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Characterization of novel heat-responsive transcription factor (TaHSFA6e) gene involved in regulation of heat shock proteins (HSPs) — A key member of heat stress-tolerance network of wheat

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ABSTRACT

Heat stress has an adverse effect on the quality and quantity of agriculturally important crops, especially wheat. The tolerance mechanism has not been explored much in wheat and very few genes/ TFs responsive to heat stress is available on public domain. Here, we identified, cloned and characterized a putative TaHSFA6e TF gene of 1.3 kb from wheat *cv*. HD2985. We observed an ORF of 368 aa with Hsf DNA binding signature domain in the amino acid sequence. Single copy number of TaHSFA6e was observed integrated in the genome of wheat. Expression analysis of TaHSFA6e under differential HS showed maximum transcripts in wheat *cv*. Halna (thermotolerant) in response to 38 °C for 2 h during pollination and grain-filling stages, as compared to PBW343, HD2329 and HD2985. Putative target genes of TaHSFA6e (*HSP17*, *HSP70* and *HSP90*) showed upregulation in response to differential HS (30 & 38 °C, 2 h) during pollination and grain-filling stages. Small *HSP17* was observed most triggered in Halna under HS. We observed increase in the catalase, guaiacol peroxidase, total antioxidant capacity (TAC), and decrease in the lipid peroxidation in thermotolerant *cvs*. (Halna, HD2985), as compared to thermosusceptible (PBW343, HD2329) under differential HS. Multiple stresses (heat - 38 °C, 2 h, and drought - 100 mL of 20% polyethylene Glycol 6000) during seedling stage of wheat showed positive correlation between the expression of *TaHSFA6e*, putative targets (*HSP70*, *HSP90*, *HSP17*) and TAC. Halna (thermotolerant) performed better, as compared to other contrasting *cvs*. TaHSFA6e TF can be used as promising candidate gene for manipulating the heat stress-tolerance network.

1. Introduction

Abiotic stresses are serious threats to our agriculture causing significant damage to the plant growth and development as evident from the economic yield (Wahid and Close, 2007; Allakhverdiev et al., 2008). Among all the abiotic stresses, high temperature is one of the major stresses which have a negative impact on the yield of the crop plants (Charng et al., 2007; Yokotani et al., 2008). Heat stress is exposure of plant to temperature above ambient causing irreversible damage (Kumar et al., 2015). Heat stress affects the cellular membrane, photosynthetic machinery, and enzymatic activities associated with metabolic pathways and defense network of plants (Kumar et al., 2012). Due to HS, there is outburst of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), etc. which is very lethal for the plants. Free oxygen radicals damage the key enzymes involved in different pathways, other than damaging the membrane of the cellular organelles. Therefore, high temperature has become a major concern for the growth and yield of agriculturally important crops all over the world (Meehl et al., 2007).

Wheat is staple food grain crop of major areas of the world and is

Abbreviations: HSF, Heat shock transcription factor; HSP, heat shock protein; SAGs, stress associated genes; PEG, polyethylene Glycol 6000

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highly sensitive to HS during the grain-filling stage (terminal heat). Even slight fluctuation in environmental temperature severely affects the growth of the plant, as evident from the decrease in the quantity and quality of the grains. Heat stress especially during the flowering and grain-filling stages severely affects the growth and reduces the yield of wheat. Heat stress causes drying of stigmatic surface, pollen sterility, improper fertilization, disintegration of photosystem II, reduction in photosynthetic rate, improper transportation of photosynthates, small and disintegrated starch granules, and ultimately shriveled seed grains. Exposure to extreme HS may also leads to cell death due to accumulation of free oxygen radials and denaturation of enzymes/ proteins (Grover et al., 2013).

Plants has inbuilt defense mechanism to cope up with the change in the environmental temperature for their survival by over-expression of stress-associated genes/ proteins (SAGs/ SAPs) involved in thermotolerance. The survival of plant under HS depends on the cellular stress-response system which involves activation of wide range of transcription factors (TFs) and SAGs (Mittler, 2006). One of the key SAPs is Heat Shock Proteins (HSPs) which is basically molecular chaperone protecting the nascent proteins from denaturation/ aggregation under HS (Richter et al., 2010). The expression of HSPs is regulated by TFs which recognize the heat shock elements (HSE) within the promoter site of HSPs (Scharf et al., 2012). Transcription factors (TFs) are the protein which interacts with specific DNA sequence, regulating the rate of transcription of HS-responsive genes. It includes one or more DNA binding domains (DBDs), which interact with the specific DNA sequence of the genes to be regulated (Ptashne and Gann, 1997). HSFs are present in inactive form under normal condition and are activated upon exposure to stress by oligomerization and bind to HSE present in the promoter sequence of HS-responsive genes. HSFs form cytoplasmic complex with HSP90/HSP70 chaperon under normal condition and is released from the chaperon complex and binds to HSE of target gene under HS (Hahn et al., 2011; Lin et al., 2001). HSEs are mainly characterized by multiple inverted repeats of AGAAn sequences and at least three HSE motifs are required for efficient HSF oligomer binding in eukaryotic organism (Kumar et al., 2009).

HSFs plays very important role in tolerance against abiotic stress by regulating the expression of SAGs (Frank et al., 2009; Chauhan et al., 2011; Jin et al., 2013). HSF has been classified into four classes - HSFI, II, III and IV. HSF1 and HSF3 are involved in regulating the expression of HSPs under HS, whereas HSF2 and HSF4 are involved in gene regulation under normal condition (Akerfelt et al., 2007). HSFs also regulate the sensing and signaling of plant under HS (Scharf et al., 2012). HSFs consist of conserved binding domain (CBD), oligomerisation domain (HR-A/B), flexible linker of variable length, nuclear localize signal (NLS) and C- terminal activation domain (AHA).

Heat stress causes proteolysis of HSF1 and increase in the expression of HSF2 followed by enhanced DNA binding activity (Prasad et al., 2007). HSFs (*HSF3* and *HSFA4a*) have been reported to upregulate the expression of HSPs (*HSP70* and *HSP17*) in wheat under HS (Kumar et al., 2013). Similarly, HSF1a and HSA1b have been reported to regulate the expression of SAGs in Arabidopsis under HS. HSFA1 was reported to act as master regulator for the HS response in tomato (Liu and Charng, 2012).

The complexity of plant HSF has not much been explored in cereal crops like wheat, rice, maize, etc. Very limited HSFs have been identified and characterized in wheat (Chauhan et al., 2012). In present investigation, we used *de novo* transcriptomic approach to identify a putative HSF gene and further it was cloned, characterized and correlated with the expression of its target genes and thermotolerance of wheat.

2. Materials and methods

Seeds of four popular wheat *cvs*. reported to have contrasting behavior for thermotolerance (Thermotolerant - HD2985, Halna; Thermosusceptible - HD2329, PBW343) were procured from the Division of Genetics, Indian Agricultural Research Institute (IARI), India. The experiment was laid down inside microprocessor regulated chambers at National Phytotron Facility, IARI. Pre-treated seeds (Bavistin @ 0.25%) were sown in 72 pots (18 pots per cultivar) for pollination and grain-filling stages (considered as critical stages for wheat growth and yield). Plants were exposed to differential HS of 30 °C, 2 h (T_1) and 38 °C, 2 h (T_2) in a sinusoidal mode inside a microprocessor regulated chamber with an increment of 1 °C per 10 min till it reaches the desired stress temperature. One set of pots kept at 22 ± 3 °C was used as control in each group. The plants were well irrigated with water before exposure to HS. The HS treatment was given at pollination (Feekes scale - 10.52), and grain-filling stages (Feekes scale - 11.2) considered as the critical stages of wheat growth and development for 4 days before collection of samples in triplicates (Large, 1954). Samples (leaves) were collected in triplicate and immediately frozen in liquid nitrogen for further downstream processing. A pilot experiment was also conducted using the contrasting wheat genotypes (HD2985, HD2329, Halna, PBW343) to analyze the expression of identified TF gene (*TaHSFA6e*) in wheat seedlings (15 days old) grown inside growth chamber in four groups (12 pots each) and exposed to heat and drought stresses [control (C) - 22 ± 3 °C, heat stress (H) - 38 °C, 2 h; drought (D) – 100 ml of 20% polyethylene Glycol 6000, and heat stress + drought (H + D) conditions]. 100 ml of water was added to the control sample. Stress treatments were continuously given for 4 days before collection of samples in triplicates. Samples were kept frozen in liquid N_2 and stored at −80 °C for further downstream application.

2.1. Identification and cloning of heat-responsive transcription factor gene

An experiment was executed in our lab for the whole transcriptome sequencing of wheat *cvs*. HD2985 (thermotolerant) and HD2329 (thermosusceptible) under control (22 ± 3 °C) and HS-treated (42 °C, 2 h) conditions during pre-anthesis stage (NCBI BioProject database acc. no. **PRJNA171754**). Data-mining using different databases such as NCBI non-redundant, EnsemblPlant, etc. showed the presence of 37 transcripts having HSF-DNA binding domain in the sequence; homology based search showed overlapping among 18 of the transcripts. Based on the digital fold expression, transcript_21 (~1100 bp long) was selected for the cloning and characterization.

