantly reducing cereal production (Vaezi et al., 2024). Heat stress perturbs multiple cellular activities by increasing cell membrane fluidity, generating reactive oxygen species (ROS), denaturing proteins and lipids, and inactivating enzymes involved in various metabolic pathways. These effects can lead to crop development and productivity issues (Chaudhary et al., 2020). Heat acclimation evolved in plants through complex mechanisms to cope with the effects of heat stress (Kan et al., 2023). The Ca^{2+} flows into the cytoplasm at high temperatures due to cell wall loosening. It binds directly to calcium-binding proteins, thereby inducing heat shock transcription factors (HSFs) in cells (Wu et al., 2018). Heat shock factors (HSFs) regulate heat shock proteins (HSPs), critical for cell survival and contribute to thermotolerance. Like other molecular chaperones, HSPs facilitate the stabilization, refolding, translocation, and degradation of endogenous proteins. Additionally, heat stress-induced accumulation of ROS releases HSFs from their chaperone complexes, thereby activating heat-responsive genes (Kan et al., 2023). *Hordeum vulgare* ssp. *spontaneum* L., a close ancestor of cultivated barley *(H. vulgare* ssp. *vulgare* L.), shares a remarkably high level of genome homology (Ellis et al., 2000; Arzani and Ashraf, 2016). Responses to abiotic stress in wild barley, assessed through morphological, physiological, and transcriptomic alteration assays, have revealed significant diversity in stress adaptation within the Iranian *H. spontaneum* germplasm (Ebrahim et al., 2020; Barati et al., 2019; Bahrami et al., 2019; Gharaghanipor et al., 2022). This diversity is further supported by our prior investigations into heat stress tolerance, focusing on photosynthesis and yield performance within this

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germplasm (Bahrami et al., 2019, 2021).

Transcriptome sequencing serves as a powerful tool for investigating transcriptional networks and identifying differentially expressed genes (DEGs) under diverse abiotic stress conditions (Mutz et al., 2013). Transcriptomic studies have revealed the molecular mechanisms behind heat tolerance in crops, highlighting the up-regulation of heat shock factors (HSFs), heat shock proteins (HSPs), antioxidants, osmoprotectants, and signaling pathways that work together to preserve cellular homeostasis. These findings have been reported in cereals such as maize (Shi et al., 2017), wheat (Su et al., 2019), and pearl millet (Sun et al., 2020). Genes involved in networking, such as HSPs and HSFs, play a crucial function in signal transduction, regulating heat-responsive genes (Cantalapiedra et al., 2017; Hassan et al., 2021). Poonia et al. (2020) found that overexpressing the *TaHsfA6b* gene (HSF) enhanced thermo-tolerance in cultivated barley by regulating major HSPs and antioxidant expression under heat stress. The accumulation of LEA proteins, proline, polyamines, and other protein protectants has been identified as an effective mechanism for maintaining cellular redox, enabling plants to survive under heat stress. Mishra et al. (2024) identified various DEGs, including HSFs, HSPs, antioxidant enzymes, Ca^{2+}-mediated signaling components, and ABC transporters, and confirmed their role in stress tolerance in barley.

To address the urgent need for improved heat tolerance in cereal crops in response to global warming and the limited understanding of molecular thermo-tolerance mechanisms in both cultivated and wild barley, this study aimed to: (*i*) compare the transcriptional responses to heat stress between heat-tolerant wild barley and a heat-sensitive barley cultivar, and (*ii*) identify potential gene candidates linked to thermo-tolerant wild genotype that could supply helpful information about the genetic mechanisms under heat stress in barley.

2. Materials and methods

2.1. Plant materials and heat treatment

This study utilized a wild barley genotype (*H. spontaneum*) originated from the salty shores of Urmia Lake in West Azarbaijan, Iran, known for its thermo-tolerance, alongside the heat-sensitive cultivar 'Mona'. The genetic materials used are derived from our previous experiments on the agro-morphological assessment of our wild barley collection and several barley cultivars, conducted under heat stress in field conditions over two growing seasons. (Bahrami et al., 2019, 2021). The seeds were germinated on moist filter paper in the Petri dish at 4 °C, after which the seedlings were transplanted into plastic pots under controlled temperature conditions, with a 16 h photoperiod with a PPFD of ~400 μmol m^{-2} s^{-1}, the temperatures of 24 ± 2 °C (day) and 18 ± 2 °C (night), and relative humidity of around 50%. Heat stress was applied by transferring half of the seedlings at the two-leaf stage to 40 ± 2 °C for 3 h over five consecutive days, while the remaining seedlings continued to grow under control conditions. Fresh leaves were harvested from 10 plants in each experimental unit and immediately frozen in liquid nitrogen for subsequent analysis.

2.2. Physiological measurement

Growth and physiological parameters, including shoot dry weight (DW), membrane stability index (MSI), the maximum quantum yield of the dark-adapted PSII photosystem (Fv/Fm), total chlorophyll content, and proline content, were evaluated using four replications of plant materials (more details are presented in Table S1). Analysis of variance for the traits was performed using the GLM procedure of SAS software, version 9.3 (SAS Institute, 2011). Furthermore, mean comparisons were conducted using Fisher's least significant difference (LSD) at a significance level of $p \leq 0.05$.

2.3. RNA isolation and cDNA library construction

Fresh leaf samples were used for RNA isolation from wild and cultivated genotypes under control and heat stress conditions, with two biological replicates for each treatment. Total RNA was extracted using the RNA Isolation Kit (Roche, Germany) according to the manufacturer's protocol. A NanoDrop 8000 spectrophotometer evaluated RNA quality and quantity. High-quality RNA samples for RNA sequencing were sent to Macrogen Company, Seoul, South Korea. Paired-end complementary DNA (cDNA) sequencing libraries with an average insert size of 151 bp were prepared from RNA samples using the TruSeq Stranded mRNA LT Sample Prep Kit and sequenced on the Illumina HiSeq2500, following the manufacturer's instructions (Illumina Part # 15031047 Rev. E).

2.4. Analysis of DEGs

RNA-seq data were utilized for transcriptome analyses, incorporating heat-tolerant and heat-sensitive genotypes grown under both control and heat-stress conditions, with two biological replicates for each treatment. After quality control using FastQC1, raw reads from eight samples were trimmed by removing the reads containing adaptor contamination and filtering the low-quality (Phred quality score ≥30) and short reads (≤60 bp) using Trimmomatic software (v.0.39; Bolger et al., 2014). Subsequently, the clean reads were aligned to the barley genome reference Morex V3 (http://plants.ensembl.org/Hordeum_vulgare/Info/Index?db=core) using the STAR software tool (Dobin et al., 2013). After determining the gene and isoform expression by RSEMv1.3.2 (Li and Dewey, 2011), expression values were estimated with Cufflinks v. 2.2.1 based on fragments per kilobase of transcript per million mapped reads (FPKM). Statistical analysis of transcriptome data was then carried out using the DESeq2 package in R (Love et al., 2014). Significantly differentially expressed genes were filtered based on FDR-adjusted *p*-value ≤0.05 with $|\log_2 FC| \geq 2$ for further analysis.

2.5. Functional annotation, gene ontology, and pathway enrichment analyses

To maximize the functional annotation of DEGs, two databases—Ensemble Plants (https://plants.ensembl.org/index.html) and Barlex (https://apex.ipk-gatersleben.de/apex/f?p=284:57)—were used in this study. GO analysis was carried out using the g: Profiler database (https://biit.cs.ut.ee/gprofiler/) on four groups of DEGs, with the Benjamini-Hochberg FDR test applied at a *p*-value threshold of ≤0.05 to describe biological processes, molecular functions, and cellular components (Raudvere et al., 2019). The annotated genes were aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database using the online KEGG Automatic Annotation Server (KAAS: https://www.genome.jp/kegg/kaas).

2.6. Quantitative real-time PCR (RT-qPCR)

To validate the RNA sequencing data, the relative expression of four candidate genes — *AvHSP20*, *HvHsp90-6*, *HvBip-70*, and *Dhn7*—was assessed; they were up-regulated in the heat-tolerant wild genotype under heat stress. The four specific pairs of primers designed using Primer 3 software (http://primer3.ut.ee) are listed in Table 1. Total RNA was isolated from wild and cultivated barley leaf samples grown under control and heat stress conditions, including two biological and two technical replicates, using the RNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For each sample, cDNA was synthesized from the isolated RNA using the Thermo Revert Aid™ First Strand cDNA Synthesis Kit. The RT-qPCR was performed using the RealQ Plus 2x Master Mix Green –Ampliqon kit to measure the relative expression of four candidate genes, including *AvHSP20*, *HvHsp90-6*, *HSP70*, and *Dhn7*, which showed up-regulation in the heat-tolerant wild genotype exposed to heat stress, as identified in our

Table 1
Primer sequences employed for RT-qPCR amplification in barley.

