



# Heat-stress-responsive *HvHSFA2e* gene regulates the heat and drought tolerance in barley through modulation of phytohormone and secondary metabolic pathways

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Received: 30 April 2024 / Accepted: 28 May 2024 / Published online: 14 June 2024  
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## Abstract

**Key message** The heat stress transcription factor *HSFA2e* regulates both temperature and drought response via hormonal and secondary metabolism alterations.

**Abstract** High temperature and drought are the primary yield-limiting environmental constraints for staple food crops. Heat shock transcription factors (HSF) terminally regulate the plant abiotic stress responses to maintain growth and development under extreme environmental conditions. *HSF* genes of subclass A2 predominantly express under heat stress (HS) and activate the transcriptional cascade of defense-related genes. In this study, a highly heat-inducible *HSF*, *HvHSFA2e* was constitutively expressed in barley (*Hordeum vulgare* L.) to investigate its role in abiotic stress response and plant development. Transgenic barley plants displayed enhanced heat and drought tolerance in terms of increased chlorophyll content, improved membrane stability, reduced lipid peroxidation, and less accumulation of ROS in comparison to wild-type (WT) plants. Transcriptome analysis revealed that *HvHSFA2e* positively regulates the expression of abiotic stress-related genes encoding HSFs, HSPs, and enzymatic antioxidants, contributing to improved stress tolerance in transgenic plants. The major genes of ABA biosynthesis pathway, flavonoid, and terpene metabolism were also upregulated in transgenics. Our findings show that *HvHSFA2e*-mediated upregulation of heat-responsive genes, modulation in ABA and flavonoid biosynthesis pathways enhance drought and heat stress tolerance.

**Keywords** Heat stress · Drought · Heat shock transcription factor · *HvHSFA2e* · Transcriptional modulation · ABA

## Introduction

The food security for global population is solely dependent on crop production which is determined by the variations in climate. The high variations in climatic factors lead to a

reduction in crop production and severely impact food supply (Ray et al. 2015). Temperature and water availability primarily determine the crop yield and the fluctuations beyond optimal range impose deleterious effects on yield potentials. It has been reported if the rise in global surface temperature continues at the current rate, then it will increase up to or beyond 1.5 °C in comparison to the pre-industrial climate by ~2040 and the warming will be higher on land than that of ocean surface (Masson-Delmotte et al. 2019). More than half of the calorific requirement of world population is fulfilled by cereal crops (Nelson et al. 2010), which are highly susceptible to heat stress during the reproductive phase (Farooq et al. 2011). The combined effect of heat and drought stress is comparatively more damaging to crop yield in comparison to the individual impact of either of the stresses (Lamaoui et al. 2018; Yashavanthakumar et al. 2021). Crop improvement is an essential step to ascertain food security under future warmer climate with water-limiting conditions (Atlin et al. 2017). It is more challenging for plant scientists to

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Communicated by Haitao Shi.

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increase the crop yield as well as abiotic stress tolerance, simultaneously, to cope up with the threat of global climate change (Battisti and Naylor 2009).

Under abiotic stress, one of the major obstacles for plants in maintaining the yield is to overcome the excessive generation and increased accumulation of ROS. The compromised ROS homeostasis results in oxidizing environment in plant cells. To avoid the oxidative damages, plants have evolved the molecular strategies in the form of stress responses. As plants cannot move and migrate to avoid unfavorable environmental conditions, they face stress conditions and develop tolerance/resistance to survive. To cope with environmental challenges, plants have evolved an intricate response system; one of the primary actions that improve stress tolerance is dynamic reprogramming of transcriptional activity. Under various stress circumstances, several transcription factor families are implicated in complex and overlapping responses. The major transcriptional regulators which regulate and reprogram the gene expression under high-temperature and drought stress are *HSFs*, *WRKY*, *MYB*, *DREB*, *MBF1C* (*multiprotein bridging factor 1c*), *bZIP* (*basic region/leucine zipper motif*), *ERF* (*ethylene-responsive factor*), and *NAC*. Plant heat shock response (HSR) is regulated by heat stress transcription factors (HSFs). The DNA-binding ability of HSFs allow them to bind with a consensus *cis*-regulatory sequence *nAGAAAnTTCT* (Heat shock element—HSE) present in the promoter of heat-responsive genes (Nover et al. 1996; Mishra et al. 2020). Predominant upregulation of class A HSFs in response to high temperature has been widely characterized. Among the class A, *HSFA2* gene showed strong inducibility in both dicot as well as monocot plants against heat shock (Charng et al. 2007; Giorno et al. 2010; Xue et al. 2014; Chauhan et al. 2011). Schramm et al. (2006) reported that the expression of ascorbate peroxidase and galactinol synthase is also regulated by *HSFA2* under heat stress. In HSR, HSFs directly regulate the transcription of HSPs, antioxidant enzymes, and other thermotolerance contributing genes (Lämke et al. 2016; Wang et al. 2017). Thus, the molecular pathways in plants are precisely reprogrammed under the regulation of stress-responsive transcription factors to minimize the damage and maintain the physiological processes.

Transcriptome-based studies showed that the expression of plant *HSF* genes shows modulation not only under high temperature but also during other abiotic stresses. Moreover, the functional characterization of various *HSF* genes showed their role in transcriptional regulation of different genes which are responsive to drought, salinity, high light, cold and heavy metal stresses. Similarly, the transcriptome profiles under different stress conditions show overlapping in gene expression patterns suggesting the cross-talk among these stress responses (Swindell et al. 2007). *HSF* genes are induced by multiple stress conditions; most of the abiotic

stresses ultimately result in oxidative stress, which arise due to excessive generation and accumulation of ROS in plant cell. Excessive ROS is harmful to physiological processes of plants, though ROS-mediated signaling plays an important role in evoking the plant defense system (Mittler et al. 2022). Previous studies showed that ROS generating conditions induce the expression of *HSF* genes (Pérez-Salamo et al. 2014; Choi et al. 2019). It has been observed that the overexpression of single *HSF* gene may have impact on tolerance against multiple abiotic stresses. Not only the thermotolerance but drought, water stress, salinity, and heavy metal stress tolerance were also enhanced in transgenic plants overexpressing the candidate genes from *HSF* Class A1 and A2 (Li et al. 2005, 2014; Bechtold et al. 2013).

To maintain optimum yield potential in the projected adverse climate, transgenic cereal plants can be developed with altered *HSF* expression levels. These transgenic plants will be able to tolerate adverse environmental conditions such as high temperature, water depletion, and saline soil, ultimately resulting in ensuring food security for the global population. Among the 23 barley HSFs, *HvHSFA2e* was not only the highest expressing HSF under 30 min of heat shock (HS) but also one of the early heat-responsive HSFs, showing heat-induced upregulation as early as 10 min of HS onset (Mishra et al. 2020). Considering these characteristics, *HvHSFA2e* gene was targeted for constitutive overexpression to reveal its potential role in enhancing the abiotic stress tolerance in barley plants in the present study.

## Materials and methods

### Plant material and growth conditions

Seeds of barley (*Hordeum vulgare* L.) Cv. Golden promise were germinated in a mixture of soil and soilrite (1:1) under controlled conditions in greenhouse 18/16 °C (day/night) temperature and 16 h photoperiod. 2-week-old wild-type (WT) plants were used to assess the stress inducible expression of *HvHSFA2e* gene. For heat shock (HS), tissue samples (Shoot and root) were harvested at 0 (control), 30 min, and 2 h time points from the plants exposed to 42 °C temperature. For drought stress, 2-week-old plants were exposed to drought stress by stopping the water supply for next 2 weeks. The shoot and root samples were harvested from 4-week-old plants grown under water-limiting conditions. For drought stress experiment, control tissue was collected from 4-week-old plants growing under optimal non-stress conditions. Harvested stress-exposed plant tissue samples were snap frozen in liquid nitrogen and used for RNA isolation.

For genetic transformation of barley, immature embryos harvested from field as well as greenhouse grown plants were used as explant.

Transgenic lines and WT plants were grown in above-described conditions, 2-week-old plants were subjected to 40 °C for 4 h to assess the thermotolerance. For drought tolerance analysis, 2-week-old transgenic and WT plants were exposed to drought stress by limiting the water supply for next 2 weeks.

### **Cloning of HvHSFA2e cDNA into binary vector and genetic transformation of barley**

A 2022 bp DNA fragment corresponding to full-length cDNA (FL- cDNA) of *HvHSFA2e* gene (AK358380) was amplified from the plasmid provided by GeneBank, NARO, Japan using gene specific primer pair (Supplementary Table S1). The PCR product was cloned in PCR cloning vector pJET1.2 (Invitrogen, ThermoFisher Scientific USA) and further subcloned in binary vector *p6oAct-UbiZm-LH* (DNA cloning services, Hamburg Germany). A restriction digestion was performed with *SwaI* and *EcoRV* enzymes to obtain the *ZmUbi1:HvHsfA2e:NosT* cassette. This cassette was cloned in modified gateway binary vector *pANIC6B* (Mann et al. 2012) and used to transform *Agrobacterium* strain *EHA105*. *Agrobacterium*-mediated genetic transformation of barley was performed according to Hensel et al. (2009) to develop the *HvHSFA2e* overexpression lines (O.E.).

### **Transactivation assay**

The yeast system was used for assessing the transactivation potential of HvHsfA2e protein. The *HvHSFA2e* ORF was amplified using the ORF specific primer pair (Supplementary Table S1) and fused to GAL4 BD in the yeast vector *pGBKT7* (Clontech, CA, USA), generating the constructs *pGBKT7:HvHsfA2e*. The empty *pGBKT7* was used as a negative control. Lithium acetate-polyethylene glycol-mediated transformation procedure was followed for transferring the *pGBKT7:HvHsfA2e* plasmid into yeast strain AH109. The transformants were allowed to grow in SD medium lacking amino acid tryptophan and histidine at 30°C. To assess if there was leaky expression of HIS3 reporter gene, dilutions were dotted on the respective dropout media supplemented with 1 mM and 5 mM of 3 AT. Each experiment was performed three times.

### **Subcellular localization through transient expression in *Nicotiana benthamiana***

The ORF of *HvHSFA2e* gene without stop codon was amplified using the *HvHSFA2e-GFP-F* and *HvHSFA2e-GFP-R* primer pair with flanking attB sites (Supplementary Table S1). The PCR product was cloned into *pDONR221* by Gateway™ BP Clonase™ II Enzyme mix (Invitrogen), and a LR reaction was performed with

*pB7FWG2* empty vector and *pDONR221-HvHSFA2e* with LR Clonase™ enzyme mix (Invitrogen) to generate the construct *pB7FWG2:35S:HvHSFA2e:GFP*. The vector *pB7FWG2-35S:HvHSFA2e:GFP* was transformed into *A. tumefaciens* GV3101. Transient expression in *N. benthamiana* through *Agrobacterium* infiltration was performed according to Li, (2011). Protein localization was analyzed 24–48 h after infiltration by confocal laser scanning microscope (TCS SP8, Leica). GFP was excited at 488 nm and the fluorescence detected between 498 and 525 nm.

### **Confirmation and transgene expression analysis of *HvHSFA2e* overexpression line**

For the confirmation of successful integration of targeted gene, leaf tissue of putative transgenic plants was used to isolate the genomic DNA using DNA-XPress™ Reagent (MB501, HiMedia Laboratories, Mumbai, India) as per manufacturer's instructions. Gene-specific primers amplifying the selection marker gene *Hygromycin phosphotransferase (HPT Full F-R)*, and reporter gene  *$\beta$ -glucuronidase (Gusplus)* were used to confirm the integration event in barley transgenic lines (Supplementary Table S1).