2.2. Reverse transcriptase PCR (RT-PCR) amplification of candidate gene

Transcript specific forward and reverse primers were designed using the Gene Fisher primer designing software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). The quality of the oligo's was checked using Oligo Analyser (IDT, USA) and was synthesized commercially. Total RNA was isolated from the collected samples by Trizol method (Invitrogen, UK); integrity of the RNA was checked on 1.2% agarose gel as well as using the Nano Drop (Thermo Fisher Scientific, USA). The optical density ratio of 260 to 280 > 2.0 was further selected for the cDNA synthesis using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, USA). The quality of the cDNA was checked on 1% agarose gel. The cDNA was used as template for the RT-PCR using the transcript specific forward and reverse primers (Table 1). The amplicon was resolved on 1% agarose gel, and further cloned in pGEMT-Easy vector (Promega, UK). The positive transformants were sequenced using Sanger's di-deoxy method (T7 and SP6 were used as universal forward and reverse primers). The full length gene was further amplified using 5' and 3' RACE (First Choice® RLM-RACE Kit, Thermo Fisher Scientific, USA) and cloned in the same maintenance vector.

Table 1
List of primers used for the cloning and expression analysis of *TaHSFA6e* TF and their putative target genes in wheat under differential heat stress.

Primers ID	Sequence (5'-3')	Tm (°C)
TaHSFA6e (F)	ATGGACGCGATGCCGCCGG	59.7
TaHSFA6e (R)	CTACTGCGGGCTAGTAGAACTCAG	59.1
qTaHSFA6e (F)	CGCAGAACATTCAGGAGCTT	60
qTaHSFA6e(R)	GGTCTCCTTCCCTCCAACTC	60
β-Act(F)	GCGGTCGAACAACTGGTATT	63.7
β-Act(R)	GGTCCAAACGAAGGATAGCA	63.8
qHSP17(F)	AGT GGGTAG CGAGTT TCCTGTGAT	65.2
qHSP17(R)	CAAACAACCACCAGTACG CACGAA	65.3
qHSP70(F)	CTTCGTCCAGGAGTTCAAGC	63.9
qHSP70(R)	GTCGATCTCGATGGTGGTTT	63.9
qHSP90(F)	TGATGATGGGTGGACTGCCAACAT	62.7
qHSP90(R)	TCTCGAAGAGCAGCATCACAAGGT	62.7

2.3. In silico characterisation of cloned TaHSFA6e TF gene

The nucleotide sequence of cloned *TaHSFA6e* TF was submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov). We retrieve the transcript sequence showing homology with *HSFA6e* from the transcriptome data of wheat *cv*. HD2329 (thermosusceptible) generated under control and HS-treated conditions. Both the nucleotide sequences from HD2985 and HD2329 was aligned using ClustalW alignment tool in order to analyze the sequence specific variations. The homology search of *TaHSFA6e* was carried out using the BLASTn and BLASTp tools of NCBI. The chromosomal localization of *TaHSFA6e* was characterized using NCBI genome blast on the chromosomes of the closely related species. Expasy translation tool (http://www.expasy.org/) was used to find out the amino acid sequence of the TaHSFA6e TF gene. Open Reading Frame (ORF) was identified using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Further, the identification of phosphorylation sites and kinase specific phosphorylation sites were predicted using the NetPhosK. Motif-based sequence analysis was carried out using the MEME suite Version 4.12.0. (http://meme-suite.org/tools/meme). We also used the Plant Transcription Factor databases (Plant TFDB) to characterize the cloned *TaHSFA6e* for its Gene Ontology, functional and homology based protein prediction (http://planttfdb.cbi.pku.edu.cn). Motif based scan analysis was carried out using Find Individual Motif Occurrence (FIMO) tool in order to find out all the positions where a protein motif matches with one or more protein sequences. We used different databases like PANTHER, SuperFamily, Gene3D, SMART, etc. to characterize the motif of predicted proteins showing homology with *TaHSFA6e* TF.

2.4. Southern blot analysis for the identification of copy number of TaHSFA6e TF gene

The cloned TaHSFA6e TF gene was used as probe for the southern blot analysis in order to identify the copy number in wheat genome. High quality genomic DNA was isolated from the leaves of wheat *cvs*. HD2985 and HD2329 through cetyl trimethylammonium bromide (CTAB) method. The isolated genomic DNA was checked for its quality through Bio Analyzer (Agilent, UK) and by electrophoretic separation on agarose gel (0.8%). 12 µg of genomic DNA from each sample were restricted with *Eco*RI, *Hin*DIII and *Bam*HI restriction enzymes following the standard protocol as mentioned in Lewandowski et al. (2015). The restricted genomic DNA was separated on 0.8% agarose gel followed by gel treatment and blotting using iBlotter (Invitrogen, UK). The other steps for the southern blotting were followed as mentioned in our earlier publication (Kumar et al., 2015). The X-ray film was developed using automatic developer machine and the result was scanned using Gel Doc Easy (Bio Rad, USA).

2.5. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the control and HS-treated samples collected during pollination and grain-filling stages by Trizol method (Invitrogen, UK). Quality of the isolated RNA was checked using Oligo Analyzer (Agilent, USA). Further, RNA integrity was verified on 1.2% agarose gel. CDNA was synthesized using oligo dT primer (Revert Aid^{TM} H minus First Strand cDNA Synthesis Kit, Fermentas, USA) following the instructions as given by the manufacturer's. CDNA used for the qRT-PCR was further diluted to 100 ng/µL and was quantified using Nano Drop (Thermo Fischer Scientific, USA). Primers for qRT-PCR were designed using the Gene Fischer primer designing software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/) (Table 1). The genes used for the expression analysis were *TaHSFA6e* TF (acc. no **KU291394**), *HSP90* (acc. no. **JN052206**), *HSP70* (acc. no. **JN561161**), and *HSP17* (acc. no. **JN572711**). Three biological and three technical replicates were used for te expression analysis. In brief, reaction mixture consist of 10 µL of 2x SYBR mix, 0.4 µL forward and reverse primers (10 mM) each, 100 ng/µL template, and finally volume was make up to 20 µL by adding nuclease free water. The PCR cycle was: 95 °C for 3 min, 39 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 5 s followed by plate read. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 3% agarose gel. β-actin gene (acc. no. **AF282634**) was used as endogenous control for normalizing the C_t value. Relative expression was calculated using the Pfaffl method (Pfaffl et al., 2002).

2.6. Expression analysis of cloned TaHSFA6e TF gene in wheat under differential HS by northern blot analysis

The total RNA isolated from the control (22 ± 3 °C) and HS-treated (30° and 38 °C for 2 h) wheat *cvs*. HD2985 and HD2329 were used for the northern blotting using radioactively labelled (^{32}p-dCTP) *TaHSFA6e* fragment as a probe. The protocol for the northern blot analysis was followed as mentioned in the Lewandowski et al. (2015) with slight modifications. The blotting of the RNA band was carried out using the iBlotter (Invitrogen, UK).

2.7. Effect of multiple stresses on expression of TaHSFA6e TF gene in wheat seedlings

The seedlings of contrasting wheat cultivars *i.e.* PBW343, Halna, HD2985 and HD2329 were sown in four groups (12 pots each) under regulated conditions (as mentioned in earlier section). Fifteen days old seedling (in triplicate) were exposed to treatments [control (C) - 22 ± 3 °C, heat stress (H) - 38 °C, 2 h; drought (D) – 100 ml of 20% polyethylene Glycol 6000, and heat stress + drought (H + D) conditions] continuously for 4 days and further samples were collected in liq. nitrogen for total RNA isolation, cDNA synthesis and qRT-PCR based expression analysis as mentioned in above section.

2.8. Protein assay of HSP90 – the target gene of TaHSFA6e TF

We selected one each of thermotolerant (HD2985) and thermosusceptible (HD2329) wheat *cvs*. for the protein assay. The samples were collected from wheat *cvs*. under control (22 ± 3 °C) and HS-treated conditions (T_1- 30 °C, 2 h; T_2 - 38 °C, 2 h). Total protein extraction was carried out using the Total Protein Extraction kit (G Bioscience, UK) and the quality of the protein was checked by 1D-SDS PAGE (Laemmli, 1970). The protein concentration was determined using the Bradford method (Bradford, 1976). Forty microgram of isolated protein was separated on 12% SDS PAGE gel, and further blotted on to the Nylon membrane using iBlotter (Invitrogen, UK). The blotted nylon membrane was developed as mentioned in our earlier publication (Kumar et al., 2016). We used the polyclonal anti-HSP90 specific antibody (Sigma, USA) and anti-rabbit HRP conjugated secondary