Gene candidate	Gene name	Description	Forward primer sequence (5′–3′)	Reveres primer sequence (5′–3′)
HORVU.MOREX.r3.3HG0226140	*AvHSP20*	HSP20-like chaperone	CAGACAACAACCTACCTACG	GGTGTCGAACGGGTCAG
HORVU.MOREX.r3.5HG0447870	*HvHsp90-6*	Heat shock protein 90-6, mitochondrial	TACTTGGCATTTCTTGTAGTGA	GGACTTCTGCTCTGGTTTC
HORVU.MOREX.r3.6HG0545620	*HvBip-70*	Heat shock protein 70kD, peptide-binding domain superfamily	TACCACCTACTCATGTGTCG	TCCTTGTCCTCAAACTTTCTTC
HORVU.MOREX.r3.6HG0622790	*Dhn7*	Dehydrin 7	AGGGCATCATGGACAAAATC	GCTCACTTCATTTCGGGAAG
Reference gene	*β-tubulin*	Constitutive proteins of microtubules	GTCCACCCACTCCCTCCTTG	CGGCGGCAGATGTCATAGATG

transcriptomic analysis of the DEGs. The five specific pairs of primers used in this study are listed in Table 1. The *β-tubulin* gene was used as an internal reference for normalizing gene expression in the RT-qPCR reactions. Each gene's relative expression level was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The significant differences in transcript expression between wild and cultivated barley genotypes relative to the control were determined using the Mann–Whitney *U* test.

3. Results

3.1. Growth and physiological traits

High temperature resulted in a significant decrease in shoot DW, MSI, Fv/Fm, and total chlorophyll content. In contrast, proline content increased in barley seedlings (Fig. 1). In response to heat stress, DW was reduced by 43% in the wild genotype and by 74% in the cultivated genotype. Moreover, cultivated barley exhibited greater membrane leakage, as indicated by the more significant reduction in MSI (66.54%) compared to the wild genotype in response to heat stress. Although both genotypes revealed no significant difference in Fv/Fm and total chlorophyll content under control conditions, wild barley exhibited a lesser reduction in the mentioned parameters when exposed to heat stress. In contrast, leaf proline content increased in response to heat stress, with the highest accumulation (up to 70%) observed in wild barley.

3.2. Transcriptome analysis of heat tolerant and heat sensitive genotypes

A deep RNA-seq analysis was performed on the transcriptome profiles of wild and cultivated barley leaf samples under control and heat stress conditions. An average of 46 million clean reads, ranging from 39 to 55 million read pairs, were obtained from the paired-end reads of the eight libraries (Table S2). High-quality trimmed reads were aligned with the reference barley genome sequence. Over 73% of clean reads from wild genotype RNA-seq data were uniquely mapped to the reference barley sequence. A mapping rate of 75–90% was achieved from the transcriptome data of the 'Mona' cultivar (Table S2).

The DEGs were analyzed to compare gene expression between the wild genotype (tolerant) and the 'Mona' cv. (sensitive) under control and heat stress treatments. In wild barley, 972 up-regulated and 1169 down-regulated genes were differentially expressed in response to heat stress. On the other hand, heat stress in the 'Mona' cv. resulted in the up-regulation of 426 and down-regulation of 1030 genes (Fig. 2a). A total of 449 DEGs were common between the tolerant and sensitive genotypes in this study. Fig. 2b and c presents frequency histograms of DEGs identified in the two genotypes, illustrating the distribution of fold changes in gene expression. Most DEGs exhibited a fold change between 2 and 3 in both genotypes. In the heat-tolerant wild barley, 40 DEGs showed an expression fold change greater than 8, whereas only eight DEGs surpassed this threshold in the heat-sensitive barley.

3.3. Gene ontology and KEGG enrichment analysis of DEGs

The GO enrichment analysis was carried out to classify the total DEGs that was significantly enriched (Benjamini-Hochberg $p \leq 0.05$) in response to heat stress across various biological processes, molecular functions, and cellular components (Table 2). In response to heat stress, 157 GO terms were significantly enriched for up-regulated genes in heat-tolerant barley, while 67 GO terms were significantly enriched in heat-sensitive barley. A total of 2099 down-regulated genes in wild and cultivated barley were significantly categorized into 202 and 196 GO terms, respectively.

In response to heat, catalytic activity (GO:0003824) and oxidoreductase activity (GO:0016491) were the top molecular functions of uniquely upregulated genes in the heat-tolerant genotype (Fig. 3). Moreover, the most significant biological processes identified in response to heat (GO:0009408), protein folding (GO:0006457), response to temperature stimulus (GO:0009266), and response to abiotic stimulus (GO:0009628) were observed in tolerant wild barley (Fig. 3). Unfolded protein binding (GO:0051082), response to heat (GO:0009408), and cellular component (GO:0005575) were the top three functional groups enriched among the uniquely up-regulated genes in the sensitive genotype (Fig. 4). The down-regulated genes in the tolerant genotype were significantly enriched for catalytic activity (GO:0003824), biological process (GO:0008150), and plastid and chloroplast (GO:0009536 and GO:009507) in response to high temperature

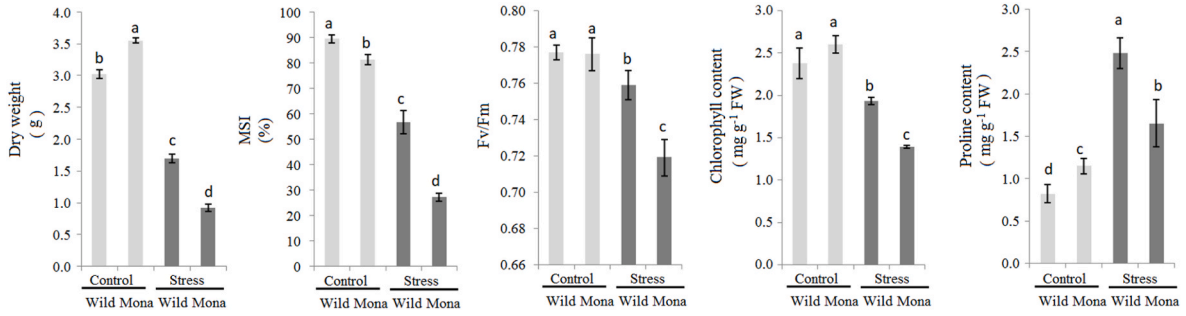


Fig. 1. Mean comparisons of growth and physiological traits of tolerant and sensitive barleys under control and heat stress conditions.
Bars represent means ± SE and bars with the same letter do not significantly differ at $p < 0.05$ among the genotypes grown under control and heat stress.