The expression level of *HvHSFA2e* gene in transgenic lines was analyzed through qRT-PCR. The expression analysis was done using QuantStudio 3 system and PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA) according to the manufacturer's instructions and primer Express 3.0 software (Thermo Scientific, USA) was used to design the primers. Leaf tissue of WT barley plant grown under control conditions was used as control and *snoR14* gene was used as internal reference control (Ferdous et al. 2015). The fold change calculation for gene expression was done using the formula  $2^{-\Delta\Delta C_t}$  (Livak and Schmittgen 2001). Similarly, the increased expression of other genes, which were found upregulated in RNA-seq data, was validated using the qRT-PCR.

### **Detection of GUS activity**

Histochemical Gus assay was performed to confirm the stable transformation. The transformed immature embryos and leaf tissue of mature transgenic plants were subjected to Gus assay. The transgenic material was transferred in a microcentrifuge tube and immersed in freshly prepared X-glc solution containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100, followed by vacuum infiltration for 10 min and overnight incubation at 37°C. All samples were washed and fixed in 70% ethanol

to remove the photosynthetic pigments prior to visualize the staining and taking photographs. GUS expression was observed under a stereo zoom microscope.

### Histochemical detection, visualization, and quantification of ROS

The formation of superoxide free radicals ( $O_2^{\bullet-}$ ) in leaves was detected by NBT staining method (Wohlgenuth et al. 2002). The leaves sections were immersed in NBT-PBS solution and incubated at room temperature under dark conditions until the blue spot of formazan appeared.

The formation and accumulation of peroxide radical in leaves was detected by DAB staining method (Thordal-Christensen et al. 1997). The leaf sections were submerged in 1 mg/mL DAB–HCl (pH3.8) for 12 h under light condition. The peroxide accumulation sites can be visualized as the brown spots generated due to the polymerization of DAB. All samples were washed and fixed in 70 % ethanol to remove the pigments prior to visualize and taking photographs.

The leaf  $H_2O_2$  content was quantified according to Chakraborty et al. (2016) using Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, USA).

### Determination of enzymatic antioxidant activities

To determine the activities of antioxidative enzymes, 500 mg leaf tissue was homogenized in 5 mL of assay buffer containing 100 mM phosphate buffer pH7.0, 2mM  $Na_2EDTA$ , 1% PVP. The solution was centrifuged at  $15,000\times g$  for 20 min at 4 °C. The clear supernatant was collected and kept at 4 °C to use as crude enzyme extract in estimation protocols.

The ascorbate peroxidase activity was assayed according to Nakano and Asada (1981). The assay mixture consisted of 150  $\mu$ L of the enzyme extract, 50 mM phosphate buffer (pH 7.0), 0.2  $\mu$ M EDTA, 0.2 mM ascorbate, and 2.0 mM  $H_2O_2$  in a total volume of 3 mL. Ascorbate oxidation was monitored by reading the absorbance at 290 nm at the moment of  $H_2O_2$  addition and 1 min later. The difference in absorbance ( $\Delta A_{290}$ ) was divided by the ascorbate molar extinction coefficient ( $2.8\text{ mM}^{-1}\cdot\text{cm}^{-1}$ ) and the enzyme activity expressed as  $\mu\text{mol of } H_2O_2\text{ min}^{-1}\text{ mg}^{-1}\text{ protein}$ , considering that 1.0 mol of ascorbate reduces 1.0 mol of  $H_2O_2$  (McKersie and Leshem 1994).

The activity of superoxide dismutase was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride, as described by Gianopolitis and Ries (1977). The assay mixture consisted of 300  $\mu$ L of the enzyme extract, 100 mM phosphate buffer (pH 7.8), 3 mM EDTA, 200  $\mu$ M methionine, 2.25  $\mu$ M nitro blue tetrazolium, and 60  $\mu$ M riboflavin (added at last) in a total

volume of 3.2 mL. The tubes were inverted for 2–3 times for proper mixing and placed under fluorescent light. After 15 min, lights were switched off to stop the reaction. Then the tubes were immediately put in dark. Absorbance was measured at 560 nm. SOD activity was estimated by the method given by Fridovich (1986), and unit activity was expressed as enzyme utilized for 50% inhibition of NBT reduction.

### Estimation of chlorophyll and carotenoid content, relative water content, solute leakage, and lipid peroxidation

For estimation of chlorophyll and carotenoid pigments, 500 mg fresh leaf tissue was homogenized in 5 mL of 80 % acetone and centrifuged at 6000 rpm for 15 min. The supernatant was collected and absorbance was measured at 480, 510 nm for carotenoid content and 645, 663 nm for chlorophyll a & b. The estimation of photosynthetic pigments was done according to Lichtenthaler and Wellburn (1983).

The relative water content (RWC) displays the hydration status of leaves. Freshly harvested leaf sections of known weight were kept in sterile distilled water for 24 h to obtain the turgid weight. The samples were then kept at 65 °C in a hot air oven until a constant weight was achieved to calculate the dry weight. RWC was calculated as  $RWC (\%) = [(FW - DW)/(TW - DW)] \times 100$  (Ings et al. 2013), where FW indicates fresh weight, DW indicates dry weight, and TW indicates turgid weight.

The extent of solute leakage is used to determine the membrane stability in plants under unfavorable conditions. To compare the membrane stability of wild-type and transgenic lines, the solute leakage percentage was determined according to Dionisio-Sese and Tobita (1998) under control and heat stress conditions.

The lipid peroxidation in plants under stressed condition is measured in the form of MDA content which was estimated according to the method described by Zheng et al. (2012). The thiobarbituric acid (TBA) reaction was used to quantify the malondialdehyde (MDA) level, a byproduct of lipid peroxidation. 5 mL of 5% trichloroacetic acid (TCA) was added to 0.5 g of fresh leaf sample, and the mixture was homogenized. The homogenized mixture was centrifuged for 20 min at 10,000 rpm. Four milliliters of 0.5% thiobarbituric acid (TBA) were added to one milliliter of supernatant, and the mixture was heated for 30 min on water bath until an orange–yellow hue developed. Thereafter, it was quickly cooled on ice and absorbance was measured at 532 nm. MDA has an extinction coefficient of 155.



## RNA-seq and data analysis

RNA isolation from leaf tissues of 5-week-old non-transformed wild-type and *HvHSFA2e* overexpression transgenic lines was done using RNeasy plant RNA isolation kit as per manufacturer's instruction (Qiagen, Germany; Cat No./ID: 74904 and 79254). High-quality RNA samples were outsourced to Bionivid Technology Private Limited (Bengaluru, India) for RNA-seq using Illumina HiSeq platform. The RNA-seq data (accession number GSE241562) used and analyzed during this study are available in the GEO, NCBI database.

Quality control of all the reads obtained for each sample was done using NGSQC Tool kit (Patel et al. 2012) and reads with Phred score >Q20 were selected for further analysis. Barley reference genome was downloaded from Ensembl Plants database ([http://ftp.ensemblgenomes.org/pub/plants/release-53/fasta/hordeum\\_vulgare/](http://ftp.ensemblgenomes.org/pub/plants/release-53/fasta/hordeum_vulgare/)) for alignment and mapping of reads using STAR pipeline (Dobin et al. 2013; <https://github.com/alexdobin/STAR>) and Trinity platform (genome guided mode) (Haas et al. 2013; <https://github.com/trinityrnaseq/trinityrnaseq>). Abundance estimation was calculated using RSEM (RNA-Seq by Expectation-Maximization) method (Li and Dewey 2011), and differential gene expression analysis was performed using DESeq2 (Love et al. 2014; <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>). Transcripts having  $\log_2$  fold change greater  $\geq 2$  were considered as DEGs. Volcano plots were constructed using Plotly (pandas) in python (<https://plotly.com/python/volcano-plot/#volcanoplot>). Functional annotation of the transcriptome was performed using the Trinotate pipeline (<https://github.com/Trinotate/Trinotate.github.io/blob/master/index.asciidoc>), employing uniprot-swissprot ([https://ftp.uniprot.org/pub/databases/uniprot/current\\_release/knowledgebase/complete/](https://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/complete/)) database for blastx and blastp searches. Cytoscape (v 3.9.1) plug-in bingo (Maere et al. 2005) was used to perform GO (Gene Ontology) enrichment analysis. Enriched GO terms were identified separately for upregulated and downregulated sets of genes in barley transgenics as compared to wild type. Obtained enriched terms were plotted using the web server Revigo (<http://revigo.irb.hr/>; Supek et al. 2011). Heatmaps were constructed using ggplots (Warnes et al. 2020) and RColorBrewer (Neuwirth 2014) packages in R.

## Statistical analysis

In present study, all the data were generated by three independent experiments; and each experiment was performed with three technical replicates. Microsoft Office Excel 2021 was used to perform the statistical analysis. Error

bars in the graphs denote standard errors of the mean values ( $\pm$ SE). The statistical significance between WT and transgenic data sets was determined by student's *t* test. The level of significant difference was set at  $P < 0.05$  ( $P$ -value; \* = < 0.05; \*\* = < 0.01; \*\*\* = < 0.001).