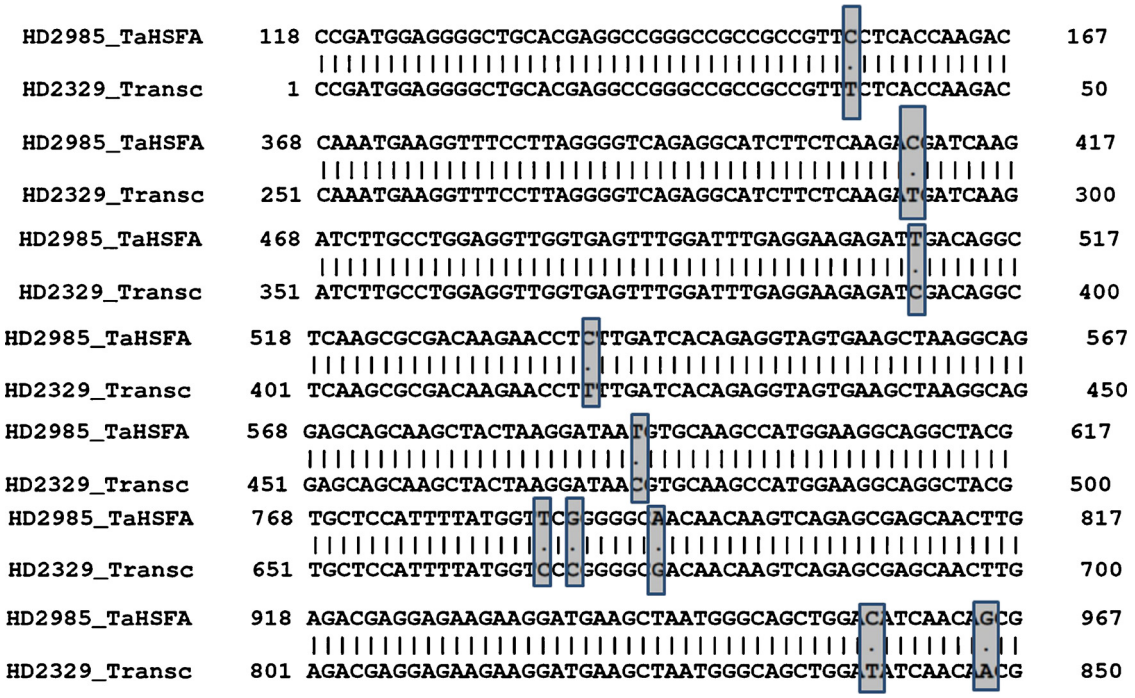

Fig. 1. Characterization of sequence specific variation in the TaHSFA6e TF gene cloned from wheat cv. HD2985 (thermotolerant) and HD2329 (thermosusceptible) under HS. Both the sequences of *TaHSFA6e* TF were retrieved through Transcriptome data mining (NCBI BioProject database acc. no. PRJNA171754).

antibody in the dilution of 1:2000 and 1:10,000 dilutions for the hybridization. The colored band developed at the specific size was captured using the Gel Doc Easy (Bio Rad, USA).

2.9. Estimation of thermotolerance-associated biochemical parameters in wheat

2.9.1. Guaiacol peroxidase (GPX) activity assay

The activity of guaiacol peroxidase (GPX) was determined following the method of Mika and Lüthje (2003) with slight modification. Fresh leaf material (1 g) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 10,000 *g* (4 °C) for 10 min. The tissue extracts were used for the quantification of soluble protein content by using Bradford method and analysis of peroxidase activity. The oxidation of guaiacol into tetraguaiacol was estimated by measuring the absorbance at 470 nm against the reagent blank, and using the extinction coefficient of 26.6 mM^{-1} cm^{-1}.

2.9.2. Catalase activity (CAT) activity assay

The activity of catalase enzyme was measured as described by Maehly and Chance (1954). Fresh leaf material (1 g) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 10,000 *g* (4 °C) for 10 min. The tissue extracts were used for the quantification of soluble protein content by using Bradford method (Bradford, 1976). For assaying CAT activity, the decomposition of H_2O_2 was followed by decline in the absorbance at 240 nm. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient, 39.4 mM^{-1} cm^{-1}) at 240 nm over a 3 min interval.

2.9.3. Estimation of lipid peroxidation

The level of lipid peroxidation was measured in terms of Thiobarbituric acid reactive substances (TBARS) content as per the protocol given by Heath and Packer (1968). Leaf sample (0.5 g) was homogenized in 10 mL of trichloroacetic acid (0.1%). The homogenate was centrifuged at 15,000 × *g* for 15 min. To 1.0 mL aliquot of the supernatant, 4.0 mL thiobarbituric acid (0.5%) in 20% TCA was added. The mixture was heated at 95 °C for 30 min in the laboratory electric oven (Scientific, India) and then cooled in an ice bath. After centrifugation at 10,000 × *g* for 10 min, the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated using the extinction coefficient value (155 mM^{-1} cm^{-1}).

2.9.4. Estimation of total antioxidant activity (TAC)

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain (1999) was followed for the total antioxidant activity assay. This method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol L^{-1} $FeSO_4$.

3. Results

3.1. Cloning of identified heat-responsive transcription factor

Transcript specific primers (forward and reverse) were used for the RT-PCR and an amplicon of ~1.1 kb was amplified. The amplified sequence was cloned in pGEMT-Easy vector (Promega, USA) and sequenced using Sanger's di-deoxy method. We observed nucleotide sequence of 1100 bp which was named as *TaHSFA6e* and further submitted to NCBI GenBank with accession no KU291394.

3.2. In silico characterization of TaHSFA6e TF gene

TaHSFA6e cloned in present investigation from thermotolerant wheat *cv*. HD2985 was aligned along with the transcript_31021 identified from wheat *cv*. HD2329 (thermosusceptible) showing homology with *HSFA6e*. We observed ~2% variations in the *HSFA6e* sequences observed from thermotolerant and thermosusceptible *cvs*. at nucleotide level (Fig. 1). This variation may be reflected in the codon optimization

of HD2985 and one of the reasons behind the thermotolerance nature compared with HD2329.

TaHSFA6e showed maximum homology with clone NIASHv2013N08 (acc. no. **AK363263.1**) reported from *Hordeum vulgare*. Open Reading Finder (ORF) showed the presence of 368 amino acids in the protein sequence. The molecular weight of protein was estimated to be ~40 kDa, and pI value of 5.03. We observed the presence of single HSF_DNA- binding domain of 97 residues starting at residue 57 and end at 150.

We observed the occurrence of 110 motifs in the sequence with p-value less than 0.0001 (Fig. S2a; Table S1). The presence of winged helix-turn-helix DNA-binding domain (InterPro ID IPR011991) and Heat Shock Factor (HSF)-type, DNA-binding domain (InterPro ID IPR000232, IPR000232, IPR000232, IPR000232, IPR000232, IPR000232) were observed in the sequence (Fig. S2b). Multiple EM for Motif Elicitation (MEME) tool was used to generate the block diagram of the occurrences of the motif in order to find out the novel and ungapped motifs (recurring, fixed-length patterns) in *TaHSFA6e* (Fig. S2c). We observed very high frequency of GA and TC in the conserved motif site displayed as sequence LOGOs. The height of the possible letter code represents the probability of the letter at that position multiplied by the total information content of the stack in bits.

Gene Ontology (GO) analysis of *TaHSFA6e* showed sequence-specific DNA-binding TF activity as molecular function and nuc as cellular component. Plant Ontology analysis showed the localization of the protein in the flag leaf. Protein structure analysis showed maximum homology with heat shock factor protein 1 (PDB ID 2ldu_A) (Fig. S2d). Functional analysis using UniProt db showed the protein to act as transcriptional activator that specifically binds to DNA sequence 5′-AGAAnnTTCT-3′ known as heat shock promoter elements (HSE). Phosphorylation analysis of TaHSFA6e protein showed the presence of serine (8 sites), threonine (3 sites), and tyrosine (1 site) above threshold level in the amino acid sequence, which might help the HSF in regulating HSPs to perform dual action of protein folding and chaperone activity (Fig. S2e).

3.3. Southern blot analysis of TaHSFA6e TF

The cloned *TaHSFA6e* TF was used as probe for the identification of copy number in wheat genome. We selected one thermotolerant (HD2985) and one thermosusceptible (HD2329) *cvs*. for the blot analysis. Genomic DNA isolated from wheat *cv*. HD2985 showed the presence of single positive band in lane restricted with *Eco*RI and *Hin*DIII, whereas *Bam*HI restricted lane showed the presence of two bands (Fig. 2a and b). Similarly, genomic DNA isolated from HD2329 and restricted with *Eco*RI and *Bam*H1 showed single band, whereas, two bands were observed in lane restricted with *Hin*DIII. The pattern of appearance of positive blot in different restricted lanes of HD2985 and HD2329 makes us to conclude that the *TaHSFA6e* has single copy number in the wheat genome. The result was further validated by mapping the sequence of *TaHSFA6e* TF on to the wheat reference genomic sequence available on URGI (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php). We observed single copy of the *TaHSFA6e* TF with homology > 98%.

3.4. Expression profiling of TaHSFA6e TF in wheat under differential HS

Expression profiling of *TaHSFA6e* TF was studied in contrasting wheat *cvs*. PBW343, HD2985, Halna and HD2329 under control (C - 22 ± 3 °C) and differential HS (T_1 - 30°, 2 h and T_2 −38 °C, 2 h) at pollination and grain-filling stages. During pollination stage, *TaHSFA6e* TF showed maximum relative fold expression (2.6 and 2.9-fold) in Halna in response to T_1 and T_2, as compared to control (Fig. 3a). The expression of *TaHSFA6e* was observed very low in PBW343 under T_1 (2.2-fold) and T_2 (2.4-fold) treatments.

Expression analysis of *TaHSFA6e* during grain-filling stage showed maximum relative fold expression in Halna (3.6 and 2.2-fold) in response to T_1 and T_2, as compared with control (Fig. 3b). Similarly, PBW343 showed minimum expression during grain-filling stage under differential HS (T_1 and T_2). Thermotolerant *cvs*. (HD2985 and Halna) showed abundance of *TaHSFA6e* transcripts, as compared to thermosusceptible cultivars (HD2329 and PBW343) under differential HS during pollination and grain-filling stages.