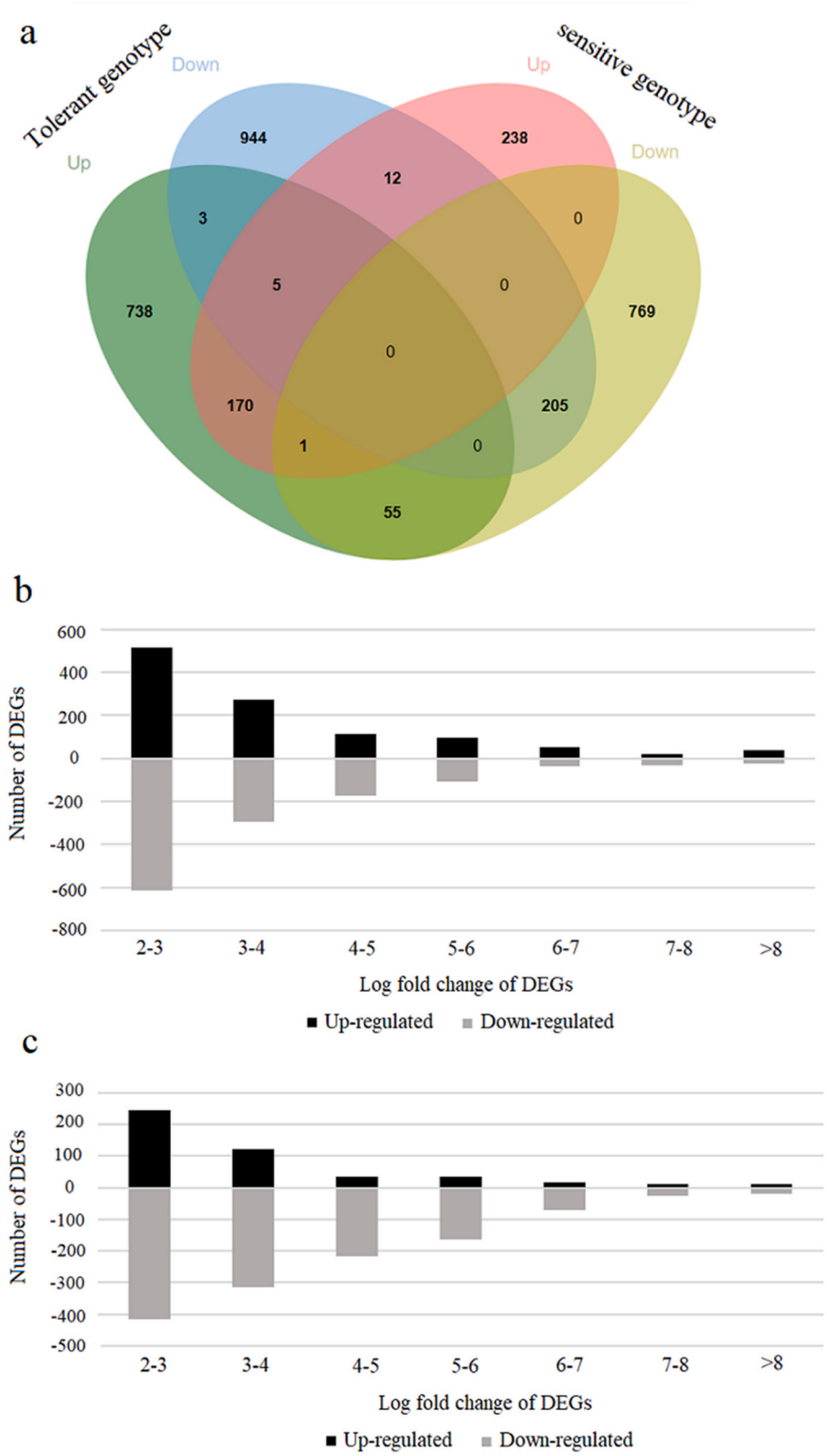


Fig. 2. (a) Venn diagrams showing the number of differentially expressed genes (DEGs) found in two barley subspecies (wild genotype and 'Mona' cultivar). Histogram of the fold change in expression of the DEGs (Log2 FC) of two barley subspecies: (b) wild genotype and (c) 'Mona' cultivar.

(Fig. 5). Cell wall organization or biogenesis (GO:0071554), catalytic activity (GO:0003824), and extracellular region (GO:0005576) were the top three processes among the down-regulated genes in the sensitive genotype (Fig. 6).

The four DEG groups were subjected to KEGG analysis to determine their roles in heat adaptation. In the wild genotype, out of 928 genes assigned to KEGG database pathways, 435 up-regulated and 493 down-regulated DEGs were mapped into 252 and 262 pathways, respectively (Fig. 7a and b). Metabolic pathways (KO 01100), biosynthesis of secondary metabolites (KO 01110), protein processing in the endoplasmic

Table 2
Unique enriched functional categories of DEGs wild and cultivated barley in response to heat stress annotating by GO term.

GO	Term name	Term ID	Term size	p-value (-Log10)	Intersection size
Wild barley: uniquely up-regulated DEGs					
MF	catalytic activity	GO:0003824	10390	13/929	404
BP	response to heat	GO:0009408	68	16/937	24
CC	intrinsic component of the membrane	GO:0031224	3878	5/770	162
Wild barley: uniquely down-regulated DEGs					
MF	catalytic activity	GO:0003824	10390	15/765	480
BP	biological_process	GO:0008150	15839	13/645	659
CC	plastid	GO:0009536	939	31/755	115
Cultivated barley: uniquely up-regulated DEGs					
MF	unfolded protein binding	GO:0051082	73	3/047	8
BP	Response to heat	GO:0009408	68	15/983	17
CC	cellular_component	GO:0005575	10651	4/105	174
Cultivated barley: uniquely down-regulated DEGs					
MF	catalytic activity	GO:0003824	10390	12/568	418
BP	cell wall organization or biogenesis	GO:0071554	310	16/868	47
CC	extracellular region	GO:0005576	682	26/468	84

reticulum (KO 04141), biosynthesis of amino acids (KO 01230), glutathione metabolism (KO 00480), and microbial metabolism in diverse environments (KO 01120) were the top pathways enriched in the up-regulated genes of wild barley. In response to heat stress, 190 up-regulated DEGs were mapped to 155 pathways, while 423 down-regulated DEGs were assigned to 223 KEGG pathways in cultivated barley (Fig. 7c and d). The key pathways associated with up-regulated genes in cultivated barley were enriched in metabolic pathways (KO 01100), biosynthesis of secondary metabolites (KO 01110), and protein processing in the endoplasmic reticulum (KO 04141). In cultivated barley, out of the 223 KEGG pathways found for down-regulated DEGs, the top pathways in response to heat were metabolic pathways (KO 01100), biosynthesis of secondary metabolites (KO 01110), amino sugar and nucleotide sugar metabolism (KO 00520), and phenylpropanoid biosynthesis (KO 00940).

3.4. Candidate genes for heat tolerance

The DEGs identified under control and heat stress conditions can be further compared between wild and cultivated genotypes to gain additional insights into the heat resilience of DEGs in wild barley. Table 3 highlights key functionally assigned candidate genes linked to heat tolerance in barley, emphasizing the statistical significance of their expression differences. Among the identified DEGs, the five most significant groups include heat shock proteins and factors, osmotic regulators, ROS inhibitors, genes involved in calcium (Ca^{2+}) signaling, and ABC transporter genes, which are discussed in detail below.

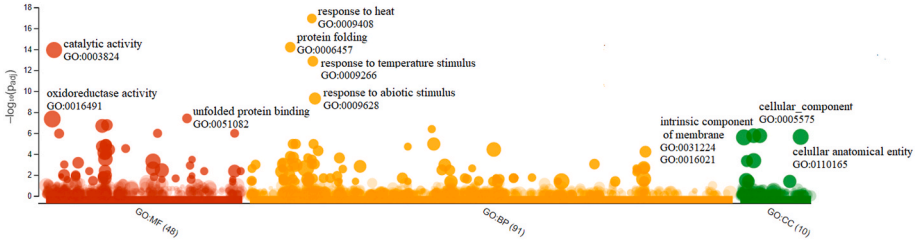


Fig. 3. GO enrichment analysis up-regulated differentially expressed genes (DEGs) in the wild barley using the g: Profiler database Benjamini-Hochberg FDR test with a *p*-value ≤0.05 as a threshold.

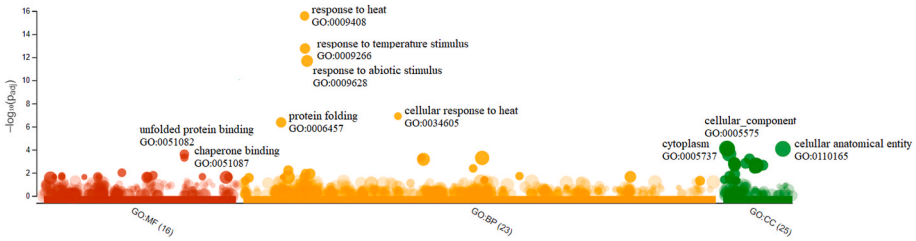


Fig. 4. GO enrichment analysis up-regulated differentially expressed genes (DEGs) in the 'Mona' cultivar using the g: Profiler database Benjamini-Hochberg FDR test with a *p*-value ≤0.05 as a threshold.