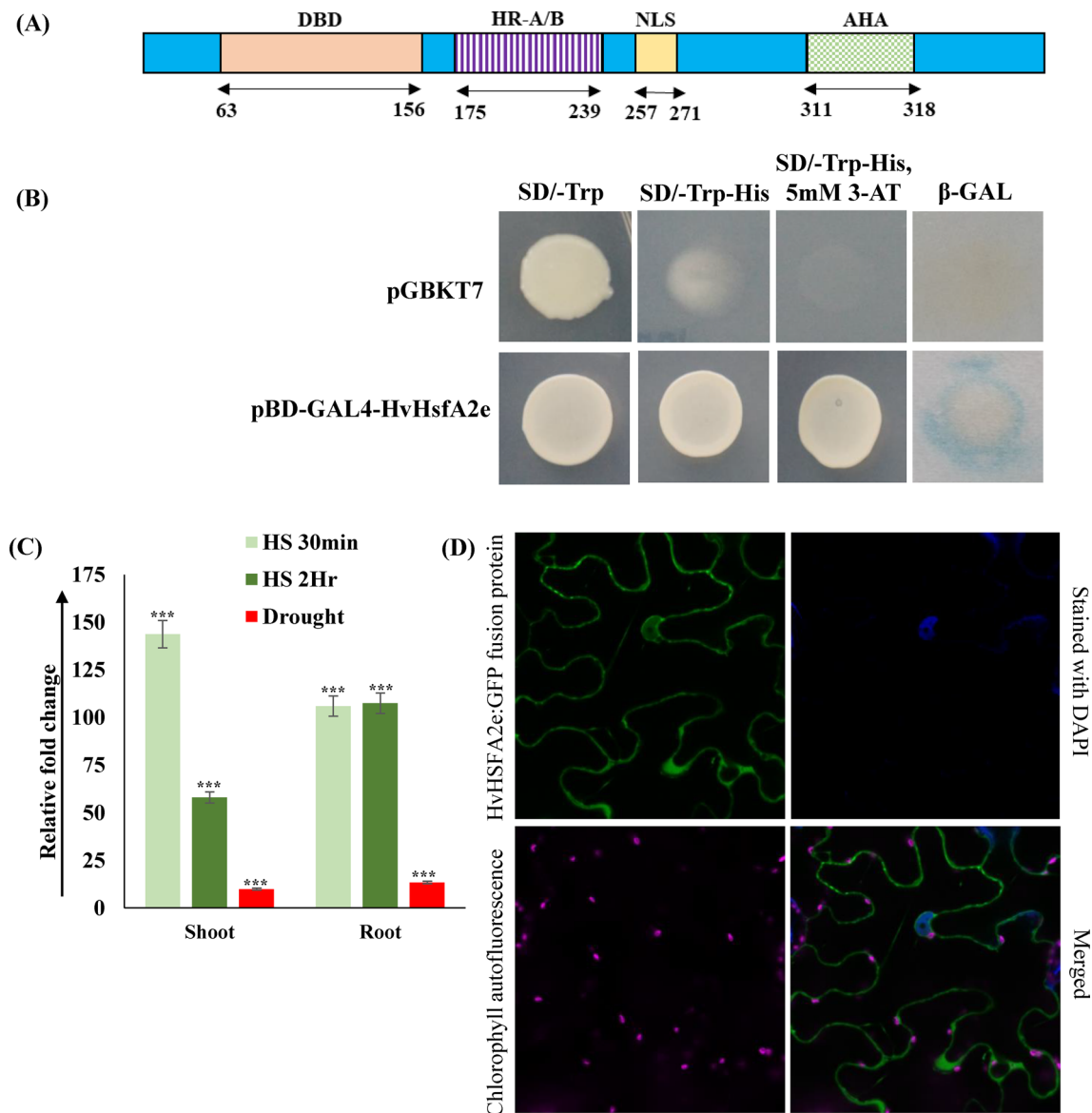
## Results

### Generation of transgenic barley lines overexpressing *HvHSFA2e* and subcellular localization of *HvHSFA2e* protein

The *HSF* gene family of barley possess 23 members, among these genes, *HvHSFA2e* showed highest expression under heat stress conditions in vegetative shoot of barley cultivar RD2786 (Mishra et al. 2020). *HvHSFA2e* gene encodes for 371 amino acid long protein with 41.1 kDa molecular weight. It displays all characteristic features of a class A HSF, including conserved HSF domain or DNA-binding domain (DBD), oligomerization domain, transactivation domain (AHA domain), and nuclear localization signal (NLS) (Fig. 1A). We analyzed the transactivation potential of *HvHSFA2e* through transactivation assay. The yeast strain AH109 cells transformed with the *pGBKT7:HvHSFA2e*, grew well on the medium of SD/-Trp, and the colonies appeared blue on X- $\alpha$ -Gal supplemented SD/-Trp medium, revealing that *HvHsfA2e* possess transactivation potential (Fig. 1B). Similar to Indian commercial barley cultivar RD2786, the *HvHSFA2e* gene of European spring barley cultivar Golden promise (suitable for tissue culture) showed the heat-shock-induced expression in shoot tissue (Fig. 1C).

The *Agrobacterium*-mediated genetic transformation was performed to generate the *HvHSFA2e* O.E. in barley Cv. Golden promise. The *HvHSFA2e* was cloned under the regulation of *Ubiquitin* promoter of *Zea mays* (*ZmUbi* promoter) in modified *pANIC6B* binary vector for its constitutive expression. *Agrobacterium*-mediated transformation of barley immature embryos through vacuum infiltration (Hensel et al. 2009) followed by the tissue culture resulted into the generation of the overexpression transgenic lines.

The putative 17 T0 transgenic lines were confirmed by the PCR amplification of *Gusplus* (reporter) and *Hpt* (selectable marker) genes. The positive results of PCR reactions using the genomic DNA isolated from T0 plants confirmed the successful-stable transformation. The primers used for PCR reactions were specific to full-length *Gusplus* (FL *Gusplus*) and *Hpt* genes present in *pANIC6B* binary vector. In PCR analysis, all the 17 T0 plants showed positive results for amplification of targeted genes; *HptIII* and *Gusplus*. The bands of desired size were visualized on the agarose gel (Supplementary figure 2 A and B).



**Fig. 1** *HvHSFA2e* gene is highly heat-inducible and encoded protein shows transactivation potential and nuclear–cytoplasmic localization. *HvHSFA2e* is a class A HSF, consisting 371 amino acids. It displays the presence of HSF domain (DNA-binding domain), oligomerization domain, transactivation domain, and nuclear localization signal (A), transactivation potential of *HvHsfA2e* in GAL4BD filter lift assay (B), qRT-PCR-based assessment showed the heat and drought inducible expression of *HvHSFA2e* gene in WT seedlings; heat shock (HS) exposure was given at 42 °C for 30 min., 2 h and drought expo-

sure was given for 2 weeks. *snoR14* gene was used as internal control reference for calculating the expression level in terms of relative fold changes by the  $\Delta\Delta C_t$  method (C), *HvHSFA2e* ORF was cloned under *CaMV35S* promoter, C-terminal GFP fused construct *GFP:HvHSFA2e* transiently expressing in *N. benthamiana* leaves was found to be localized in nucleus and cytoplasm (D). (HS heat shock, DBD DNA binding domain, HR-A/B hydrophobic repeats A and B, NLS nuclear localization signal, AHA aromatic–hydrophobic-acidic amino acid domain)

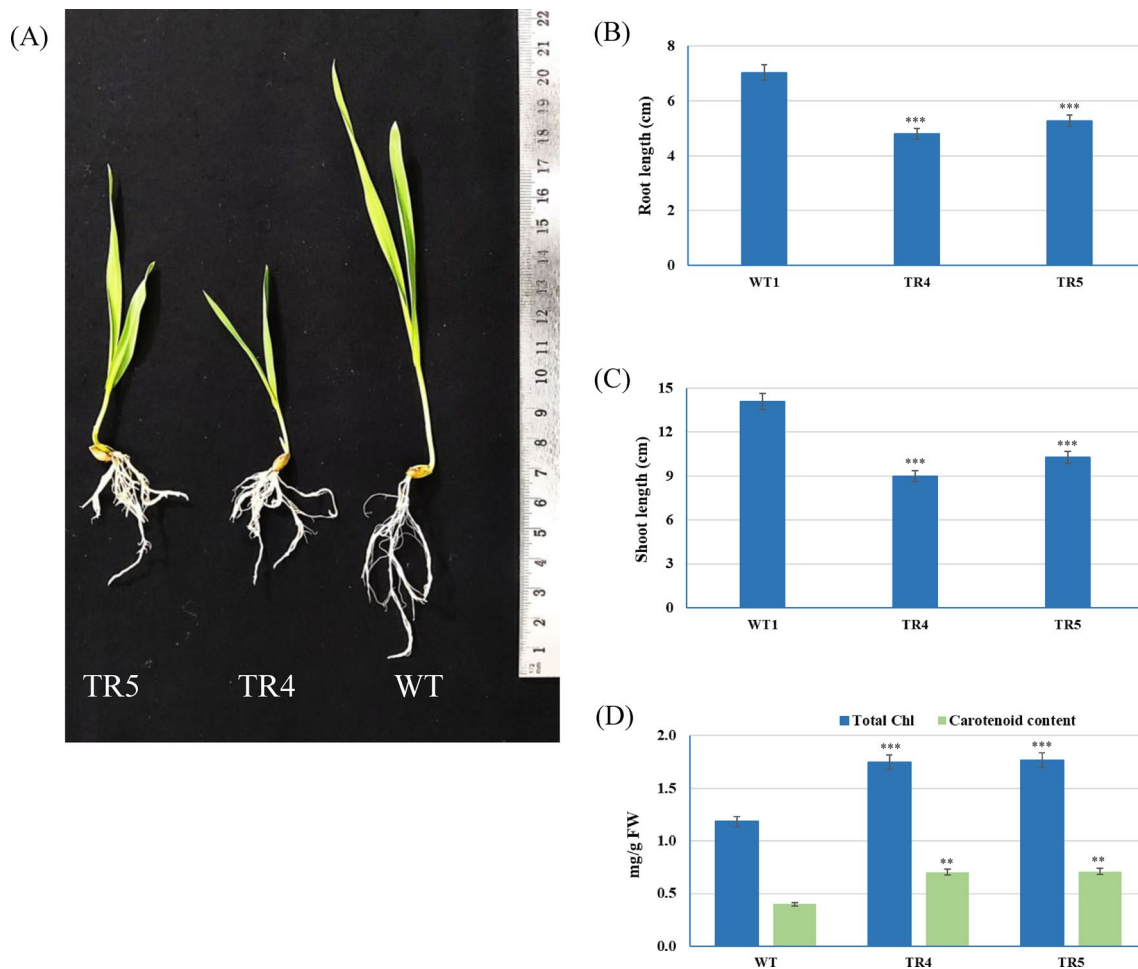
The expression of *Gusplus* reporter gene was assessed through histochemical analysis at different stages of tissue culture as well as in mature transgenic lines. The callus, regenerants as well as the mature plants of T0 progeny showed the expression of reporter gene (Supplementary

figure 3). Further, expression of *HvHSFA2e* was assessed till T2 progeny and the TR4 and TR5 lines which showed stable constitutive expression were selected for biochemical and transcriptome analysis (Supplementary Figure 4).

The subcellular localization of HvHSFA2e protein was analyzed by constructing a fusion gene with GFP at C-terminal and transiently expressing it in *N. benthamiana* leaves. The confocal laser scanning microscopy showed the uniform localization of fusion protein in cytoplasm as well as nucleus, suggesting the cytoplasmic and nuclear localization of the HvHSFA2e protein (Fig. 1D).

## Overexpression of HvHSFA2e resulted in reduced growth rate and increased photosynthetic pigment formation in seedlings

The *HSF* genes have been reported for their role in plant growth and development. To investigate the effect of *HvHSFA2e* overexpression on plant growth, we observed the growth pattern of WT and transgenic lines (15 plants each) under controlled optimal conditions. The transgenic lines showed slow shoot and root growth during seedling and early vegetative phase in comparison to WT plants. The 10-day-old transgenic seedlings showed significant reduction in growth rate in the form of reduced shoot and root length (TR4: shoot-9.0 cm, root-4.8 cm; TR5: shoot-10.3 cm, root-5.3 cm) when compared to WT seedlings



**Fig. 2** Plant growth and photosynthetic pigments. After 10 days of germination, the impact of *HvHSFA2e* overexpression on seedling growth was observed. WT and *HvHSFA2e* overexpression lines were grown under optimal conditions (18/16 °C day/night temperature and 16 h photoperiod) for 10 days and photographed for morphological assessment (A), the graphs show the average shoot and root length of