3.5. Expression profiling of heat-responsive chaperones regulated by A6 subclass of HSF in wheat under HS

Three heat shock protein (HSP) genes were selected as targets of *TaHSFA6e* by mapping the sequence on to Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/) and PlantRegMap. Even the report of Xue et al. (2013) was also considered for the target prediction. The targets *HSP90*, *HSP70*, and *HSP17* were selected for the expression analysis under control (C - 22 ± 3 °C) and differential HS (T_1 - 30°, 2 h and T_2 −38 °C, 2 h) at pollination and grain-filling stages.

Expression analysis of *HSP90* during pollination stage showed maximum relative fold expression in Halna (3.2-fold) followed by HD2985 (3.0-fold) in response to T_2 (Fig. 4a). *HSP90* expression was observed very low in PBW343 in response to T_1 and T_2 during pollination stage. During grain-filling, HSP90 showed maximum relative fold expression (2.4-fold) in HD2985 followed by Halna under T_2, whereas minimum was observed in PBW343 in response to T_1 (Fig. 4b). The *HSP90* transcript was observed abundant during pollination stage compared with grain-filling under differential HS.

Transcript profiling of *HSP70* during pollination stage showed maximum relative fold expression in Halna in response to T_1 (3.0-fold) and T_2 (3.8-fold), as compared with other cultivars. PBW343 showed very low *HSP70* expression under T_1 (2.2-fold) and T_2 (2.9-fold) (Fig. 4c). Similarly, expression profiling of *HSP70* during grain-filling showed maximum expression in Halna (3.1 and 2.7-fold) and minimum in HD2329 (2.8 and 2.5-fold) under T_1 and T_2 treatments (Fig. 4d).

Expression analysis of *HSP17* during pollination stage showed maximum expression in Halna (14.6 and 17.5-fold) and minimum in PBW343 (12.2 and 14.8-fold) in response to T_1 and T_2 treatments (Fig. 4e). Transcript profiling of *HSP17* during grain-filling showed maximum relative fold expression in Halna (24.9-fold) and minimum in HD2329 (17.2-fold) in response to T_2 (Fig. 4f). We observed significant increase in the expression of *HSP17* under differential HS; percent increase was observed maximum in Halna, as compared to other wheat *cvs*.

3.6. Validation of TaHSFA6e TF expression through northern blotting

In order to validate the expression of *TaHSFA6e* TF under HS, we selected one thermotolerant (*cv*. HD2985) and thermosusceptible (*cv*. HD2329) wheat *cvs*. for the northern blot analysis. Northern blot analysis showed significant increase in the accumulation of *TaHSFA6e* transcripts in response to differential HS (C - 22 ± 3 °C, T_1 - 30 °C, 2 h and T_2 - 38 °C, 2 h) in HD2985, as evident from the intensity of the band (Fig. 5). In case of HD2329, we observed maximum accumulation of transcript in response to T_1, and further decrease in the transcript was observed under T_2 treatment. Maximum accumulation of transcript was observed in HD2985 in response T_2 (38 °C, 2h).

3.7. Comparative analysis of thermotolerance in wheat using different biochemical parameters

3.7.1. Catalase activity assay

Catalase assay during pollination stage showed significant increase in the activity in all the four *cvs*. under differential HS treatment (C - 22 ± 3 °C, T_1 - 30 °C, 2 h and T_2 - 38 °C, 2 h). Catalase activity was observed maximum in Halna in response to T_1 (15.8 U/mg protein) and T_2 (16.4 U/mg protein), as compared to other cultivars (Fig. 6a). The

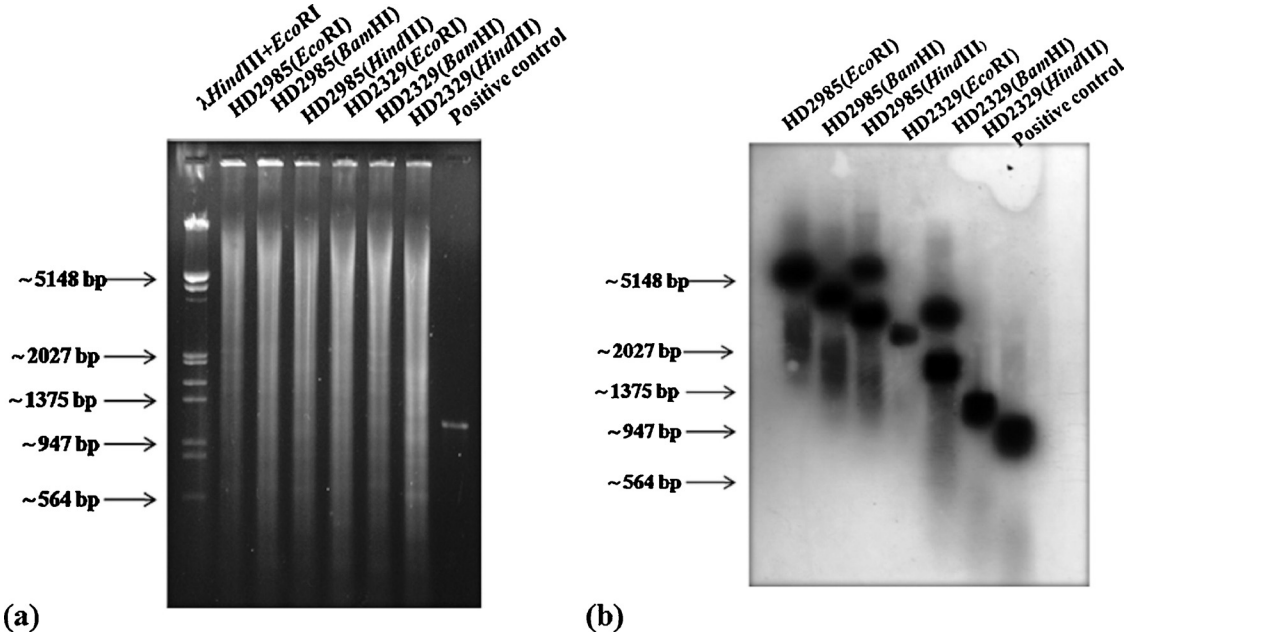

Fig. 2. Southern blot analysis of *TaHSFA6e* TF gene identified and cloned from wheat. (a) Restriction analysis of isolated genomic DNA, (b) Blot developed after probing with *TaHSFA6e* TF; 0.8% agarose gel was used for the RE.

CAT activity was observed very low in HD2329 under T_1 (8.12 U/mg protein) and T_2 (8.65 U/mg protein). Similarly, we observed maximum activity of CAT in Halna (18.4 U/mg protein) and minimum in PBW343 (9.2 U/mg protein) during grain-filling in response to T_2 (Fig. 7a).

Guaiacol peroxidase (Gpx) assay during pollination stage showed very high activity in Halna in response to T_1 (50.2 U/mg protein) and T_2 (58.4 U/mg protein), as compared with other cultivars. The activity was observed minimum in PBW343 under T_1 (33.8 U/ mg protein) and T_2 (38.2 U/ mg protein) (Fig. 6b). Similar findings of Gpx activity was observed during grain-filling stage in response to T_1 and T_2 (Fig. 7b).

Lipid peroxidation was estimated during pollination stage in terms of TBARS and we observed very high lipid peroxidation in wheat *cv*. HD2985 in response to T_1 (102.2 µmol/ g fresh weight) and T_2 (78.8 µmol/ g fresh weight) (Fig. 6c). The TBARS were estimated very low in PBW343 under HS of T_1 and T_2 during pollination stage. Similarly, TBARS accumulation was observed maximum in PBW343 and minimum in Halna during grain-filling stage under T_1 and T_2 treatments (Fig. 7c).

Total antioxidant capacity (TAC) estimation during pollination stage showed maximum TAC in Halna under T_1 (42.8 mM/g fresh weight) and T_2 (64.6 mM/g fresh weight), whereas minimum was observed in PBW343 (31.1 and 45.2 mM/g fresh weight) under T_1 and T_2 (Fig. 6d). Similar findings of TAC were observed during grain-filling stage in response to T_1 and T_2 (Fig. 7d). Halna was observed more thermostable, as compared with other cultivars.