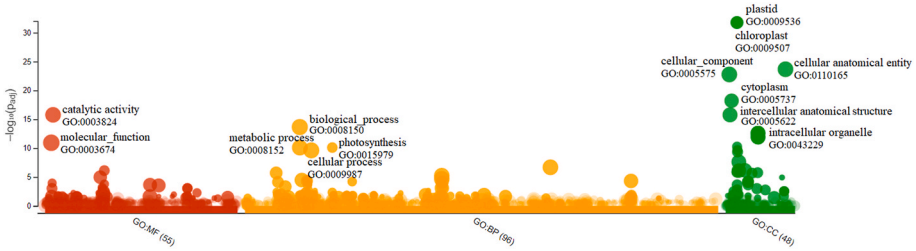


Fig. 5. GO enrichment analysis down-regulated differentially expressed genes (DEGs) in the wild barley using the g: Profiler database Benjamini-Hochberg FDR test with a *p*-value ≤0.05 as a threshold.

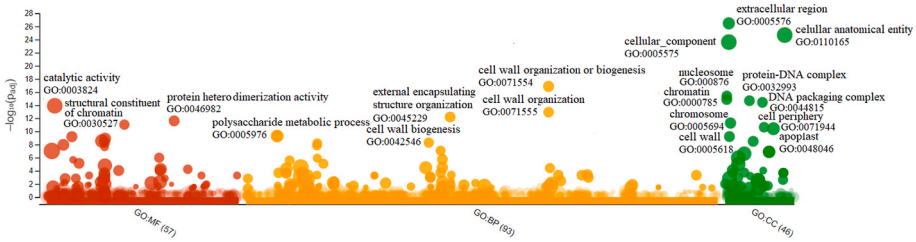


Fig. 6. GO enrichment analysis down-regulated differentially expressed genes (DEGs) in the 'Mona' cultivar using the g: Profiler database Benjamini-Hochberg FDR test with a *p*-value ≤0.05 as a threshold.

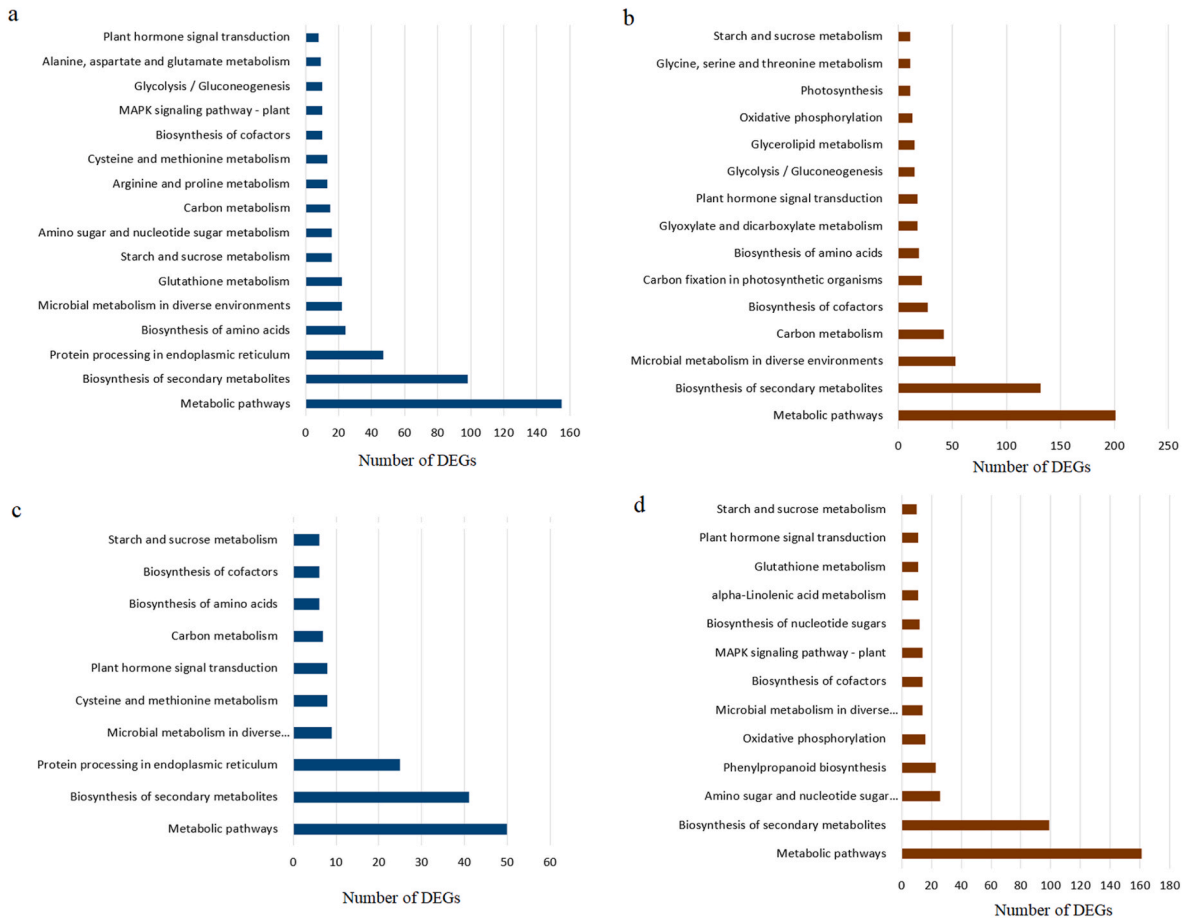


Fig. 7. KEGG pathway classification of the differentially expressed genes (DEGs) (a) up-regulated and (b) down-regulated in the wild genotype; (c) up-regulated and (d) down-regulated in the 'Mona' cultivar grown under control and heat stress conditions.

3.4.1. Genes encoding protection proteins

In the wild genotype, a total of 59 genes associated with protective proteins were up-regulated in response to heat stress. In this study, the small HSP (*sHSP*) genes, *HSP70*, *HSP90,* and *HSP100* gene families, as well as chaperone proteins 60 kDa, ClpB, DnaK, DnaJ, and htpG exhibited significantly higher expression levels under heat stress compared to normal conditions (Table 3). *HvHsp70Mt70-3*, *HvHsp90-6*, and *HvBip-1*, along with 15 heat shock and chaperone genes, were uniquely expressed in the wild barley in response to heat stress (Table 3). The heat stress triggered over 10-fold increase (log2 FC ≥ 10) in the expression of eight HSP transcripts in wild heat-tolerant barley. Additionally, the HvHsp100-1, HvHsp100-2, and HvHsp100-3 genes were up-regulated in response to heat stress across both barley genotypes. Five *HSF* genes, including *HvHSFC-2a* and *HsfC2b*, were triggered by heat stress, particularly in the tolerant genotype, while the *HsfAc2* gene was significantly up-regulated in both genotypes (Table 3).

3.4.2. Genes involved in redox homeostasis maintenance and accumulation of compatible solutes

Compared to the wild genotype, the 'Mona' cultivar showed either no change or down-regulation in the expression of LEA protein genes in response to heat stress. The *HvLEA protein18* was the only LEA gene induced by heat stress in both genotypes (Table 3). Notably, in response to heat stress, the most up-regulated LEA protein genes in wild barley were linked to integral membrane components and anchored elements of the plasma membrane.

Transcripts associated with proline accumulation, *HvP5CS1* and *HvP5cr3,* were significantly induced under heat stress in wild barley, while their differential expression was insignificant in the cultivar (Table 3). Both barleys had significantly elevated expression of *HvP5CSB* in response to heat. Sugars will eventually be exported transporter (SWEET) genes, including *HvSWEET15a*, *HvSWEET3*, and *HvSWEET1*, which were up-regulated in the wild heat-tolerant barley but down-

Table 3
Functional annotations of some of the differentially expressed genes in wild (tolerant) and cultivated (sensitive) genotypes of barley grown under control and heat stress conditions.