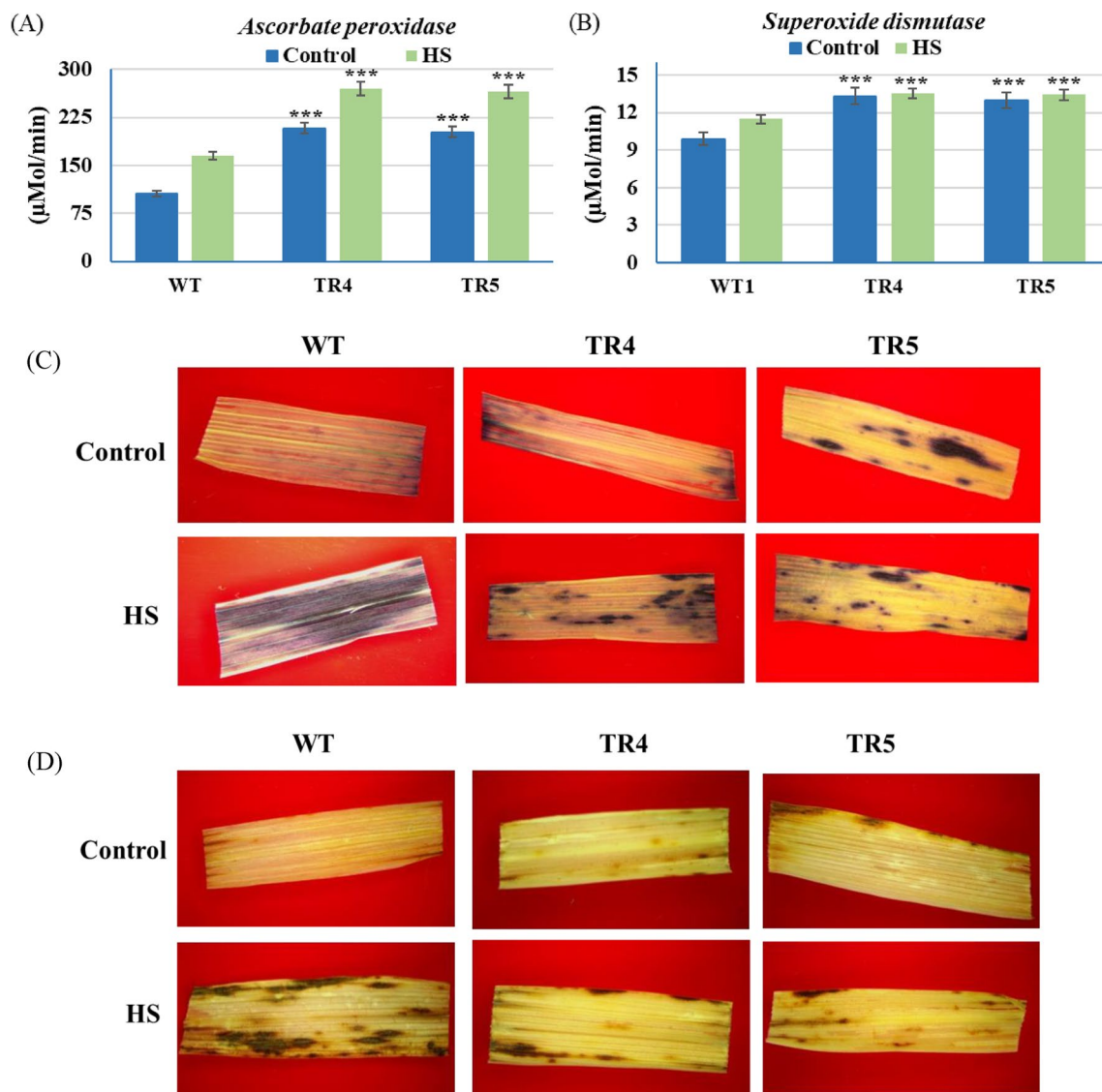
15 seedling each WT and transgenic (TR4, TR5) lines (B,C), transgenic plants show increased chlorophyll and carotenoid content in comparison to WT (D). Data represent mean  $\pm$  SE. Asterisks (\*, \*\*, \*\*\*) were used to indicate significant differences between WT and transgenic lines calculated using Student's *t* test, \**P* value < 0.05, \*\**P* value < 0.01, and \*\*\**P* value < 0.001

(Shoot–14.1 cm, root–7.04 cm) (Fig. 2A, B, and C). However, there were no phenotypic differences in WT and transgenic plants grown under control conditions during late vegetative and reproductive phase (data not shown).

The estimation of photosynthetic pigments in WT and transgenic plant leaves showed that the total chlorophyll and carotenoid contents in transgenic plants were significantly higher (TR4—Chl: 1.75, carotenoid: 0.704; TR5—Chl: 1.77, carotenoid: 0.71 mg/g FW) than that in WT (total Chl: 1.18, carotenoid: 0.399 mg/g FW) plants (Fig. 2D), indicating an enhanced synthesis of photosynthetic pigments in *HvHSFA2e* O.E.

### Overexpression of *HvHSFA2e* improves the activity of antioxidant enzymes and reduces the ROS accumulation and lipid peroxidation

To protect the plants from oxidative damage, the antioxidant enzymes determine the concentration of ROS species in plant cells by eliminating the excessive accumulation of ROS. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) are the two major components of enzymatic antioxidant defense system. The activities of these two enzymes were measured in transgenic lines (TR4 and TR5) as well as wild-type plants under control conditions. The transgenic



**Fig. 3** Antioxidant enzyme activity and ROS accumulation analysis. Activity of ROS-scavenging enzymes viz APX and SOD in WT and transgenic lines (TR4 and TR5) (A, B), visualization of Superoxide radical accumulation by NBT staining (C) and peroxide radical accumulation by DAB staining (D) in the leaves of wild-type (WT) and

*HvHSFA2e* overexpression lines (TR4 and TR5) under control and HS conditions. Data represent mean  $\pm$  SE. Asterisks (\*, \*\*, \*\*\*) represent significant differences between WT and transgenic lines calculated using Student's *t* test, \**P* value < 0.05, \*\**P* value < 0.01, and \*\*\**P* value < 0.001



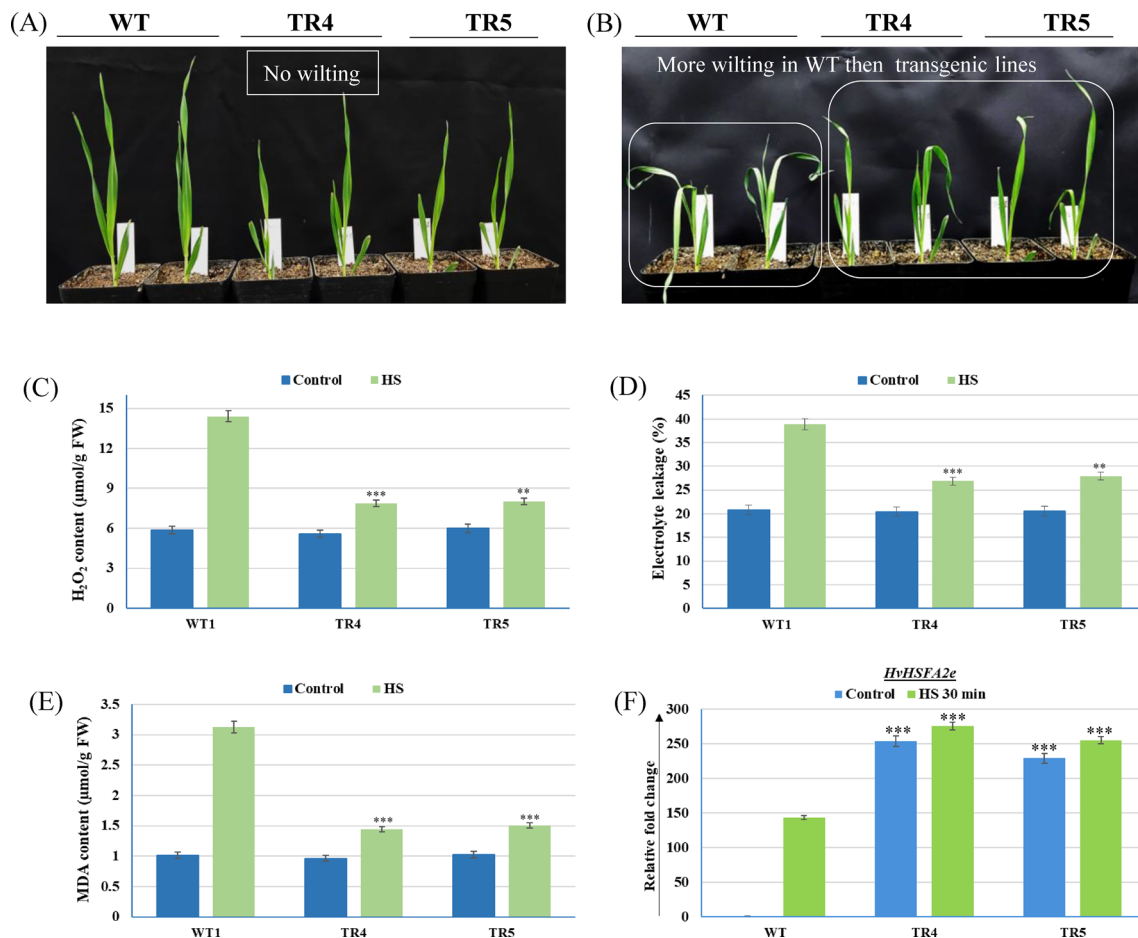
lines showed significantly enhanced activity for both the enzymes in comparison to wild-type barley plants (Fig. 3A, B).

The high temperature stress causes increased generation and accumulation of ROS species. The unchecked accumulation of ROS species results in oxidative damage to plant cells at molecular and physiological level. The accumulation of peroxide and superoxide anions was investigated in the leaf tissue of wild type as well as transgenic lines (TR4 and TR5) under control and heat stress conditions. The accumulation of superoxide free radical in leaf tissue was displayed through the NBT staining (Wohlgemuth et al. 2002), whereas the peroxide radical accumulation was visualized by DAB staining (Liu and Friesen 2012). Under control conditions, no significant difference was observed in ROS accumulation in transgenic lines and wild-type plants; however, the transgenic lines displayed significantly less

accumulation of peroxide as well as superoxide anions in leaf tissue in comparison to wild-type barley plants under heat stress (Fig. 3C, D).

### Overexpression of *HvHSFA2e* improves the thermotolerance in transgenic barley plants

To investigate the impact of *HvHSFA2e* overexpression on plant thermotolerance, we analyzed the performance of transgenic lines under heat stress. Two-week-old WT and transgenic plants were subjected to a heat shock at 40 °C for 5 h, WT plants displayed severe leaf damage in the form of wilting, whereas transgenic lines showed resistance in comparison to the WT (Fig. 4A, B). Moreover, the quantification of H<sub>2</sub>O<sub>2</sub> content in leaf tissue also revealed the more deleterious impact on WT plants in comparison to *HvHSFA2e* O.E., as under heat stress conditions, H<sub>2</sub>O<sub>2</sub> content



**Fig. 4** Physiological response of wild-type (WT) and *HvHSFA2e* overexpression lines under heat stress. 2-week-old WT and overexpression lines were subjected to heat stress (40 °C for 4 h), WT plants show comparatively more negative impact than transgenic lines in terms of visible symptoms (A,B), H<sub>2</sub>O<sub>2</sub> content (C), electrolyte leakage (D), and MDA content (E). Relative expression of *HvSFA2e* gene

in WT and transgenic lines under control and heat shock conditions. Error bars represent standard error (SE). Data represent mean  $\pm$  SE. Asterisks (\*, \*\*, \*\*\*) were used to indicate significant differences between WT and transgenic lines calculated using Student's *t* test, \**P* value < 0.05, \*\**P* value < 0.01, and \*\*\**P* value < 0.001

was significantly higher in WT plants (14.41  $\mu\text{mol/g}$  FW) in comparison to transgenic lines (TR4-7.86, and TR5-8.04  $\mu\text{mol/g}$  FW) (Fig. 4 C).

The measurement of MDA content and electrolyte leakage showed the same trend as there was no significant difference among the WT and transgenic plants under control condition, though under heat stress, WT plants showed significantly high MDA content (3.12  $\mu\text{mol/g}$  FW) and electrolyte leakage (38.88%) in comparison to transgenic lines (MDA content—TR4: 1.44, TR5: 1.50  $\mu\text{mol/g}$  FW; electrolyte leakage—TR4: 26.82%, TR5: 27.89%) (Fig. 4D, E). These results show that the *HvHSFA2e* overexpression lines are comparatively better in maintaining the membrane integrity and limiting the lipid peroxidation than WT plants under heat stress conditions.