3.8. HSP90 protein assay in wheat under heat stress

HSP90 was observed as one of the main targets of *TaHSFA6e* TF. In order to characterize the accumulation pattern of HSP90 protein, we collected the control and HS-treated (C - 22 ± 3 °C, T_1 - 30 °C, 2 h and T_2 - 38 °C, 2 h) leaves samples of two contrasting wheat *cvs*. HD2985 (thermotolerant) and HD2329 (thermosusceptible). Protein profiling through SDS-PAGE showed uniform separation of protein bands on gel; not much differences in the protein bands were observed in response to the differential HS treatments in both the *cvs*. (Fig. S3a). Immunoblot analysis using anti-HSP90 specific antibody showed decrease in the accumulation pattern of HSP90 in response to T_1 and T_2 treatments, as

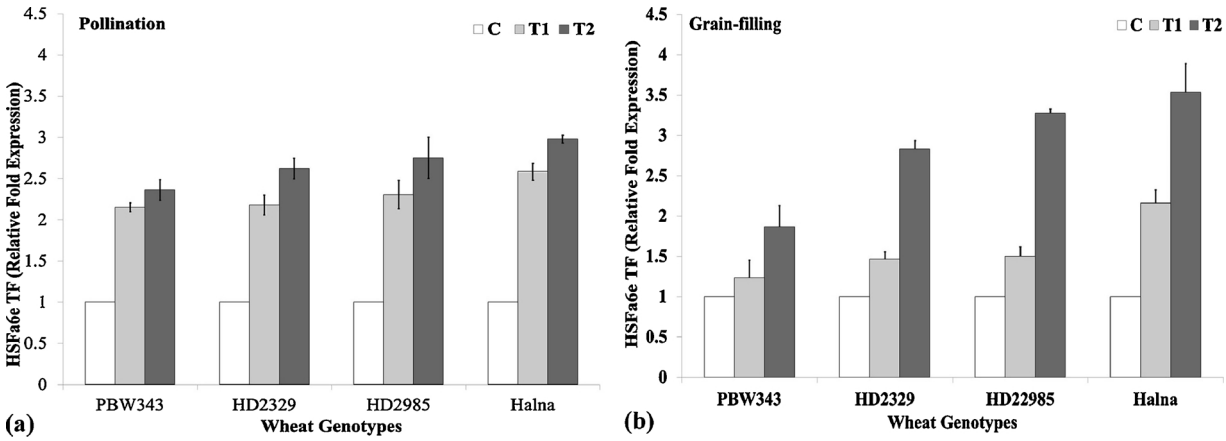

Fig. 3. Transcript profiling of *TaHSFA6e* TF in contrasting wheat *cvs*. under differential HS. (a) Expression of *TaHSFA6e* TF in response to HS during pollination stage, (b) Expression of *TaHSFA6e* TF in response to HS during grain-filling stage; C- 22 ± 3 °C, T1- 30 °C, 2 h, T2- 38 °C, 2 h, Thermotolerant (HD2985 & Halna) and thermosusceptible (PBW343 & HD2329) *cvs*. were used for the expression analysis; vertical bars indicate *s.e* (n = 3).

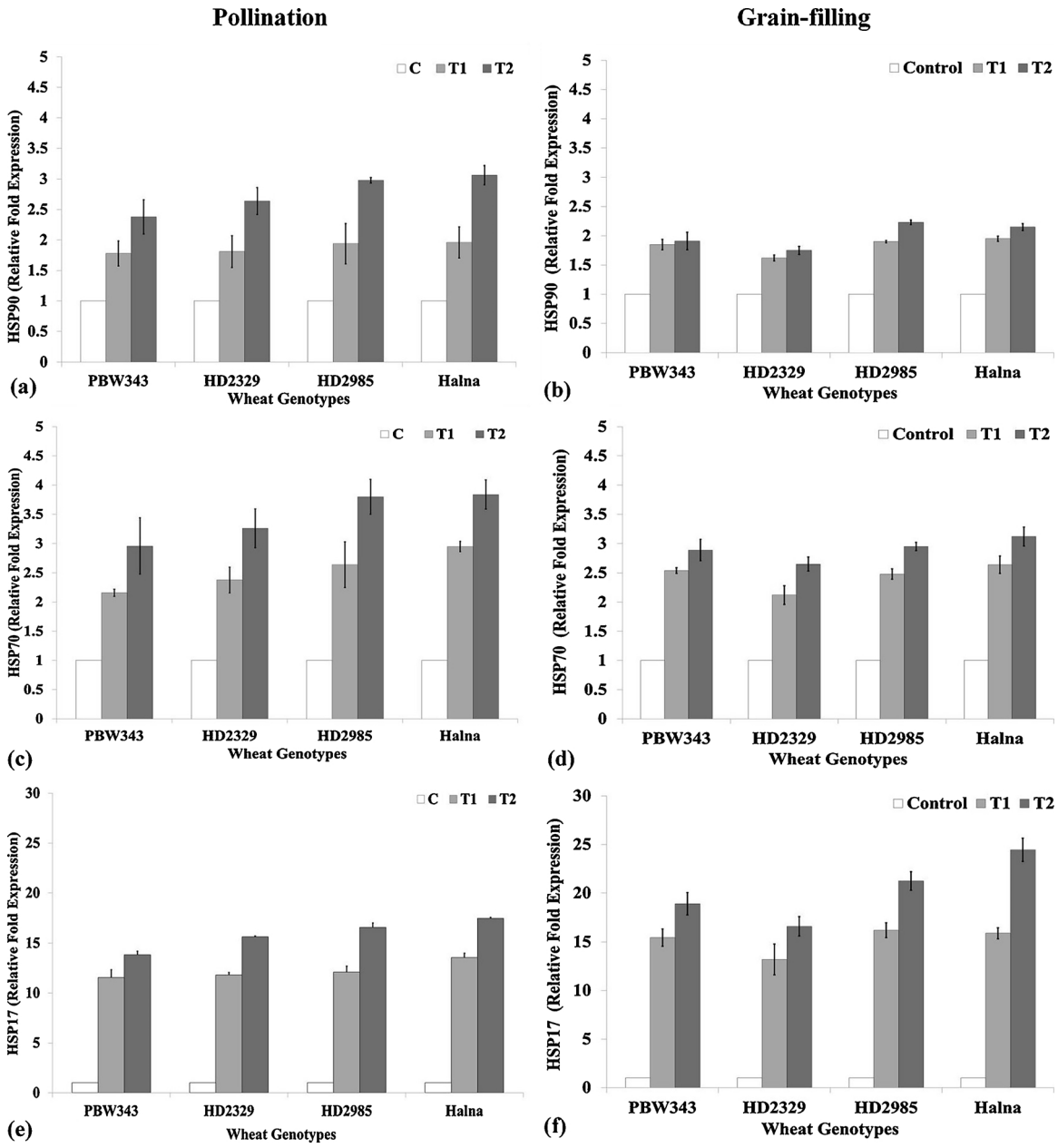

Fig. 4. Expression analysis of target genes of *TaHSFA6e* TF in contrasting wheat *cvs*. under differential HS. Transcript profiling of targets (a & b) *HSP90*, (c & d) *HSP70*, and (e & f) *HSP17* in response to HS during pollination and grain-filling stages. C- 22 ± 3 °C, T1- 30 °C, 2 h, T2- 38 °C, 2 h, β-Actin gene was used as endogenous control for normalizing the data, Pfaffl method was used for the relative fold expression calculation, Thermotolerant (HD2985 & Halna) and thermosusceptible (PBW343 & HD2329) *cvs*. were used for the expression analysis; vertical bars indicate *s.e* (n = 3).

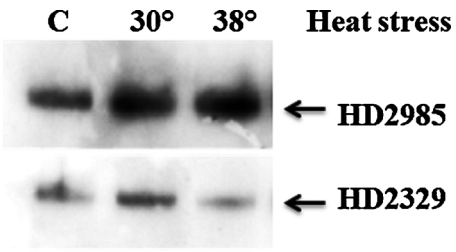

Fig. 5. Northern blot analysis of *TaHSFA6e* TF in contrasting wheat *cvs*. under differential HS. C- 22 ± 3 °C, T1- 30 °C, 2 h, T2- 38 °C, 2 h, Thermotolerant (HD2985) and thermosusceptible (HD2329) *cvs*. were used for the expression analysis.

compared to control (Fig. S3b). Wheat *cv*. HD2985 (thermotolerant) showed more abundance of HSP90 protein, as compared with HD2329 (thermosusceptible) under different treatments (Fig. S3b).

3.9. Effect of multiple stresses on expression of TaHSFA6e and alteration in biochemical parameters associated with thermotolerance

We exposed the wheat seedling (15 days old) of all the four selected contrasting wheat *cvs*. to heat stress (H - 38 °C, 2 h), drought (D – 100 ml of 20% PEG 6000), and heat stress + drought (H + D) conditions. The relative expression of *TaHSFA6e* was observed maximum in Halna under D (2.3-fold), H (2.75-fold) and H + D (3.19-fold), as compared with other cultivars during seedling stage (Fig. 8a). The expression of *TaHSFA6e* was observed minimum in PBW343 under individual treatment of D and H. Similar pattern of expression of *HSP70*