Gene ID	Gene function	Gene symbol	Wild barley (Log2)	Cultivated barley (Log2)
HORVU.MOREX.r3.1HG0028320	70 kDa heat shock protein	*HvHsp110-4*	4.48**	2.15**
HORVU.MOREX.r3.1HG0032990	Chaperone DnaK		2.74**	0.52^{ns}
HORVU.MOREX.r3.1HG0040610	60 kDa chaperonin		3.02**	−0.21^{ns}
HORVU.MOREX.r3.1HG0073350	70 kDa heat shock protein	*HvHspc70-5a*	7.53**	6.89**
HORVU.MOREX.r3.1HG0077030	ATP-dependent chaperone ClpB	*HvHsp100-1/ClpB1-Cyt*	8.22**	8.63**
HORVU.MOREX.r3.2HG0170930	Heat-shock protein, putative		11.44**	5.17[a]
HORVU.MOREX.r3.2HG0210630	Heat shock protein		4.80**	2.41^{ns}
HORVU.MOREX.r3.3HG0225040	Heat shock protein		9.25**	5.39[a]
HORVU.MOREX.r3.3HG0226110	Heat shock protein		15.21**	6.62**
HORVU.MOREX.r3.3HG0226120	Heat shock protein		9.30**	1.51^{ns}
HORVU.MOREX.r3.3HG0226140	Heat-shock protein, putative	*AvHSP20*	11.33**	5.22**
HORVU.MOREX.r3.3HG0237580	Heat-shock protein, putative		7.26**	4.91**
HORVU.MOREX.r3.3HG0237670	Heat-shock protein, putative		9.99**	5.85**
HORVU.MOREX.r3.3HG0237690	Heat shock protein		5.24**	1.22^{ns}
HORVU.MOREX.r3.3HG0237670	Heat-shock protein, putative		11.87**	8.51**
HORVU.MOREX.r3.3HG0237680	Heat shock protein		9.78**	4.89**
HORVU.MOREX.r3.3HG0288370	Chaperone clpb, putative	*HvHsp100-2/ClpB2-Cyt*	6.57**	3.75[a]
HORVU.MOREX.r3.3HG0302810	70 kDa heat shock protein	*HvHspc70-5b*	5.52**	5.10**
HORVU.MOREX.r3.4HG0344210	Heat-shock protein, putative		10.58**	5.01**
HORVU.MOREX.r3.4HG0384590	Chaperone protein DnaJ		3.59**	3.85[a]
HORVU.MOREX.r3.4HG0387610	70 kDa heat shock protein	*HvHspc70-4*	9.69**	6.44**
HORVU.MOREX.r3.4HG0387870	70 kDa heat shock protein	*HvHspc70-2*	3.08[a]	3.14[a]
HORVU.MOREX.r3.4HG0389360	Heat-shock protein, putative		8.55**	5.86**
HORVU.MOREX.r3.4HG0389370	Heat-shock protein		12.85**	8.95**
HORVU.MOREX.r3.4HG0392240	Heat shock protein, putative		15.31**	9.06**
HORVU.MOREX.r3.4HG0416940	Chaperone DnaK	*HvHsp70Mt70-2*	5.51**	3.26[a]
HORVU.MOREX.r3.4HG0418530	ATP-dependent chaperone ClpB	*HvHsp100-4/ClpB-C*	3.51**	2.31[a]
HORVU.MOREX.r3.5HG0447870	Chaperone protein htpG family protein	*HvHsp90-6*	2.48**	0.44^{ns}
HORVU.MOREX.r3.5HG0488480	Chaperone protein htpG family protein		3.49**	0.57^{ns}
HORVU.MOREX.r3.5HG0491130	Chaperone DnaK	*HvHsp70Mt70-3*	2.64**	1.78^{ns}
HORVU.MOREX.r3.5HG0517090	Chaperone protein dnaJ, putative		3.08**	3.19**
HORVU.MOREX.r3.6HG0540200	60 kDa chaperonin		2.53**	−0.40^{ns}
HORVU.MOREX.r3.6HG0545620	Heat shock 70 kDa protein	*HvBip-70*	2.82**	−0.71^{ns}
HORVU.MOREX.r3.6HG0568470	ATP-dependent chaperone ClpB		4.85**	2.86**
HORVU.MOREX.r3.6HG0578520	Small heat-shock protein, putative		4.83[a]	1.08^{ns}
HORVU.MOREX.r3.6HG0616950	Heat-shock protein, putative		11.75**	3.51[a]
HORVU.MOREX.r3.6HG0621200	Heat-shock protein, putative		8.90**	8.26**
HORVU.MOREX.r3.7HG0641550	Chaperone protein dnaJ		2.85**	1.09[a]
HORVU.MOREX.r3.7HG0664900	Heat-shock protein, putative		6.00**	0.84^{ns}
HORVU.MOREX.r3.7HG0664900	Heat-shock protein, putative		4.48**	−1.3 ^{ns}
HORVU.MOREX.r3.7HG0664850	SHSP domain-containing protein		5.48**	2.46 ^{ns}
HORVU.MOREX.r3.7HG0664840	Heat-shock protein, putative		4.59**	0.24 ^{ns}
HORVU.MOREX.r3.7HG0678460	Heat shock protein 90		5.49**	2.77**
HORVU.MOREX.r3.7HG0746770	Chaperone protein htpG family protein		2.59**	−1.10[a]
HORVU.MOREX.r3.7HG0669480	Heat-shock protein, putative		9.43**	7.28**
HORVU.MOREX.r3.1HG0083950	Heat shock transcription factor	*HsfAc2*	2.45**	3.08**
HORVU.MOREX.r3.4HG0402170	Heat shock transcription factor		0.26^{ns}	2.16**
HORVU.MOREX.r3.4HG0417910	Heat shock transcription factor	*HvHSF C-2a*	3.13**	1.84 ^{ns}
HORVU.MOREX.r3.5HG0486440	Heat shock transcription factor		2.64**	1.37 ^{ns}
HORVU.MOREX.r3.5HG0497990	Heat shock transcription factor		4.35**	5.47**
HORVU.MOREX.r3.7HG0686980	Heat shock transcription factor		4.73**	0.38 ^{ns}
HORVU.MOREX.r3.7HG0717290	Heat shock transcription factor		3.36[a]	0.60 ^{ns}
HORVU.MOREX.r3.7HG0718540	Heat shock factor C2b, Predicted protein	*HsfC2b*	2.01[a]	−2.93**
HORVU.MOREX.r3.1HG0046460	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein		2.26[a]	0.72 ^{ns}
HORVU.MOREX.r3.1HG0031210	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	*LEA-2*	2.00**	−2.2**
HORVU.MOREX.r3.1HG0038750	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family		−1.52^{ns}	−2.5**
HORVU.MOREX.r3.2HG0135210	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family		1.88^{ns}	−7.1**
HORVU.MOREX.r3.2HG0140470	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family		4.95**	1.97 ^{ns}
HORVU.MOREX.r3.2HG0167300	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family		−1.63^{ns}	−6.00**
HORVU.MOREX.r3.2HG0191620	Late embryogenesis abundant family protein		6.89**	0.47 ^{ns}
HORVU.MOREX.r3.2HG0209950	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein		3.83[a]	1.02 ^{ns}
HORVU.MOREX.r3.5HG0527260	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family		1.72^{ns}	−6.30**
HORVU.MOREX.r3.6HG0569090	Late embryogenesis abundant hydroxyproline-rich glycoprotein family	*LEA-2*	3.27[a]	−1.61[a]
HORVU.MOREX.r3.7HG0637810	Seed maturation protein	*HvLEA18*	2.59**	3.71[a]
HORVU.MOREX.r3.1HG0073870	Delta-1-pyrroline-5-carboxylate synthase	*HvP5CS1*	2.32[a]	0.63 ^{ns}
HORVU.MOREX.r3.3HG0319810	Pyrroline-5-carboxylate reductase	*HvP5cr3*	2.78**	−0.10 ^{ns}
HORVU.MOREX.r3.3HG0302060	Delta-1-pyrroline-5-carboxylate synthase	*HvP5CSB*	3.53**	2.70[a]
HORVU.MOREX.r3.1HG0031940	Bidirectional sugar transporter SWEET	*HvSWEET3*	2.13**	−1.50[a]
HORVU.MOREX.r3.1HG0081000	Aldehyde dehydrogenase	*HvALDH12A1*	2.46**	−1.00 ^{ns}
HORVU.MOREX.r3.7HG0648500	Sugar transporter 1	*HvSWEET1*	4.10**	−0.10 ^{ns}
HORVU.MOREX.r3.7HG0658900	Bidirectional sugar transporter SWEET	*HvSWEET15a*	4.70**	−1.63 ^{ns}
HORVU.MOREX.r3.2HG0100060	Arginase		2.27**	0.01^{ns}