### Overexpression of *HvHSFA2e* improves the drought tolerance in transgenic barley lines

To analyze the impact of *HvHSFA2e* overexpression on plant drought tolerance, WT and transgenic plants were grown up to 2 weeks under optimal controlled condition, then subjected to drought condition by stopping the watering for next 2 weeks. As shown in Fig. 5, severe drought-induced leaf wilting was observed in WT plants after a drought treatment of 2 weeks, although the symptoms were less severe in case of transgenic plants. Transgenic lines maintained higher RWC and low electrolyte leakage, whereas WT plants showed loss in RWC and increase in electrolyte leakage under stress conditions. These findings indicate the role *HvHSFA2e* in enhancement of water retention capacity and cell membrane stability under water-limiting condition for improved plant performance.

### Global transcriptome analysis to reveal the *HvHSFA2e*-mediated modulation in major stress-related transcriptional cascades

The HSFs of subclass A2 have been reported for their role in transcriptional regulation of the genes related to heat stress response (HSR) (Ogawa et al. 2007; Xin et al. 2017; Li et al. 2018). RNA-seq analysis of overexpression lines TR4, TR5, and wild-type barley plants WT1, WT2 revealed the modulations in transcriptome (Fig. 6A).

A total of 893 genes were differentially expressed in *HvHSFA2e* overexpression lines, with 584 upregulated and 309 downregulated. Gene Ontology (GO) enrichment analysis identified the functional distribution of these transcripts. The enriched GO terms, visualized using REVIGO (Fig. 6B), were categorized into biological processes (stress response, thermotolerance, transport, development, macromolecule biosynthesis, and photosystem), molecular

functions (nucleic acid binding, transferase activity, oxidoreductase activity, terpene synthesis, vacuolar traffic, metal ion binding, and transmembrane transport), and cellular components (plasma membrane, nucleus, chloroplast, mitochondria, Golgi apparatus, ubiquitin ligase complex).

Major differentially expressed genes (DEGs) included heat shock factors (HSFs), heat shock proteins (HSPs), ABC transporters, antioxidant enzymes,  $\text{Ca}^{2+}$ -mediated signaling, and genes related to terpene and flavonoid biosynthesis (Fig. 6A). Specifically, ten HSF genes and various HSP family genes (*HvHSP20*, *HvHSP70*, *HvHSP90*, *HvHSP100*), along with molecular chaperones and chaperonins involved in protein homeostasis and stress tolerance, were upregulated.

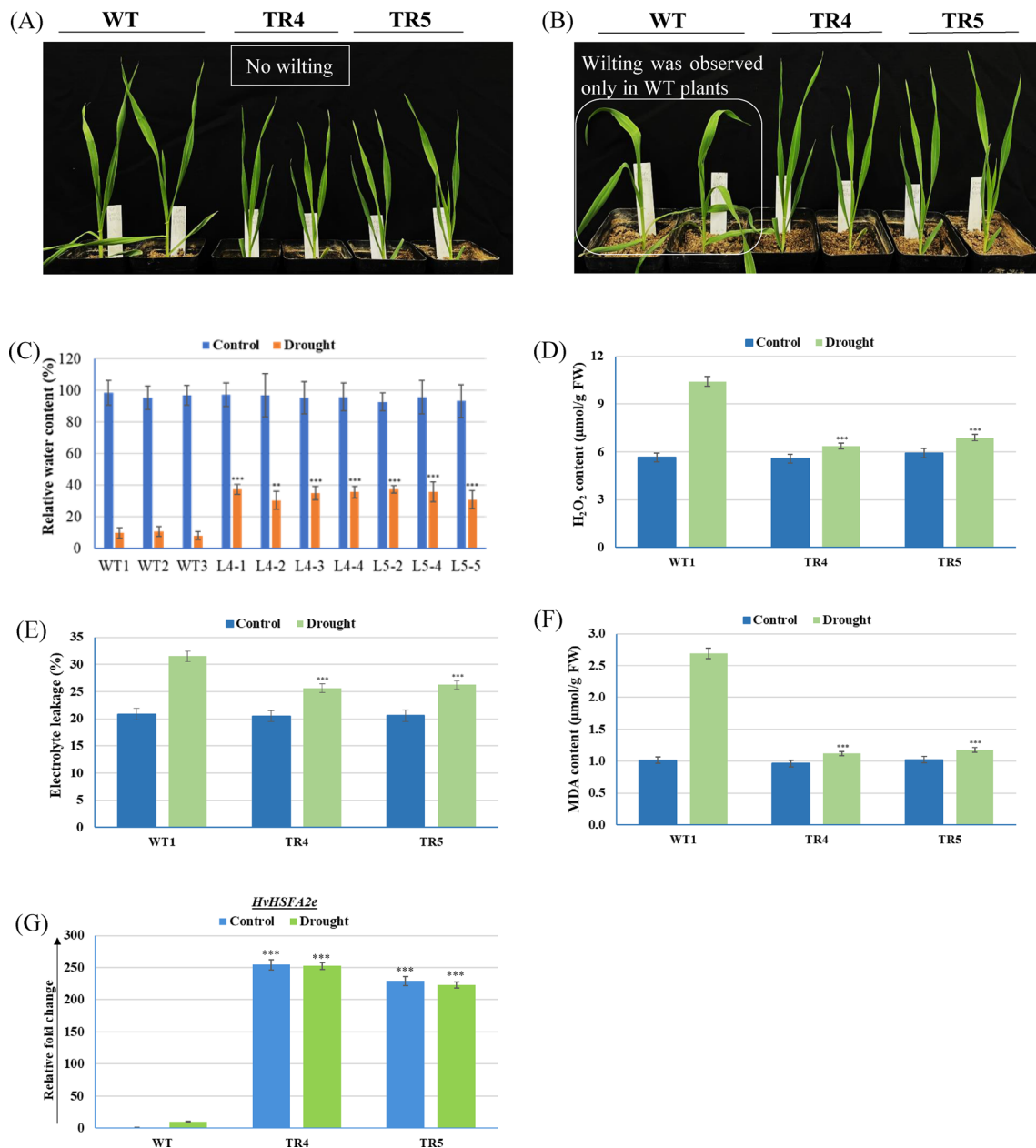
Antioxidant enzyme genes like *HvMDAR*, peroxidase, *HvGSHB*, *HvGSTF*, and *HvGPX* showed increased expression, helping to mitigate ROS accumulation under stress.  $\text{Ca}^{2+}$ -mediated signaling genes, including calcium-binding proteins, CIPK19, calcium uniporter protein, calcium sensing receptor, and CBPK, were also upregulated, highlighting their role in stress tolerance.

In addition, the overexpression of *HvHSFA2e* resulted in upregulation of ABA-related genes (NDR1/HIN1-like proteins, NCED3, bHLH transcription factors), terpene biosynthesis genes (*HvTPS1*, *HvTPS10*, *HvLIS*, *E-beta-caryophyllene synthase*, *zeta-carotene desaturase*), and flavonoid biosynthesis genes (*HvCHS2*, *HvCHI3*, WD repeat-containing protein, flavonoid O-methyltransferase, flavanone 3-dioxygenase 2, flavonoid 3'-monooxygenase).

These findings suggest that *HvHSFA2e* overexpression influences a wide range of stress response mechanisms, involving the secondary metabolism and regulatory pathways in barley.

### Validation of differential expression of HSP and HSF genes through qRT-PCR

The HSP and HSF family genes which play major role in HSR and thermotolerance showed differential expression in transcriptome analysis. The validation of their differential expression was done using qRT-PCR; the genes selected for qRT-PCR validation were *HvHSP70-2*, *HvHSP90-1*, *HvHSP100-2*, *HvHSP100-3*, *HvHSFA6b*, and *HvHSFB2c*. The result of qRT-PCR experiment showed consistency with RNA-seq data (Fig. 7), confirming the differential expression of these genes in *HvHSFA2e* lines as well as good quality of transcriptome data.



**Fig. 5** Response of wild-type (WT) and *HvHSFA2e* overexpression lines under drought stress. 2-week-old WT and overexpression lines were subjected to drought stress by stopping the watering for next 2 weeks. WT plants started showing early leaf wilting in comparison to transgenic lines (A, B), and transgenic lines maintained RWC under water-limiting condition (C), H<sub>2</sub>O<sub>2</sub> content (D), electrolyte leakage

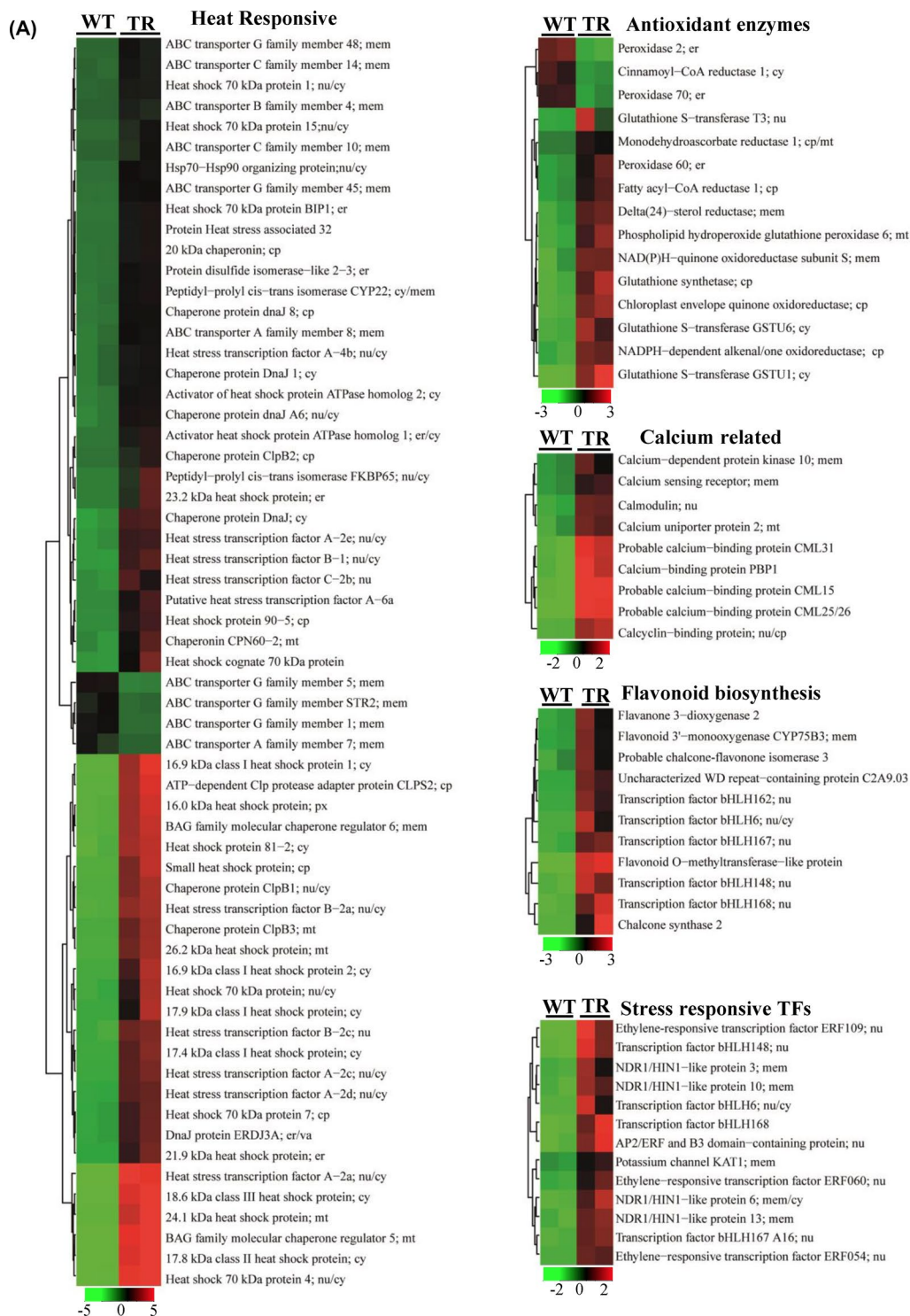
(E), and MDA content (F), relative expression of *HvSFA2e* gene in WT and transgenic lines under control and drought stress conditions (G). Data represent mean ± SE. Asterisks (\*, \*\*, \*\*\*) were used to indicate significant differences between WT and transgenic lines calculated using Student's *t* test, \**P* value < 0.05, \*\**P* value < 0.01, and \*\*\**P* value < 0.001