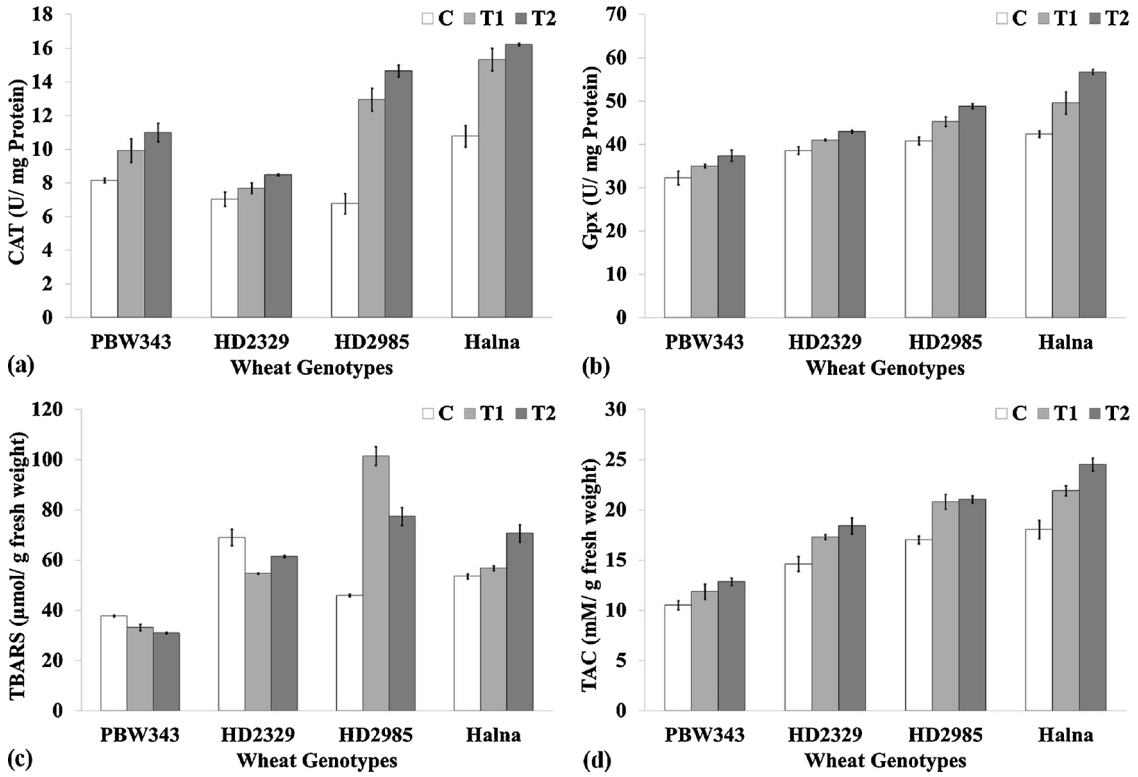

Fig. 6. Characterizing the biochemical parameters associated with thermotolerance in contrasting wheat *cvs*. in response to differential HS during pollination stage. (a) Catalase activity assay in contrasting wheat *cvs*. in response to differential HS, (b) Guaiacol peroxidase activity assay in contrasting wheat *cvs*. under differential HS, (c) TBARS estimation in contrasting wheat cvs. in response to differential HS, (d) Total antioxidant capacity in contrasting wheat *cvs*. under differential HS; C- 22 ± 3 °C, T_1- 30 °C, 2 h, T_2- 38 °C, 2 h; Thermotolerant (HD2985 & Halna) and thermosusceptible (PBW343 & HD2329) *cvs*. were used for the biochemical analysis; samples were collected during pollination stage; vertical bars indicate *s.e* (n = 3).

and *HSP90* were observed in response to D, H and D + H in all the *cvs*. during seedling stage (Fig. 8b & c). Expression analysis of *HSP17* showed very high fold increase in the expression in response to H (12.2-fold), as compared to D (7.1-fold) in Halna; transcript abundance was observed maximum in Halna in response to combined treatment of D + H compared with other cultivars (Fig. 8d).

Catalase assay showed maximum activity in PBW343 in response to H (16.1 U/ mg Protein), whereas Halna showed maximum catalase activity (15.1 and 16.1 U/ mg protein) in response to D and combined treatment of D + H, compared with other cultivars (Fig. 9a). The catalase activity was observed minimum in HD2329 in response to D, H and combined treatments.

Gpx showed maximum activity in wheat *cv*. HD2985 under control (48 U/ mg protein), D (55.1 U/mg protein), and H (56.2 U/mg protein) (Fig. 9b). Under combined treatment of D + H, maximum Gpx activity was observed in wheat *cv*. Halna (53.9 U/mg Protein). Thermotolerant *cvs*. showed maximum activity of Gpx under D, H and combined stresses of both, as compared to thermosusceptible cultivars. Lipid peroxidation analysis showed maximum peroxidation in wheat *cv*. PBW343 in response to D (33.5 µmol/ g fresh weight), H (36.2 µmol/ g fresh weight) and combined stresses of both (35.9 µmol/ g fresh weight), whereas minimum peroxidation was observed in Halna (24.8, 24.6, and 25.2 µmol/ g fresh weight) in response to D, H and combined treatments (Fig. 9c).

TAC analysis showed maximum antioxidant potential in Halna under D (22.8 mM/g FW), H (25.1 mM/ g FW) and D + H (24.5 mM/ g FW), whereas minimum TAC was observed in PBW343 in response to multiple stresses during seedling stage (Fig. 9d). All the four cultivars showed maximum TAC in response to H followed by combined treatment of both the stresses.

4. Discussion

Terminal heat stress is one of the major problems in wheat growth and yield. It causes severe reduction in the quality and quantity of the grains, as evident from the decrease in the yield with increase in the environmental temperature. HS during grain-filling cause's denaturation of enzymes associated with source and sink, defragmentation of granules, formation of empty pockets in the endospermic tissues and shrivelled seeds (Kumar et al., 2016). The sensitivity of the plants to HS depends on the stage of the plants, species and genotypes. Flowering and grain-filling are considered as critical stages in case of cereals (Kumar et al., 2015). Heat stress causes abrupt production of ROS which has damaging effect on the key cellular organelles and membranes. Although, ROSs are clearly a direct cause of cellular damage on multiple levels, several studies have also shown that ROS play a key role as molecular signals, linking plant responses to pathogen infection, environmental stresses, programmed cell death (PCD), and even developmental stimuli (Gechev et al., 2006). Expression of heat shock proteins (HSPs) is also considered as an important adaptive strategy of plants under HS (Feder and Hofman, 1999).

Heat-shock TFs plays key role in modulating the stress-tolerance of plants by regulating the expression of SAGs (Xue et al., 2013). They identified 56 TaHsf members from wheat using Bioinformatics tools and classified them into A, B, and C classes. Here, a putative *TaHSFA6e* TF gene was identified, cloned and characterized to be belongs to subclass A6. Single copy number of the respective TF was observed integrated in the genome which was further validated by mapping the sequence on *Triticum* database. The transcript of *TaHSFA6e* TF was observed abundant in leaves during vegetative, pollination and grain-filling stages of growth under control and HS-treated conditions. Xue et al. (2013) reported over-expression of subclass A6 members of TF in the endospermic tissue under normal and HS-treated conditions. Similarly,

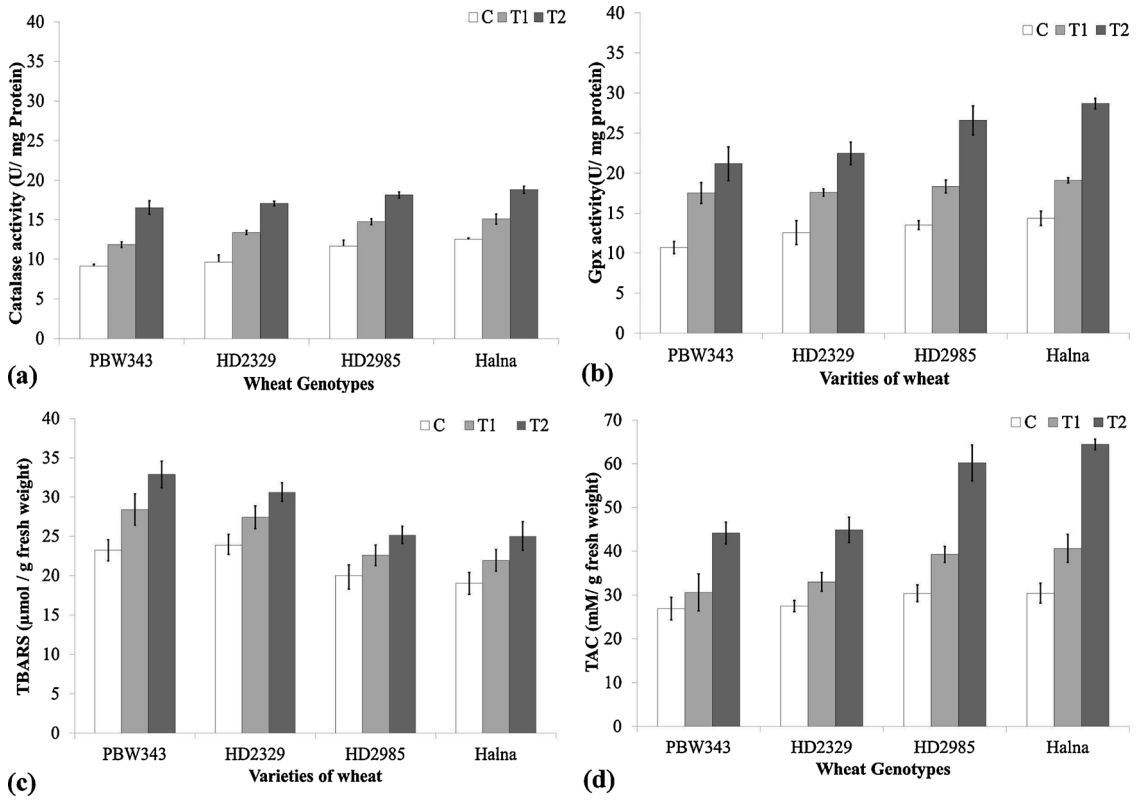

Fig. 7. Alteration in the thermotolerance associated biochemical parameters in contrasting wheat *cvs*. in response to differential HS during grain-filling stage. (a) Catalase activity assay, (b) Guaiacol peroxidase activity assay, (c) TBARS estimation, (d) Total antioxidant capacity in contrasting wheat *cvs*. under differential HS; C- 22 ± 3 °C, T_1- 30 °C, 2 h, T_2- 38 °C, 2 h; Thermotolerant (HD2985 & Halna) and thermosusceptible (PBW343 & HD2329) *cvs*. were used for the biochemical analysis; samples were collected during grain-filling stage; vertical bars indicate *s.e* (n = 3).