HORVU.MOREX.r3.2HG0200920	Agmatine coumaroyltransferase-2		3.12**	−1.10 ns
HORVU.MOREX.r3.5HG0501420	Ornithine decarboxylase	*HvODC1*	4.66[a]	−0.10 ns
HORVU.MOREX.r3.5HG0501580	Agmatine coumaroyltransferase-2		4.27[a]	−0.31 ns
HORVU.MOREX.r3.7HG0708730	Spermidine synthase	*HvSPMS2*	2.66**	−0.50 ns
HORVU.MOREX.r3.7HG0720280	Polyamine oxidase	*HvPAO2*	5.18**	1.75[a]
HORVU.MOREX.r3.1HG0017720	Glutathione S-transferase		3.90**	1.21[a]
HORVU.MOREX.r3.1HG0051750	Glutathione S-transferase		4.77**	1.18 ns
HORVU.MOREX.r3.1HG0051870	Glutathione S-transferase	*Tau GST*	2.99**	2.96[a]
HORVU.MOREX.r3.1HG0051860	Glutathione S-transferase		3.95**	−0.70 ns
HORVU.MOREX.r3.1HG0051890	Glutathione S-transferase		2.80**	0.48 ns
HORVU.MOREX.r3.1HG0051880	Glutathione S-transferase		5.30**	0.64 ns
HORVU.MOREX.r3.1HG0051910	Glutathione S-transferase	*GSTU6*	3.71**	−1.80 ns
HORVU.MOREX.r3.2HG0139310	Glutathione S-transferase		2.01[a]	−1.51[a]
HORVU.MOREX.r3.2HG0214680	Glutathione S-transferase		4.79**	−0.60 ns
HORVU.MOREX.r3.3HG0281500	Glutathione S-transferase		4.23**	0.99 ns
HORVU.MOREX.r3.3HG0291040	Glutathione S-transferase		3.09[a]	0.91 ns
HORVU.MOREX.r3.5HG0516720	Glutathione S-transferase		3.52**	1.43**
HORVU.MOREX.r3.7HG0648120	Glutathione S-transferase		4.51**	0.24 ns
HORVU.MOREX.r3.7HG0737960	Glutathione-S-transferase		2.48**	−2.10[a]
HORVU.MOREX.r3.2HG0153470	Ferredoxin–NADP reductase		5.49**	2.14**
HORVU.MOREX.r3.3HG0300590	Ferredoxin-3	*HvFdx3*	6.86**	0.32 ns
HORVU.MOREX.r3.5HG0530190	Ferredoxin-3	*HvFdx3*	3.07**	0.47 ns
HORVU.MOREX.r3.2HG0115300	Superoxide dismutase [Cu-Zn]	*HvSOD1*	2.00**	−0.31 ns
HORVU.MOREX.r3.5HG0484640	Superoxide dismutase [Cu-Zn]		2.58[a]	−0.30 ns
HORVU.MOREX.r3.5HG0485300	Superoxide dismutase [Cu-Zn]		2.73**	−0.00 ns
HORVU.MOREX.r3.2HG0103920	L-ascorbate peroxidase	*HvAPX*	3.68**	−0.30 ns
HORVU.MOREX.r3.7HG0716920	L-ascorbate oxidase		3.87[a]	1.24 ns
HORVU.MOREX.r3.2HG0215300	Peroxidase		2.59**	−0.30 ns
HORVU.MOREX.r3.3HG0291910	Peroxidase	*HvPOD*	2.50[a]	2.18[a]
HORVU.MOREX.r3.7HG0723200	Peroxiredoxin, putative		2.15**	−0.20 ns
HORVU.MOREX.r3.7HG0751550	Catalase	*HvCAT1*	2.41**	0.11 ns
HORVU.MOREX.r3.7HG0740290	Homogentisate phytyltransferase	*HvHPT*	2.96**	−0.90 ns
HORVU.MOREX.r3.2HG0181160	Caleosin		8.91**	−1.41 ns
HORVU.MOREX.r3.2HG0181170	Caleosin		5.89**	1.64 ns
HORVU.MOREX.r3.7HG0669590	Caleosin		3.97**	−1.50[a]
HORVU.MOREX.r3.4HG0381750	Calmodulin3	*HvCaM3*	4.46**	2.06[a]
HORVU.MOREX.r3.5HG0433130	Calmodulin11	*HvCaM11*	4.35**	−0.63 ns
HORVU.MOREX.r3.5HG0444870	Calmodulin-binding protein		2.18**	−0.60 ns
HORVU.MOREX.r3.1HG0077650	Non-specific serine/threonine protein kinase		2.96**	0.55 ns
HORVU.MOREX.r3.2HG0112440	Non-specific serine/threonine protein kinase		2.51**	1.31[a]
HORVU.MOREX.r3.3HG0246590	Non-specific serine/threonine protein kinase		2.99**	3.13**
HORVU.MOREX.r3.3HG0306490	Serine/threonine-protein kinase		7.86**	1.33 ns
HORVU.MOREX.r3.3HG0309380	Protein kinase family protein		2.86[a]	0.16 ns
HORVU.MOREX.r3.3HG0311790	Kinase family protein		2.21**	0.14 ns
HORVU.MOREX.r3.4HG0352300	Non-specific serine/threonine protein kinase	*HvSnRK3*	4.41**	3.34**
HORVU.MOREX.r3.4HG0353330	Protein kinase		3.47**	−0.4 ns
HORVU.MOREX.r3.6HG0596480	Kinase family protein		3.01**	2.16 **
HORVU.MOREX.r3.1HG0016710	Annexin	*HvAnn5*	1.25ns	3.09**
HORVU.MOREX.r3.7HG0665280	Annexin	*HvAnn1*	3.10**	−0.30 ns
HORVU.MOREX.r3.2HG0099650	ABC transporter B family protein		8.45**	0.65 ns
HORVU.MOREX.r3.3HG0270800	Pleiotropic drug resistance ABC transporter	*HvABCG36*	4.30**	1.02 ns
HORVU.MOREX.r3.3HG0282720	ABC transporter B family protein		2.66**	−0.50 ns
HORVU.MOREX.r3.3HG0302160	ABC subfamily C transporter		4.19**	−1.43[a]
HORVU.MOREX.r3.4HG0331260	ABC transporter ATP-binding protein ARB1	*HvABCB1*	2.51**	1.03 ns
HORVU.MOREX.r3.5HG0524470	Multidrug resistance protein ABC transporter family	*HvABCC14*	2.00**	0.99 ns
HORVU.MOREX.r3.7HG0654450	ABC transporter, putative	*HvABCC10*	3.03**	0.11 ns
HORVU.MOREX.r3.7HG0717840	ABC transporter-like family-protein	*HvABCC8*	2.78**	1.66**
HORVU.MOREX.r3.4HG0334970	Plasma membrane ATPase	*HvAHA1*	3.11**	−0.4ns

[a] $P < 0.05$; $^{**}P < 0.01$; ns, non-significant.

regulated in cultivated barley (Table 3).

3.4.3. ROS inhibiting and scavenging genes

Comparative transcriptome analysis of ROS inactivators of wild and cultivated barleys under control and heat-stress conditions exhibited several crucial genes involved in ROS disruption. In response to heat stress, the polyamine metabolic pathway genes, including one ornithine decarboxylase (*HvODS*), two agmatine coumaroyl transferase-2, one arginase, one polyamine oxidase (*HvPAO2*), and one spermidine synthase (*HvSPMS2*), were significantly up-regulated in wild barley (Table 3).

Comparative transcriptome analysis of antioxidants in wild and cultivated barleys under two environmental conditions identified crucial transcripts involved in heat tolerance. In response to heat stress, the expression of 14 glutathione S-transferase genes and duplicate ferredoxin (*HvFdx3*) genes was significantly induced in wild barley (Table 3). The genes encoding enzymatic antioxidants, including *HvSOD1*, *HvAPX*, *HvPOD*, and *HvCat1*, were significantly up-regulated in response to heat stress in the wild barley genotype. The homogentisate phytyltransferase (*HvHPT*) gene, involved in tocopherol biosynthesis, was expressed in response to heat in the tolerant wild genotype.

3.4.4. Genes involved in Ca^{2+} signaling

Calcium sensors, including caleosins, calmodulins, and protein

kinases, were significantly up-regulated in response to heat in the wild genotype (Table 3). The expression of three caleosin transcripts was uniquely observed with $\log_2$ FC > 4 in the wild-tolerant barley. *Calmodulin3*, *Calmodulin11*, and another calmodulin-binding protein gene were up-regulated in the wild genotype exposed to heat stress. Both barleys showed significantly elevated expression of the *HvCaM3* and *HvSnRK3* genes in response to heat. Nine kinase protein genes —functioning as serine/threonine-protein kinases, kinase family proteins, and non-specific serine/threonine protein kinases—were significantly induced by heat stress in tolerant barley. The expression of *HvAnn1*, another calcium-binding protein gene, was highly altered in the wild genotype (Table 3). On the other hand, the *HvAnn5* transcript was up-regulated threefold in cultivated barley but showed no significant change in wild barley (Table 3).