## Discussion

### HvHSFA2e is a heat-inducible transcription regulator

The *HSF* genes from model plants have been characterized for their role in plant heat stress response; however, there

is a scarcity of information about the role of these genes in stress response of cereal crops. Investigating the *HSF* gene family in barley (Mishra et al. 2020), we realized that these genes may contribute to the enhancement of multi-stress tolerance in crop plants. To evaluate this hypothesis, we cloned *HvHSFA2e*, a heat and drought inducible gene, to functionally characterize it through overexpression



**Fig. 6** **A** Heat maps showing modulations in transcriptome of *HvHS-FA2e* overexpression lines in comparison to WT control. Red color represents upregulation and green color shows down regulation, the color legend depicts the expression levels. **B** Illustration of GO terms enrichment for DEGs utilizing the BiNGO plugin within Cytoscape and visualized via the REVIGO web server. Subsections **A–B** denote

biological process terms (both upregulated and downregulated), sections **C–D** depict cellular component terms (both upregulated and downregulated), and segments **E–F** showcase molecular function terms (both upregulated and downregulated). Enriched significant GO terms are represented using corrected *P* values (color scale) and log values (bubble size). (DEGs: differentially expressed genes)



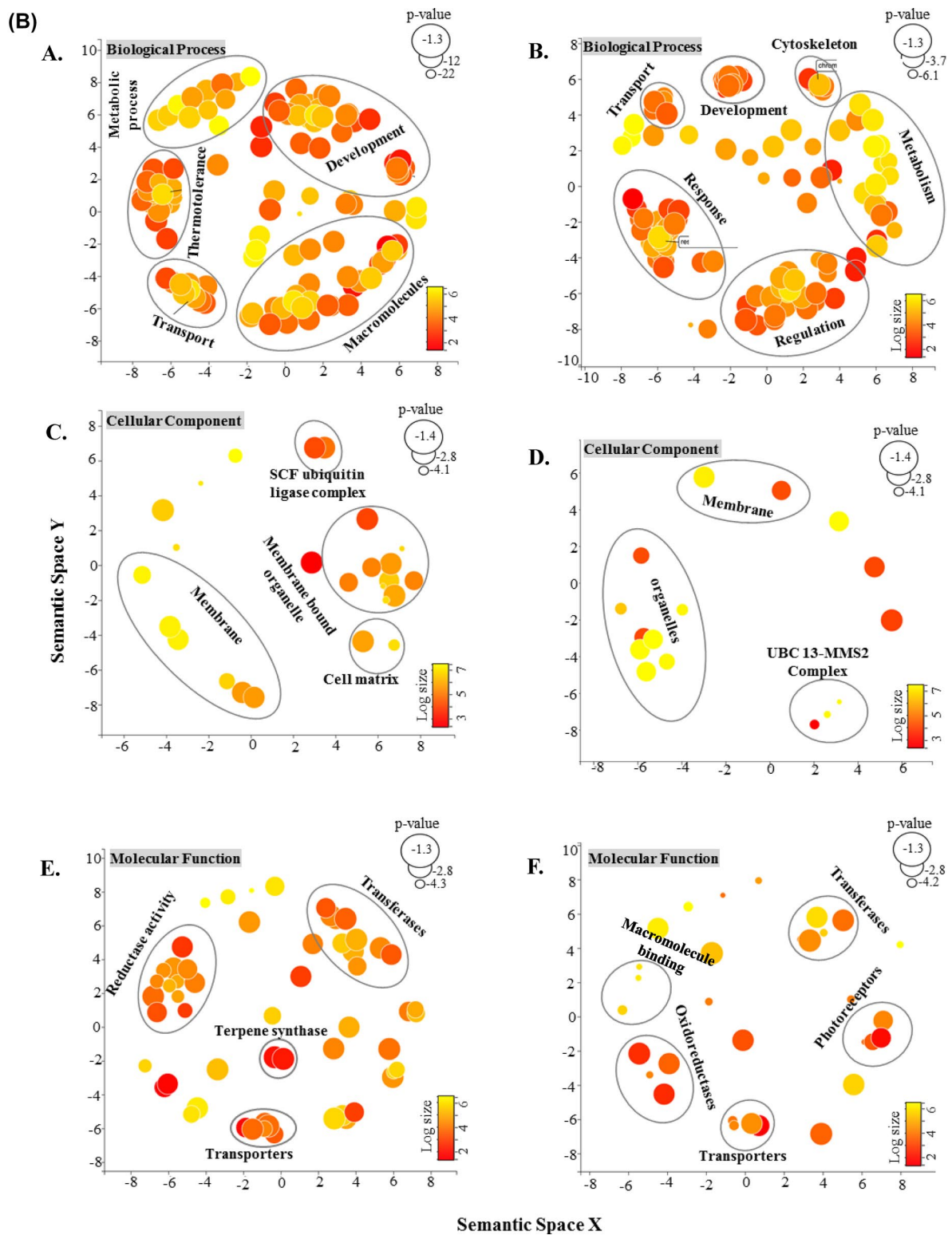
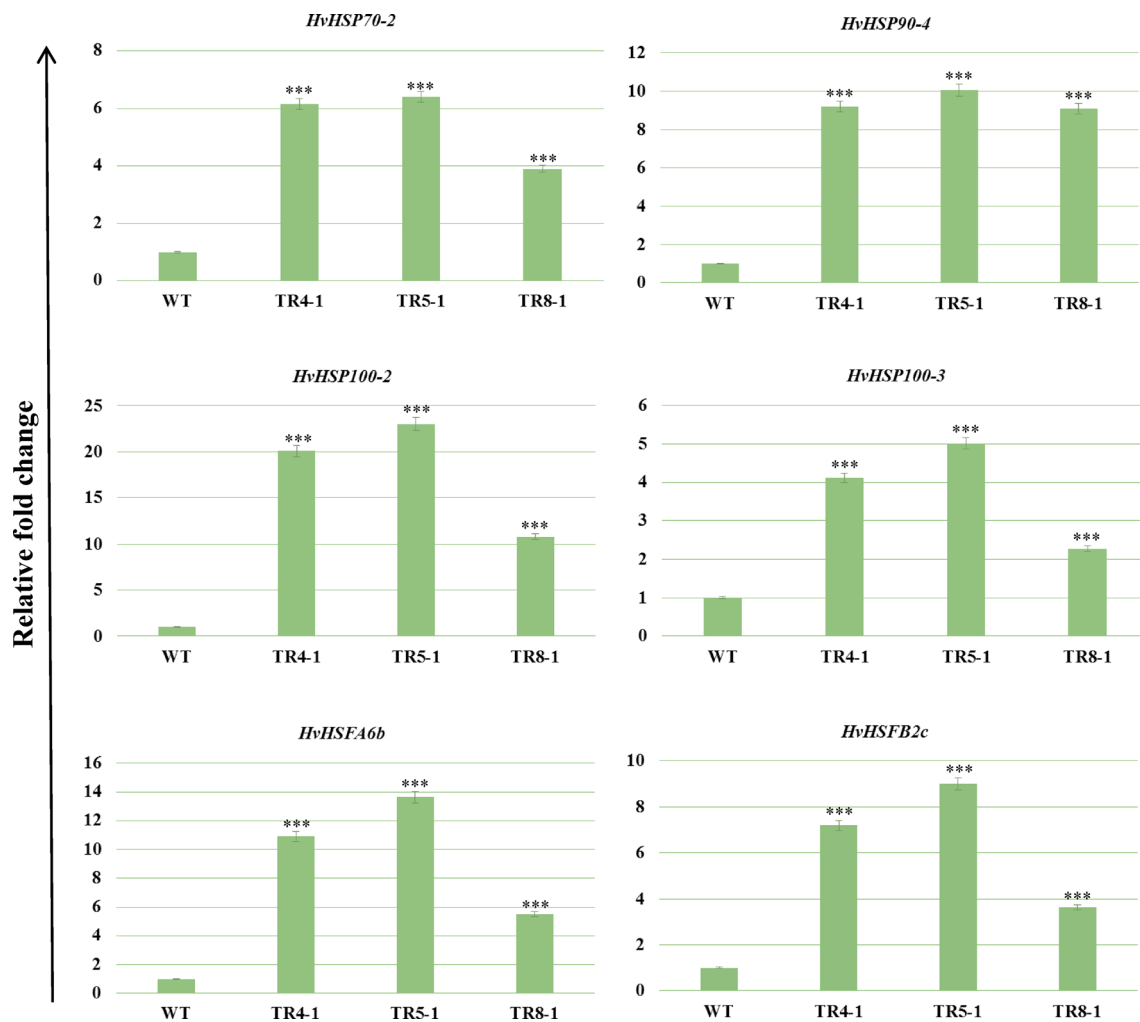


Fig. 6 (continued)

barley plants. The subcellular localization study revealed the nuclear-cytoplasmic nature of HvHSFA2e protein; the presence of HvHSFA2e protein in the nucleus suggests

its transactivation activity and regulatory role. The localization of FvHSFA2a-GFP and AtHSFA6b-YFP proteins showed a similar trend as within the plant cell, these were



**Fig. 7** Validation of differential gene expression in RNA-seq data through qRT-PCR analysis. qRT-PCR analysis of some upregulated abiotic stress-responsive genes in RNA-seq data in transgenic lines (TR4-1, TR5-1, and TR8-1) and wild-type (WT) plants. y-axis represent the relative fold change in gene expression level, *snoR14* gene

was used as internal control reference for calculating the expression by the  $2^{-\Delta\Delta Ct}$  method. Data represent mean  $\pm$  SE. Asterisks (\*, \*\*, \*\*\*) were used to indicate significant differences between WT and transgenic lines calculated using Student's *t* test, \**P* value < 0.05, \*\**P* value < 0.01, and \*\*\**P* value < 0.001

also distributed among nucleus and cytosol (Hu et al. 2015; Huang et al. 2016).

In wild-type plants, the expression of the *HvHSFA2e* gene is inducible in nature; hence for the functional characterization, we cloned full-length CDNA of this gene under the regulation of a constitutive promoter (*ZmUbi*) for overexpression. The *ZmUbi* promoter has been well studied and employed to achieve the constitutive expression of the targeted genes in cereal crops such as wheat, rice, and maize (Zang et al. 2018; Achary et al. 2020; Li et al. 2022a). The genetic transformation of barley cv. Golden promise was performed using the binary vector *pANIC6B* (Mann et al. 2012). *A. tumefaciens* does not naturally infect the monocot plants, yet the *Agrobacterium*-mediated genetic transformation has been successfully used to develop the stable transgenics in many monocotyledonous

species including barley, rice, wheat, maize, and triticale (Ziemenowicz et al. 2012; Zhang et al. 2016; Char et al. 2017; Hayta et al. 2019; Poonia et al. 2020). In our study, we used immature embryos of barley as explant and generated stable transgenic lines overexpressing *HvHSFA2e* gene through *Agrobacterium*-mediated genetic transformation approach. The transgenic lines showed stable constitutive expression of *HvHSFA2e*, which is otherwise inducible in wild-type plants. To investigate the transcription regulatory role of *HvHSFA2e* gene in thermotolerance as well as other abiotic stress responses, the impact of *HvHSFA2e* overexpression on barley global transcriptome was accessed through RNA-seq analysis.