Huang et al., (2016) reported that expression of HsfA6b, a class A HSF, extensively increase under HS and operate as a downstream regulator of the ABA-mediated stress response.

HSFs recognized the HS elements (HSE: 5′-AGAAnnTTCT-3′) conserved in the promoter regions of HS-responsive genes in all the eukaryotes (Pelham, 1982). Group A1 heat shock transcription factors (HsfA1s) have been reported as master regulators of the HS-response (HSR) in plants. HSFs are basically present in inactive form in plants and are activated upon exposure to HS through trimerization and high-affinity DNA binding (Lin et al., 2011). Different functions have been assigned to HSFs in different crops. Almoguera et al. (2015) reported that HaHsfA9 and HaHsfA4a modulate the seed longevity and desiccation tolerance of sunflower seeds. HSFs have been well characterized in Arabidopsis and tomato plant system. HSFs reported in plant system have been reported to be more diverse and complex as compared to vertebrates and yeast (Baniwal et al., 2004). HsfA1 has been reported to be the master regulator of induced thermotolerance in tomato (Mishra et al., 2002). Perez-Salamo et al. (2014) observed that HsfA4a confers salt tolerance in Arabidopsis and is regulated by oxidative stress and the mitogen-activated protein kinases (MPK3 and MPK6).

We observed HSF DNA binding signature domain in the amino acid sequence of *TaHSFA6e* TF. Ohama et al. (2017) executed domain analysis of HsfA1d in *Arabidopsis thaliana* and reported that the central region of HsfA1d is a key regulatory domain that represses HsfA1d transactivation activity through interaction with HSP70 and HSP90. Jung et al. (2013) reported that HSFA1D, HSFA2, and HSFA3 are key factors regulating the *APX2* expression under excess light in Arabidopsis. We observed increase in the expression of *TaHSFA6e* in response to drought, heat and combined stress of drought and heat in wheat seedling. Thermotolerant wheat *cvs*. (Halna and HD2985) showed maximum transcript of *TaHSFA6e* under HS, as compared to thermosensitive *cvs*. (PBW343 and HD2329). HSFs plays very important role in signal transduction pathway modulating the expression of SAGs in response to both HS and various chemical stressors (Schoffl et al., 1998). Expression analysis of targets of *TaHSFA6e* TF showed very high relative fold expression of *HSP70*, *HSP90* and *HSP17* under HS. Even the biochemical marker like TAC was observed very high in *cvs*. having high expression of *TaHSFA6e* and their targets. The findings is in conformity with the observation of Hwang et al. (2014) who reported that over-expression of *HSFa6a* modulates the salt tolerance level of Arabidopsis by increasing the expression of SAGs.

TaHSFA6e TF and their targets (*HSP70*, *HSP90* and *HSP17*) showed up-regulation under HS, which has significant effect on the tolerance level of the wheat at different stages of growth. Hu et al. (2018) reported that TaHsfC2a-B is a transcriptional activator of heat-responsive genes and serves as a proactive mechanism for heat protection in developing wheat grains via the ABA-mediated regulatory pathway. Chao et al. (2017) reported that overexpression of *SPL1* or *SPL12* enhanced the thermotolerance in both *Arabidopsis* and tobacco.

We observed positive correlation between the expression of *TaHSFA6e* TF and their targets (*HSP70*, *HSP90* and *HSP17*) under differential HS in contrasting wheat genotypes. The relative fold expression of *HSP17* was observed very high in all the *cvs*. under differential HS at different stages of growth. Double knockout experiments were executed to prove that Hsfs are responsible for the HS induced transcription of a subset of genes encoding small heat shock proteins (sHSPs), *HSP70*, *HSP101*, *Hsfs* B1, B2a and A7a as well as SAGs encoding metabolic enzymes, such as inositol-3-phosphate synthase 2 (IPS2) and galactinol synthase 1 (GolS1) (Busch et al., 2005).

Here, we observed alteration in the expression of *TaHSFA6e* TF and their target genes in wheat at different stages of growth and development which is in conformity with the observation of Xue et al. (2013) who reported expression of heat transcription factor genes from ‘A’

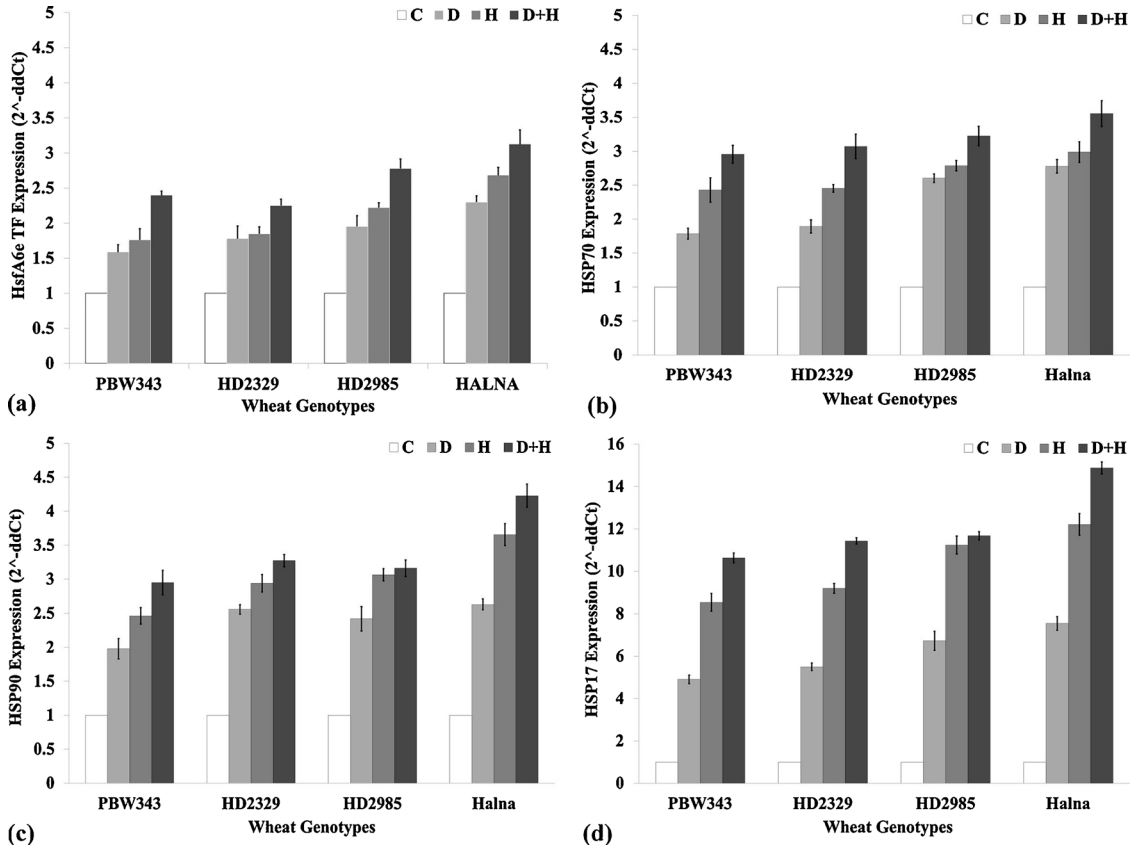

Fig. 8. Expression analysis of *TaHSFA6e* TF and its target in wheat under multiple stresses. (a) Expression of *TaHSFA6e* TF, (b) Expression of *HSP70*, (c) Expression of *HSP90*, and (d) Expression of *HSP17* in contrasting wheat cultivars under heat, drought and combined stresses; C - 22 ± 3 °C, H - 38 °C, 2 h; D – 100 ml of 20% polyethylene Glycol 6000; Wheat *cvs*. HD2985 & Halna (thermotolerant) and PBW343 & HD2329 (thermosusceptible) were used for the analysis; vertical bars indicate *s.e* (n = 3).

family (HsfA) in wheat under HS at different stages of growth. HSFs belongs to 'A' family has been reported to have constitutive expression in wheat (Mishra et al., 2002; Liu and Charng, 2012). HsfA1a has been shown to maintain its inactive monomer state by association with HSP90/HSP70 in tomato under non-heat stress conditions (Hahn et al., 2011).

Xue et al. (2013) reported that expression of *TaHsfA6f*is constitutive in green organs of wheat and showed up-regulation during HS which is in accordance with the present findings. Over-expression of *TaHsfA6f* in transgenic wheat using a drought-inducible promoter resulted in up-regulation of HSPs and a number of other heat-responsive genes like Golgi anti-apoptotic protein (GAAP) and the large isoform of RuBisCo activase. In recent years, studies on the HSF family had been thoroughly characterized in *Arabidopsis*, rice, poplar, Chinese cabbage, maize, and *Malus* (Giorno et al., 2012; Guo et al., 2008; Lin et al., 2011; Song et al., 2014), whose genomes have been sequenced. Lin et al. (2011) reported that HSF showed very high fold increase in the expression in poplar under HS, as compared to oxidation and cold. In present investigation, we observed very high relative fold expression of *HSP17* (small HSPs), as compared to other HSPs in contrasting wheat *cvs*. under differential HS. Nishizawa et al. (2006) observed three subclasses of HSF - HsfA2 (PtHsf-14), HsfA6 (PtHsf-17, PtHsf-19), and HsfA7 (PtHsf-05, PtHsf-10) and reported higher expression of these HSFs under HS-treatment (Schramm et al., 2006). Tang et al. (2016) reported 27 StHsf members from potato identified by bioinformatics and phylogenetic analyses and were classified into A, B, and C groups according to their structural and phylogenetic features.