3.4.5. ABC transporters

The ATP-binding cassette (ABC) transporters belong to a large protein family localized in cell membranes. *HvABCG36, HvABCB1*, *HvABCC10*, *HvABCC14*, and two ABC transporter B family protein genes were uniquely up-regulated in the tolerant genotype exposed to heat stress (Table 3). The highest expression increase was observed for the ABC transporter B family protein transcript (*HORVU.MOREX.r3.2HG0099650*), which had a $\log_2$ FC > 8 in wild-tolerant barley. In response to heat stress, wild barley showed a 4-fold increase in the *HvABCG36* transcript, while no significant induction was observed in cultivated barley. The expression of the *HvABCC8* gene was significant in both types of barley (Table 3).

3.5. Validation of candidate genes through real-time expression analysis

The real-time PCR analysis was performed to evaluate the expression profiles of four selected genes (listed in Table 1) to verify the accuracy of the sequencing expression results. The expression profiles of the candidate genes confirmed a similar pattern between the results obtained from RT-qPCR and transcriptome analysis in response to heat stress (Fig. 8).

4. Discussion

4.1. Plant growth and physiology

Plant growth analysis, which encompasses parameters such as dry weight (DW), photosynthesis, and yield, is a foundational step for subsequent physiological, biochemical, or transcriptional investigations. It aims to elucidate differences in plant growth, whether arising from genetic variation among genotypes under identical environmental conditions or from environmental variation affecting a single genotype. In the present study, heat stress significantly reduced plant dry weight, with wild barley outperforming cultivated barley in both DW and photosynthetic efficiency. These results provide a valuable foundation for analyzing our physiological and transcriptional data.

Wild barley demonstrated greater resilience to heat stress in physiological traits than cultivated barley. The significant 66.54% reduction in MSI observed in the 'Mona' cultivar highlights increased membrane damage and electrolyte leakage relative to wild barley. Similarly, Bahrami et al. (2019) reported that heat stress negatively affects the membrane composition of barley leaves. Maintaining membrane integrity is vital to prevent membrane leakage caused by plasma membrane disruption and subsequent cell damage due to heat stress.

Photosynthesis, an essential physiological process, is significantly affected by heat stress in barley (Bahrami et al., 2019), wheat (Mahdavi et al., 2021), and maize (Wang et al., 2018). The Fv/Fm value and leaf chlorophyll content are widely regarded as reliable indicators for evaluating damage to a plant's photosynthetic activity (Zhou et al., 2015). In this study, the wild barley exhibited only minor effects of heat stress on photosynthesis, as reflected in the smaller reductions in its Fv/Fm ratio and leaf chlorophyll content compared to the 'Mona' cultivar. These observations, which align with our dry weight (DW) data, underscore the presence of stress affecting both growth and physiological traits.

Heat stress led to an increase in the proline content in the wild genotype, suggesting that elevated proline accumulation in leaves significantly contributes to enhanced heat tolerance. This observation aligns with the results of Bahrami et al. (2019), who reported increased proline levels in *H. vulgare* ssp. *spontaneum* under heat stress. Under high-temperature conditions, greater proline accumulation may stabilize membrane structures and protect redox potential by neutralizing reactive oxygen species (ROS) generated due to heat stress, thereby contributing to thermo-tolerance (Kishor et al., 2005; Wahid et al., 2007; Bita and Gerats, 2013).

4.2. Transcriptome analysis

The primary aim of this study was to evaluate transcriptional alterations and investigate tolerance mechanisms by identifying key genes linked to heat tolerance in barley. Heat tolerance and adaptive strategies in plants, which encompass the activation of various defense-related genes, are governed by intricate signaling and transduction pathways that interact dynamically within plant cells under heat stress (Hassan et al., 2021). Protein stability and enzyme functions in cells are negatively affected by heat stress in crop plants (Chaudhary et al., 2020). In response to the disruption of the cell's homeostasis due to heat stress in crops, transcriptional alterations of many stress-responsive genes occur in the cells. HSPs, known as protective molecular chaperones, play a crucial role in restoring cellular homeostasis under heat-stress conditions (Lavania et al., 2015; Reddy et al., 2016). They are responsible for refolding misfolded proteins, preventing protein aggregation, and

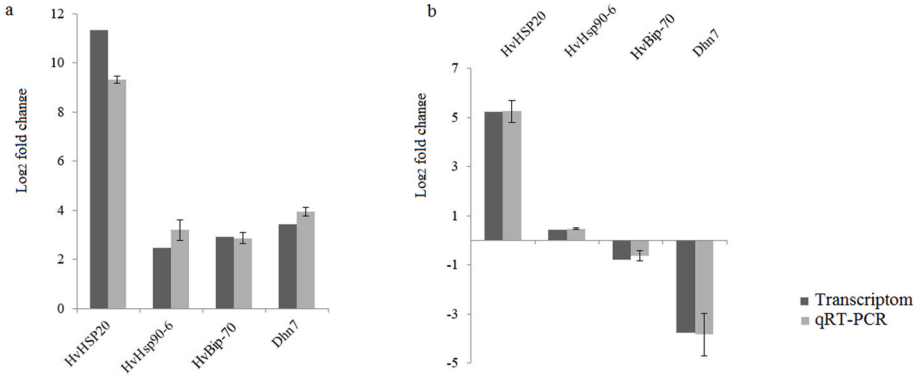


Fig. 8. Comparison of RT-qPCR and RNAseq gene expression results using four genes (*AvHSP20*, *HvHsp90-6*, *HvBip-70*, and *Dhn7*) (a) wild genotype and (b) 'Mona' cultivar.

degrading unstable proteins (Reddy et al., 2016). The up-regulation of the *Hsp70*, *Hsp90*, and *Hsp100* gene families in our study aligns with the findings of Chaudhary et al. (2019), indicating their important role in enhancing heat tolerance in barley. The up-regulation of HSP and proteins with chaperone functions suggests these genes are crucial to enhance stability, and stress tolerance in wild barley. In response to heat, a broad range of HSP (HSP70, HSP90, and sHSP) products could be up-regulated in wild barley leaves as reported by Ashoub et al. (2015), corroborating our findings.

The HSP chaperones maintain expressed HSFs in an inactive state under normal conditions. However, under heat stress, the accumulation of unfolded proteins in cells leads to the release of HSFs by the HSP chaperones. After the release of HSFs, the transcription of heat-responsive genes, such as HSPs, is initiated. Prolonged stress conditions can further amplify the expression of additional target genes regulated by HSFs (Bakery et al., 2024). Significant up-regulation of *HsfAc2*, *HvHSFC-2a*, and *HsfC2b* genes, accompanied by four other HSFs in the wild barley genotype, emphasizes that these types of TFs could be responsible for the transcriptional regulation of downstream genes for heat tolerance. The heat-responsive HSF gene of wheat, *TaHsfA6b*, in transgenic barley lines, indicated that the overexpression of *TaHsfA6b* positively impacted HSP expression and other main stress-related genes to enhance thermo-tolerance (Poonia et al., 2020). Mishra et al. (2024) reported that the *HvHSFA2e* gene enhanced drought and heat stress tolerance by up-regulating heat-responsive genes in the 'Golden' barley cultivar.

The up-regulation of genes involved in osmotic homeostasis, through the activation of oxidative stress mechanisms and the stabilization of proteins and membrane structures, enhances plant adaptation to heat. Conversely, the downregulation of genes belonging to LEA proteins suggests that the heat sensitivity observed in the barley cultivar may be linked to a reduction in these proteins as a response to heat stress. In the current study, seven LEA genes were up-regulated in the tolerant genotype. These findings may imply that these proteins are linked to heat tolerance in barley through chaperone-mediated protection, and preventing the deactivation and aggregation of different enzymes due to heat (Goyal et al., 2005). Poonia et al. (2020) indicated that high LEA gene expression could explain the improved performance of barley transgenic lines containing the *TaHsfA6b* gene. The up-regulation of genes belonging to LEA proteins in *H. vulgare* ssp. *spontaneum,* exposed to salt stress, was also reported by Gharaghanipor et al. (2022).