The *HSF* genes are the key transcriptional regulators under adverse environmental conditions; not only in heat but also under other abiotic stresses, the *HSFs* modulate the

transcriptional cascades very precisely to confer stress tolerance in plant (Nover et al. 2001; Andradi et al. 2021). The transactivation assay revealed that HvHSFA2e possesses the transcriptional activation potential and may regulate the transcription of multiple downstream genes to modulate the stress response. In transcriptome analysis, several abiotic stress response-related genes were shown to be positively regulated in barley *HvHSFA2e* overexpression lines. Earlier, the overexpression studies showed the similar results for different *HSF* genes belonging to subclass A2 (Ogawa et al. 2007; Nishizawa-Yokoi et al. 2009; Banti et al. 2010; Li et al. 2024). In our study, we found that most of the DEGs were involved in heat shock response, drought stress response, Ca<sup>2+</sup>-mediated signaling pathway, ABA biosynthesis and signaling pathway, ethylene biosynthesis, flavonoid and terpene metabolism.

### **HvHSFA2e is a key regulator of Ca<sup>2+</sup>-mediated abiotic stress signaling and response pathways in barley**

Ca<sup>2+</sup> acts as a macronutrient and secondary messenger in plant growth, development, and stress response processes. It has a significant role in maintaining the membrane stability, hormonal regulation, and enzymatic reactions (Ahmad et al. 2016). The upregulation of calcium sensing receptor (CAS), calcium uniporter protein, calcium-dependent protein kinase 10 (CDPK10), Calmodulin, calcium-binding protein PBP1, and calyculin-binding protein (CYBP) was found in *HvHSFA2e* overexpression lines. The CDPKs are involved in phosphorylation-based activation of stress-responsive proteins. Zhao et al. 2021 reported that maize CDPK7 interacts and phosphorylates a HSP17 protein under high temperature stress. In the same study, they found that maize transgenics overexpressing *ZmCDPK7* showed enhanced thermotolerance in terms of reduced ROS content and improved concentration of antioxidants. We also noted the upregulation of *Respiratory burst oxidase homolog (RBOH)* gene which encodes a Ca<sup>2+</sup>-dependent NADPH oxidase. The NADPH oxidases generate the ROS under adverse environmental conditions to initiate the ROS-dependent stress response mechanism in plants (Wang et al. 2018b; Navathe et al. 2019). Sun et al. 2019 reported that RBOH-generated superoxide is necessary to maintain the heat stress memory, and strong suppression of RBOH resulted in reduced acquired thermotolerance in tomato plants. Several studies based on identification and expression analysis of *RBOH* genes in crop and model plants depicted their essential role in ROS-dependent signaling and abiotic stress tolerance (Torres and Dangel 2005; Miller et al. 2008; Sun et al. 2019).

Most of the class A *HSF* genes possess transactivation activity and activate the expression of other *HSFs*, *HSPs*, and antioxidative defense system-related genes (Xin et al.

2010; Lavania et al. 2018; Singh et al. 2021; Li et al. 2022b). The *HSFA2a*, *A2c*, *A2d*, *A2e*, *A2f*, *A3*, and *A7* genes showed significant upregulation in rice plants under heat stress conditions (Chauhan et al. 2011). Out of 23 barley *HSFs*, a total of 10 genes, namely *HvHSFA2a*, *HvHSFA2e*, *HvHSFA4a*, *HvHSFA6a*, *HvHSFA6b*, *HvHSFA7b*, *HvHSFB1*, *HvHSFB2b*, *HvHSFB2c*, and *HvHSFC2b* showed upregulation in barley *HvHSFA2e* overexpression lines. These observations suggest that along with constitutively expressing *HvHSFA1a*, heat-inducible *HvHSFA2e* acts as a key modulator of transcriptional cascades under high-temperature stress. These upregulated HSFs have been reported to improve abiotic stress tolerance when overexpressed in plants. Meena et al. (2022) reported that *TaHSFA6b-D* is localized to nucleus under heat stress and links the unfolded protein response with HSR to regulate the protein homeostasis under stress conditions. Overexpression of *LlHSFA4* improves the basic thermotolerance through the modulation in expression of downstream genes of HSR (Wang et al. 2022). The higher expression level of *HSF* genes has been reported to upregulate the *HSP* gene of different classes. Poonia et al. (2020) reported the upregulation of Small *HSPs*, *HSP70*, *HSP90*, and *chaperonin60* genes in barley transgenic lines overexpressing a wheat heat stress transcription factor, *TaHSFA6b*. The *HSPs* have been well characterized for their role in structural and conformational maintenance of heat susceptible proteins. Most of the overexpression studies of *HSF* genes have reported the constitutive upregulation of *HSP* genes. Xin et al. 2010 found that *HSP25*, *HSP70*, and *HSP101* genes expressed constitutively in *Arabidopsis* transgenic plants overexpressing the lily *HSFA2* gene. Recently, Li et al. (2024) also reported that overexpression of *TaHSfA2-11* upregulated many heat response genes, including *HSP16.6* and *HSP21*. Similarly, we found the upregulation of *HvHSP20*, *HvHSP70*, *HvHSP90*, and *HvHSP100* genes in barely *HvHSFA2e* overexpression lines. Among the *HSP90* genes, *HvHSP90-4* and *HvHSP90-5* showed significant upregulation in our study. The *HSP90* gene from soybean ameliorates the negative impacts of abiotic stress when overexpressed in *Arabidopsis* plants (Xu et al. 2013). *HSP100/ClpB* proteins show environmental conditions and development-specific expression in plants and devoid of these proteins make the plants extremely sensitive to heat stress (Mishra and Grover 2016). The four *HSP100* genes (*HvHSP100-1*, *HvHSP100-2*, *HvHSP100-3* and *HvHSP100-4*) showed an increase ranging from 8- to 11-folds in their expression in barely transgenic lines in present study. Keeler et al. (2000) reported that *HSP100/ClpB* proteins contribute to acquire thermotolerance in lima bean plants, based on their HSR-associated expression patterns. *HSP70* proteins are essential for maintaining the native conformation of heat sensitive proteins; as soon as heat shock strikes, these proteins dissociate from the HSF/HSP complex to bind

with heat-labile proteins, releasing the HSFs for activation of HSR (Sung et al. 2001). We observed the upregulation of *HvHSP70-2*, *HvHSP70-4*, *HvHSP70-5* genes in barley transgenic lines. The higher expression levels of *HSP70* genes have been reported to contribute in thermotolerance enhancement; such as *BcHSP70*, showing the maximum similarity with *AtHSP70-4* gene and *Paeonia lactiflora HSP70* conferred the thermotolerance when overexpressed in tobacco and Arabidopsis plants, respectively (Wang et al. 2016; Zhao et al. 2019).

In addition to HSPs, antioxidative defense systems, including the enzymatic and non-enzymatic antioxidants, play a pivotal role in protecting the molecular and physiological processes which are jeopardized by oxidative damage caused by heat stress in plants (Caverzan et al. 2016). Glutathione (GSH) and ascorbate are two key regulators of a cell's redox homeostasis. In Halliwell–Asada pathway (Ascorbate–Glutathione cycle), the GSH-mediated recycling of ascorbate occurs and the enzymes involved are ascorbate peroxidase (APX), monodehydroascorbate reductase (*MDHAR*), dehydroascorbate reductase (*DHAR*), and glutathione reductase (*GR*). GSH is one of the major thiol antioxidants found in plants, the capability interchange in reduced and oxidized forms enables the glutathione to maintain the redox homeostasis (Noctor et al. 2011). GSH-mediated redox homeostasis and glutathione biosynthesis-related genes—*Glutathione synthetase (GSH2)*, *Glutathione peroxidases (GPX4 and GPX6)* and *Glutathione S transferases (GSTF1, GSTT3, and GSTU6)*—notably upregulated in *HvHSFA2e* overexpression lines. *GSH2* gene catalyzed the biosynthesis of glutathione in plants and *GPX* genes mediate the glutathione-dependent scavenging of peroxide species to protect the plant cell from oxidative damage (Park et al. 2017; Li et al. 2021; Madhu et al. 2023). *GST* genes have been reported to express differentially under abiotic stress conditions as well as the enzyme encoded by these genes facilitate the glutathione conjugation to reduce the ROS accumulation (Liang et al. 2018; Kumar et al. 2018). The *GPX* genes have been reported to differentially express under stress conditions, the *GPX6* strongly upregulated in stress-exposed *Arabidopsis* plants and Filiz et al. (2019) suggested this might be the source of mitochondrial ROS generation to initiate the stress response pathways.

Ascorbate is an important ROS eliminating member of the plant antioxidant defense system as it can directly convert peroxide species into water molecules (Arora et al. 2002). The enzymes which regulate the cellular ascorbate pool are APX, MDHAR, and DHAR. We found upregulation of *MDHAR1* gene, encoding the enzyme monodehydroascorbate reductase 1 in *HvHSFA2e* overexpression lines. MDHAR enzyme catalyzes the conversion of monodehydroascorbate to ascorbic acid and the increased expression of corresponding gene has been observed to enhance the

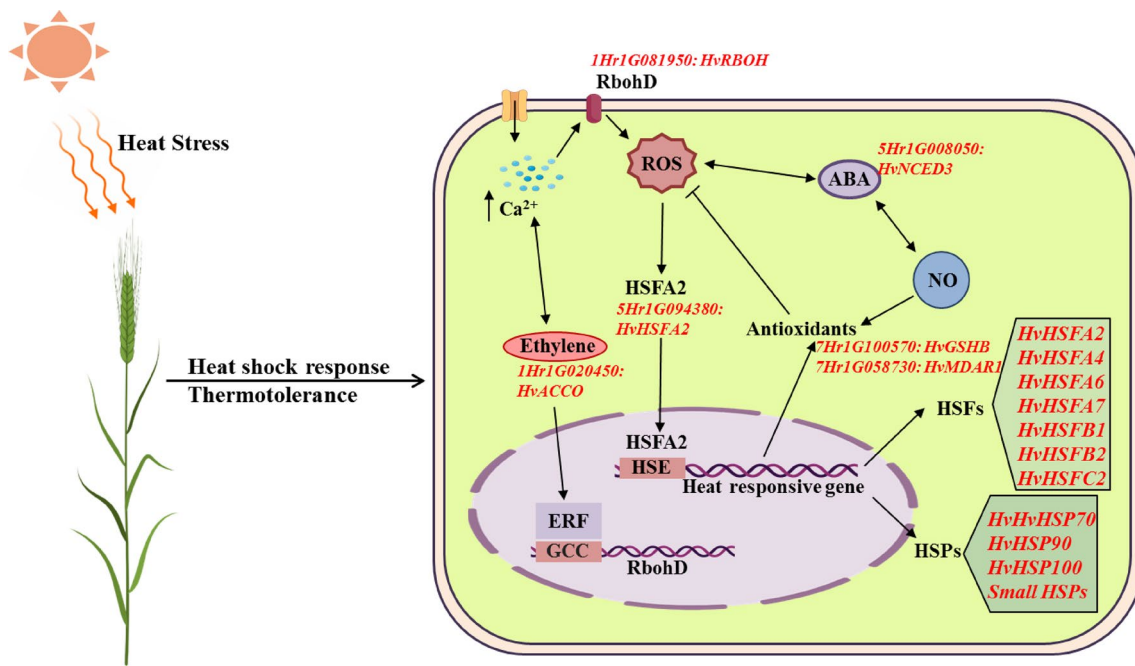
abiotic stress tolerance in plants (Yeh et al. 2019; Tanaka et al. 2021).