In response to heat stress, the accumulation of proline and sugars serves as an effective mechanism for plants to maintain cell turgidity and ensure survival (Hussain et al., 2019). In the current study, genes associated with proline biosynthesis and sugar transport were found to be induced in thermo-tolerant wild barley. The expression of genes such as *HvP5CS1, HvP5cr3*, and *HvP5CSB* leads to increased proline accumulation, which contributes to the stability of protein and membrane structures, thereby promoting heat tolerance in wild barley. Our findings align with those of Dawood et al. (2020) regarding heat stress and Gharaghanipor et al. (2022) concerning salt stress in barley. Indeed, sugars are considered the most suitable sources to provide energy for cellular metabolic processes. SWEET proteins, functioning as uniporters, enable the transport of sucrose into the cell wall (apoplast). Up-regulation of three SWEET family genes (*HvSWEET15a*, *HvSWEET3*, and *HvSWEET1*) provided insights into their roles as sources of carbon and osmotic regulators in reducing the consequences of high temperatures in wild barley. High temperatures inhibit the import and export of sucrose in the phloem from the leaves (Julius et al., 2017). SWEET family genes have also been shown to contribute to abiotic stress tolerance in plants (Gautam et al., 2022), particularly in wheat (Gautam et al., 2019; Qin et al., 2020). In rice, OsSWEET13 and OsSWEET15 have been recognized as the primary SWEET transporters affected by drought and salt stress (Mathan et al., 2021). Dawood et al. (2020) found significantly high expression of the sugar transporter 1 (*HORVU1Hr1G013480*) gene, which is involved in sugar transport, in the heat-tolerant barley genotype.

Polyamines not only reduce the production of reactive oxygen species (ROS) but also serve as transcriptional regulators for genes involved in antioxidant production and the activation of antioxidant enzymes (Blázquez, 2024). The expression of polyamine metabolic pathway genes, such as *HvODS*, agmatine, arginase, *HvPAO2*, and *HvSPMS2*, as observed in this study, suggests their role in mitigating ROS and regulating translation to enhance heat stress tolerance in wild barley. In rice, the functions of spermidine (Fu et al., 2019) and agmatine (Liu et al., 2020) genes have been illustrated to enhance thermos-tolerance by modulating polyamine metabolism. Gholizadeh and Mirzaghaderi (2020), by evaluating 30 polyamine oxidase genes in common wheat, suggested that these genes might be involved in responding to environmental stresses, particularly temperature extremes such as heat and cold.

Heat-induced accumulation of ROS triggers oxidative stress, causing molecular alterations and damage that can disrupt cellular functions or lead to cell death. Antioxidant activity plays a crucial role in preserving a balanced redox environmentthat is essential for cellular health (Fortunato et al., 2023). In our study, the up-regulation of 26 genes encoding enzymatic antioxidants, including glutathione S-transferase (GST), superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and duplicate ferredoxin, displays that oxidative damage has been resolved through an antioxidant defense system in the wild-tolerant barley exposed to heat. These findings are coordinated with the observations that antioxidant transcripts were induced in response to heat stress (Ashoub et al., 2018) and salt stress (Gharaghanipor et al., 2022) in wild-tolerant barley. Identification of highly abundant GST genes in this study suggests that they may enhance heat tolerance in wild barley by acting as scavengers within the antioxidant system. Overexpression of *HvGST4* increases glutathione content involved in H_2O_2 degradation and regulates ROS scavenging (Pan et al., 2022). GST enzymes decrease ROS accumulation by facilitating glutathione conjugation in response to abiotic stress (Liang et al., 2018; Kumar and Trivedi, 2018). Mishra et al. (2024) reported that the up-regulation of three glutathione S transferases (*GSTF1*, *GSTT3*, and *GSTU6*) overexpressed *HvHSFA2e* in barley lines. This suggests a potential role of these enzymes in enhancing stress tolerance in barley.

The *HvHPT* gene, involved in tocopherol biosynthesis, exhibited increased expression in wild-tolerant barley following heat stress. Tocopherols, essential lipid-soluble antioxidants in chloroplasts, play a role in the redox-active pathway (Sattler et al., 2004). Mutation in the *HvHPT* gene (*hpt*) in barley significantly reduced tocopherol levels, thereby negatively affecting cellular homeostasis (Zeng et al., 2020). The intracellular Ca^{2+} concentration rises sharply in response to elevated temperatures, establishing Ca^{2+} signaling as one of the earliest plant responses to heat stress (Kang et al., 2024). This surge in cytoplasmic Ca^{2+} levels triggers a complex signal transduction cascade, engaging downstream effector proteins such as calmodulins and Ca^{2+}-dependent protein kinases. These processes subsequently lead to alterations in gene expression, enzyme activity, and/or ion channel functionality (Mohanta et al., 2019).

The *AtCaM3* (Calmodulin) gene in *Arabidopsis* has been identified as a key component in Ca^{2+} signaling under heat stress (Zhang et al., 2009). The role of Caleosin genes in responding to abiotic stress has also been explored in various crops and model species, such as *Arabidopsis* (Aubert et al., 2010; Blee et al., 2014), rice (Wei et al., 2011), and cotton (Fu et al., 2022). Additionally, Chen et al. (2024) reported that the up-regulation of *HvMOREXann7* in drought-tolerant wild barley, compared to cultivated varieties, highlights the remarkable environmental adaptability in wild genotypes.

The up-regulation of multiple genes encoding calcium-binding proteins (calmodulin, caleosin, kinase family proteins, and annexin) in response to heat demonstrates the functional role of genes involved in Ca^{2+} signaling, which is crucial for triggering heat tolerance in wild barley.

ABC-transporters facilitate the import and export of various metabolites across cellular membranes and play a crucial role in regulating biological processes in plant cells (Hwang et al., 2016; Dahuja et al., 2021). In the current study, eight ABC-transporter genes were found to be induced in response to heat stress in the wild-tolerant barley. This induction may enhance thermo-tolerance by maintaining cell membrane integrity and managing lipid composition in plant cells (Dahuja et al., 2021). Similarly, Singh et al. (2024) reported the expression of genes in the leaf tissues of heat-tolerant pearl millet. Zhang et al. (2020) reported 15 ABC-transporter DEGs responding to abiotic stress through genome-wide analysis of the ABC gene family in barley.

The changes in the induction of heat tolerance genes, whether through up-regulation or down-regulation of DEGs, were more pronounced in wild barley compared to the 'Mona' cultivar. These findings underscore the adaptive mechanisms of wild barley genotype in our study, which involve a complex network of defenses that regulate and protect against abiotic stresses. This includes heat tolerance, as demonstrated in this study, as also salt tolerance, ensuring its survival under challenging environmental conditions. Future research is needed to provide deeper insights into the functions and synthesis pathways of the heat response genes identified in this study. Understanding these pathways will deepen our knowledge of how plants adapt to heat stress, potentially guiding breeding strategies to develop more resilient cultivars.

5. Conclusion

The current transcriptomic study revealed that thermal stress in wild barley induced extensive gene expression modifications, highlighting the diverse regulatory mechanisms underlying heat tolerance. Our comparison of DEGs between heat-tolerant wild barley and sensitive 'Mona' cultivar reveals key genes involved in heat tolerance, with wild barley showing a stronger genetic adaptation to heat stress. Among the DEGs, heat shock proteins, antioxidants, osmoprotectants, and regulatory networks were identified as the most prominent. Indeed, heat stability in wild barley is induced by cellular homeostasis and integrity, the presence of protective macromolecules, and the maintenance of a sustained biosynthesis process. Consequently, this research could enhance the understanding of the heat tolerance mechanisms in *H. vulgare*. Nonetheless, we acknowledge that future research incorporating analyses of additional genotypes would strengthen the conclusions and further confirm the relevance of the identified genes in diverse genetic contexts.

Author contributions

A.A. and F.B. conceptualized the study and developed the methodology. F.B. carried out the stress treatments, agro-physiological experiments, transcriptome analysis, RT-qPCR, data analysis, and drafting of the initial manuscript. A.A., M.R., and F.A. supervised, assisted with software development and validation, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability

The RNA-seq datasets generated during the current study have been deposited in the NCBI Sequence Read Archive (SRA) and assigned BioProject accession PRJNA1178137.

Ethics approval and consent to participate

This study does not include human or animal subjects.

Consent for publication

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109699.

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