## Role of transcription factors in *HvHSFA2e*-mediated abiotic stress response

Transcription factors determine the transcriptome by regulating the gene expression in a condition specific manner (Chen and Zhu 2004). To survive in adverse environment, highly specific and precisely programmed modulations take place in transcription profiles in plants. The RNA-seq analysis of WT and *HvHSFA2e* overexpression lines showed the differential expression of HSFs, WRKY, MYB, MBF1C (multiprotein bridging factor 1c), bZIP (basic region/leucine zipper motif), ERF (ethylene-responsive factor), and NAC transcription factors, which are the major transcriptional regulators participating in reprogramming transcriptome during abiotic stress response. Among the HSFs, mainly the class A genes were upregulated which are known for their role in thermotolerance (Xin et al. 2010). WRKY transcription factors are exclusive to the plant kingdom; these are involved in plant growth, development, and response to environmental stress. The *WRKY* gene family members, *WRKY19*, *WRKY28*, and *WRKY71*, showed upregulation in present study. Arabidopsis *WRKY25* and *26* upregulate the transcription of thermotolerance-related genes including HSPs and ZATs (Li et al. 2009, 2011). Contrary to *WRKY* genes, *MYB* genes are present in all the eukaryotic genomes. *MYB20* and *MYB59* genes showed upregulated transcription in *HvHSFA2e* overexpression lines. *MYB20*-overexpressing *Arabidopsis* plants showed enhanced salt tolerance (Cui et al. 2013), whereas overexpression of rice *MYB55* gene in maize upregulated the stress-related genes, consequently enhancing the performance of transgenics under high-temperature stress (Casaretto et al. 2016).

The MBF1 genes show differential expression under abiotic and biotic stresses. The MBF1C gene was highly upregulated in *HvHSFA2e* overexpression lines in comparison to WT plants. The heat stress exposure has been reported to induce the accumulation of MBF1C protein leaf tissue; the *mbf1c* mutants showed a decrease in basal thermotolerance. Though, *MBF1C* overexpressing *Arabidopsis* transgenics showed increased basal and acquired thermotolerance in comparison to wild-type plants (Suzuki et al. 2008). In Arabidopsis triple *mbf1abc*, the seedlings showed the upregulation of *HSP70* and when this mutation was complemented with *MBF1C*, the expression level of *HSP70* was found similar to wild-type plants, showing that *MBF1C* is a negative regulator of *HSP70* (Arce et al. 2010). Recently, Tian et al. (2022) showed that wheat *MBF1C* regulates the translation of heat HSR-specific genes and it localizes along with stress granules.





**Fig. 8** Schematic representation displaying the role of *HvHSA2e* gene in abiotic stress response and tolerance. *HvHSA2e* overexpression upregulated the expression of *RbohD* gene which is responsible for the initiation of ROS-mediated stress signaling. Moreover, *HvHSA2e* positively regulates the expression of stress-responsive

transcription factors and defense-related proteins through binding to the heat shock element (HSE) present in the promoter of these genes. These genes activate and maintain different stress mitigation pathways (including HSR) to enhance the stress tolerance in transgenic lines

These observations show that the upregulation of these transcription factors in *HvHSA2e* overexpression lines play major role in transcriptional modifications which improves the abiotic stress response of transgenic lines.

### ***HvHSA2e* overexpression modulates the ABA and flavonoid biosynthetic pathways in barley**

The phytohormones play an important role in various signaling pathways. The involvement of abscisic acid (ABA) has been reported in acquired abiotic stress tolerance of plants. The modulation in endogenous ABA concentration determines the expression of several stress-responsive genes. The *abi-1* mutants, which are ABA insensitive due to the mutated ABA sensing protein phosphatase (ABA insensitive), showed increased heat sensitivity in comparison to wild-type plants; the *abi-1* mutants showed retarded growth and increased mortality at 37 °C (Larkindale and Knight 2002). Three major genes identified for their role in ABA biosynthesis are 9-*cis*-epoxycarotenoid dioxygenase (*NCED3*), zeaxanthin epoxidase (*ZEP*), and abscisic aldehyde oxidase (*AAO*) (Seo et al. 2000; Iuchi et al. 2001). The wild-type barley plants showed upregulation of *HvHSA2e* gene in vegetative root and shoot tissues when treated with ABA (Mishra et al. 2020). In present study, the *HvHSA2e* overexpression lines showed the constitutive upregulation

of zeta-carotene desaturase (*ZDS*), *NCED1*, *NCED3*, *AAO*, *NDR1/HIN1*-like proteins, and *bHLH* transcription factors which are key genes for ABA biosynthesis and signaling. Zeta-carotene desaturase (*ZDS*) is an essential enzyme for carotenoid biosynthesis which catalyzes the desaturation of zeta-carotene and forms neurosporene. The *zds* mutant showed hampered carotenoid biosynthesis (Dong et al. 2007). Carotenoids not only work as antioxidants but also act as a precursor for ABA biosynthesis in plants; thus, the impaired carotenoid biosynthesis may lead to a lack of endogenous ABA levels. The constitutive expression of *AgZDS* gene resulted in the increased level of carotenoids in transgenic *Arabidopsis* plants (Ding et al. 2021). The *NCED* gene encodes 9-*cis*-epoxycarotenoid dioxygenase enzyme which catalyzes the oxidative cleavage in a rate-limiting step of ABA biosynthesis. The *RiNCED1* gene in raspberry showed induction under heat, cold, salt stress as well as upon ABA treatment. The higher ABA content and improved stress tolerance was observed in the transgenic *Arabidopsis* plants overexpressing the *RiNCED* gene (Yang et al. 2021). Similarly, the ectopic expression of the rice *NCED3* gene in *Arabidopsis* conferred drought tolerance through increased ABA content and reduction in water loss (Hwang et al. 2010). The multiple aldehyde oxidase (*ALDO*) genes are involved in ABA biosynthesis in plants; among these genes, the *AAO3/ALDO3* gene has major contribution

in maintaining the endogenous ABA levels (Gonzalez-Guzman et al. 2004). Other than ABA biosynthesis-related genes, we observed the upregulation of *NDR1/HINI*-like protein 6 in *HvHSFA2e* overexpression lines. *NDR1/HINI*-like genes show strong induction under ABA treatment as well as these genes assist the ABA-signaling mediated abiotic stress responses in plants (Bao et al. 2016).

Ethylene is known as fruit ripening and senescence-related plant hormone; however, it also has an important role in plant stress response (Iqbal et al. 2017). The three enzymes, *S*-adenosyl-*l*-methionine synthetase (SAM synthetase), 1-aminocyclopropane-1-carboxylic synthase (ACC synthase), and 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), regulate the ethylene biosynthesis process. *ACO* gene-encoded ACC oxidase is exclusively dedicated to catalyze the final step of ethylene biosynthesis from *S*-adenosyl-*l*-methionine (Ruduś et al. 2013; Pattyn et al. 2021). In our study, *HvHSFA2e* overexpression lines showed significantly high expression of *ACO*, *ERF109*, *ERF54*, *ERF60* genes in comparison to wild-type barley plants. Recently, Huang et al. (2021) reported that ethylene response factors (ERFs) may directly enhance the thermotolerance by regulating the transcription of *HSFA2* gene and subsequently increasing the expression of other abiotic stress-responsive genes. In the same study, they found that the mutation in multiple *ERF* genes results in compromised basal thermotolerance.

The plant secondary metabolism is greatly influenced by the environmental cues. Metabolic reprogramming is one of the acclimation methods in plants to survive the environmental stress. The secondary metabolites have diverse physiological functions such as ROS homeostasis, protection from photo inhibition, enzyme activation, regulation of osmolarity and signal regulation (Zandalinas et al. 2017; Delfin et al. 2019). Flavonoids are present across the plant kingdom and these secondary metabolites have been studied for their antioxidant properties. Under the environmental stresses, the increase in flavonoid biosynthesis and accumulation has been reported in vegetative plant tissues (Winkel-Shirley 2001, 2002). The chalcone synthase (CHS) and chalcone isomerase (CHI) are the genes responsible for flavonoid biosynthesis regulated by Myb/bHLH transcription factors (Xu et al. 2015). The transcription of *CHS2*, *CHI3*, *bHLH* (*bHLH6*, *bHLH148*, *bHLH162*, *bHLH167* and *bHLH168*), and Myb (*Myb20* and *Myb59*) genes was higher in *HSFA2e* overexpression lines in comparison to wild-type barley plants. Many previous and recent studies have reported the stress inducible nature of *CHS* genes; moreover, the characterization of an okra *CHS* gene in *Arabidopsis* revealed its role in enhancing the abiotic stress tolerance (Wang et al. 2018a, 2022; Yao et al. 2019; Singh and Kumaria 2020).

Terpenes are the volatile secondary metabolites which enable the plants to interact directly with biotic and abiotic

both kinds of environmental factors. A wide and diverse range of terpenes is generated from geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) precursors by the activity of terpene synthase enzymes, encoded by *TPS* gene. In *HvHSFA2e* overexpression lines, the upregulation of *TPS1* (*acyclic sesquiterpene synthase*), *TPS23* (*beta-caryophyllene synthase*), *LIS* (*linalool synthase*) genes was observed. In previous studies, the differential expression of *TPS* genes has been reported under high temperature and other abiotic stress conditions (Zhang et al. 2019). Lee et al. (2015) reported the conversion of GPP into limonene and other monoterpenes by rice *TPS20* protein; moreover, when subjected to abiotic stress, the rice *TPS20* gene showed an upregulated transcription. Based on their experiments on *Quercus* seedlings, Peñuelas and Llusia (2002) suggested that monoterpenes may contribute to thermotolerance by increasing the photochemical efficiency and reducing the photodamage at high temperatures.

## Conclusion

Functional characterization of stress response regulating genes in crop plants is essential to mitigate the yield reductions caused by heat stress. In this study, we characterized *HvHSFA2e* gene, which exhibited the highest expression in the vegetative shoot of wild-type barley exposed to a 30-min heat shock. Transcriptome analysis of transgenic lines revealed differential expression of genes related to plant stress response and ABA biosynthesis. The findings of present study suggests that *HvHSFA2e* plays a key role as transcriptional regulator in plant stress responses (Fig. 8). Biochemical analysis showed increased activation of enzymatic antioxidants and a subsequent reduction in ROS accumulation, contributing to enhanced drought and heat tolerance in the transgenic lines. Based on these findings, we propose that the *HvHSFA2e* gene is a promising target for genetic manipulation to develop the stress-tolerant varieties in temperate cereals to sustain the adverse environmental conditions such as high temperature and water depletion.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00299-024-03251-6>.

**Acknowledgements** This work was supported by a grant (CRG-002579) to HC by SERB-DST, Government of India. SKM and AKP thank MHRD and SB, MK thank UGC for providing the research fellowships. We also thank GeneBank, NARO Japan for providing plasmid harboring Fl-cDNA of *HvHSFA2e*.

**Author contributions** SKM raised and confirmed transgenic lines overexpressing *HvHSFA2e*. SKM, CC, SB, AKP, PS, MK, SG, and AK performed the experiments and analyzed data. HC, DS, and HG guided the study and finalized the manuscript. All authors read and approved the manuscript.

**Funding** This study was supported by SERB INDIA,CRG-002579,Harsh Chauhan

**Data availability** The RNA-seq data (accession number GSE241562) generated and analysed during this study is available in the GEO, NCBI database.

## Declarations

**Conflict of interests** Authors declare no conflict of interest.

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