The functional diversification of HSF genes has been observed in several plant species. HsfA1a has been reported as a single master regulator gene in tomato (Mishra et al., 2002). AtHsfA1a and AtHsfA1b are known to be involved in the early response to HS in Arabidopsis (Busch et al., 2005). AtHsfA2 enhances and maintains the HS-response when plants are subjected to long-term or repeated cycles of HS (Meiri and Breiman, 2009).

Heat stress tolerance analysis of contrasting wheat *cvs*. in our earlier observation showed abundance of HSP70 protein, H_2O_2, proline, guaiacol peroxidase activity, and TAP during the seed-hardening stage under HS. Accumulation of SAPs was observed higher in the thermotolerant wheat *cv*. than in thermosusceptible (Kumar et al., 2016). Xin et al. (2016) reported that transcriptome and proteome changes are associated with the enhanced post-anthesis high temperature tolerance induced by pre-anthesis heat priming in wheat.

Seedlings exposed to drought, HS and combined drought and heat stresses also showed very high activity of CAT in Halna (thermotolerant) followed by PBW343 (thermosusceptible). Keleş and Öncel (2002) reported that HS-treatment increased the CAT activity in *T. aestivum* genotypes, whereas decreased activity was observed in *T. durum* genotypes. We observed very high antioxidant potential in thermotolerant wheat genotypes in response to differential HS during grain-filling, as compared to thermosusceptible. Screening of seedlings for thermotolerance using TAC showed very high antioxidant potential in thermotolerant *cvs*., as compared to thermosusceptible. An increased antioxidant capacity reduces the oxidative damage, thereby increasing membrane thermal stability and reducing the leaf dark respiration rates (Mohammed and Tarpley, 2011).

TaHSFA6e TF was observed to regulate the expression of key SAGs like *HSP70*, *HSP90* and *sHSP17* and need to be further functionally validated in order to use it as potential TF gene for modulating the expression of SAGs. This will pave the way for mitigating the problem of terminal HS in wheat through development of 'climate-smart' crop.

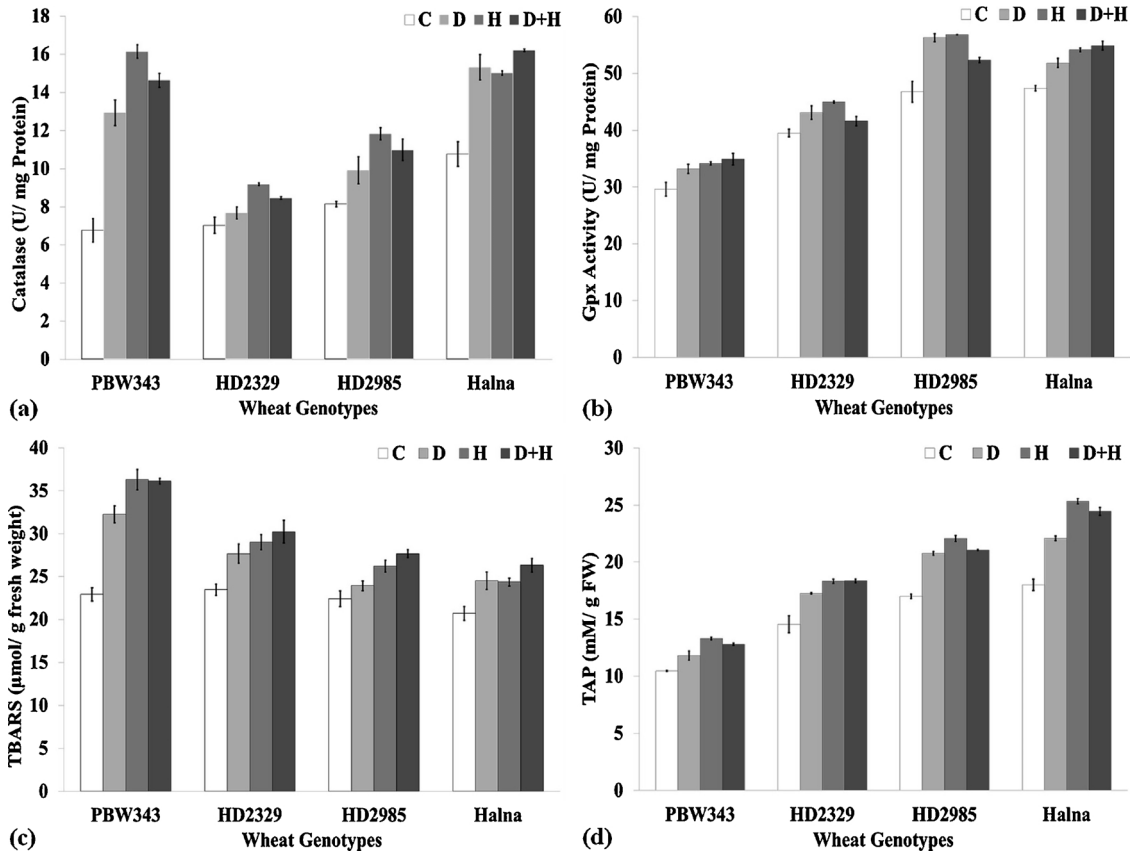

Fig. 9. Alteration in the biochemical parameters associated with thermotolerance of wheat under multiple stresses. (a) Catalase activity assay, (b) Guaiacol peroxidase activity assay, (c) TBARS content, and (d) Total antioxidant potential (TAP) estimation in contrasting wheat cultivars under heat, drought and combined stresses; C - 22 ± 3 °C, H - 38 °C, 2 h; D – 100 ml of 20% polyethylene Glycol 6000; Wheat *cvs*. HD2985 & Halna (thermotolerant) and PBW343 & HD2329 (thermosusceptible) were used for the analysis; vertical bars indicate *s.e* (n = 3).

5. Conclusion

We identified and cloned a putative heat-responsive *TaHSFA6e* TF gene from wheat which belongs to subclass A6 of HSF family. We observed HSF DNA-binding domain in the amino acid sequence. *TaHSFA6e* showed increase in the expression in response to differential HS; transcript was observed abundant in thermotolerant *cvs*. during grain-filling stage. A positive correlation was established between the expression of *TaHSFA6e* and its targets (*HSP70*, *HSP90* and *HSP17*) under HS. Thermotolerance related biochemical parameters like CAT, Gpx, and TAC were observed higher in thermotolerant *cvs*., as compared to thermosusceptible. This makes us to conclude that the expression of *TaHSFA6e* regulates the expression of HSPs under HS, which in turn modulate the activity and accumulation of biochemical entities associated with thermotolerance. The identified putative *TaHSFA6e* TF can be used as candidate gene for manipulating the HS-tolerance potential of desirable wheat lines through genetic engineering or breeding approaches.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.05.008.

References

Akerfelt, M., Trouillet, D., Mezger, V., Sistonen, L., 2007. Heat shock factors at a crossroad between stress and development. Ann. N.Y. Acad. Sci. 1113, 15–27. http://dx.doi.org/10.1196/annals.1391.005.

Allakhverdiev, S.I., Kreslavski, V.D., Klimov, V.V., Los, D.A., Carpentier, R., Mohanty, P., 2008. Heat stress: an overview of molecular responses in photosynthesis. Photosynth. Res. 98 (1–3), 541–550. http://dx.doi.org/10.1007/s11120-008-9331-0.

Almoguera, C., Personat, J.M., Prieto-Dapena, P., Jordano, J., 2015. Heat shock transcription factors involved in seed desiccation tolerance and longevity retard vegetative senescence in transgenic tobacco. Planta 242 (2), 461–475. http://dx.doi.org/10.1007/s00425-015-2336-y.

Baniwal, S.K., Bharti, K., Chan, K.Y., Fauth, M., Ganguli, A., Kotak, S., Mishra, S.K., Nover, L., Port, M., Scharf, K., Tripp, J., Weber, C., Zielinski, D., von Koskull-Döring, P., 2004. Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J. Biosci. 29 (4), 471–487. http://dx.doi.org/10.1007/BF02712120.

Benzie, I.F.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27. http://dx.doi.org/10.1016/S0076-6879(99)99005-5.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Busch, W., Wunderlich, M., Schöffl, F., 2005. Identification of novel heat shock factor-dependent genes and biochemical pathways in Arabidopsis thaliana. Plant. J. 41 (1), 1–14. http://dx.doi.org/10.1111/j.1365-313X.2004.02272.x.

Chao, L.M., Liu, Y.Q., Chen, D.Y., Xue, X.Y., Mao, Y.B., Chen, X.Y., 2017. Arabidopsis transcription factors SPL1 and SPL12 confer plant thermotolerance at reproductive stage. Mol. Plant 10 (5), 735–748. http://dx.doi.org/10.1016/j.molp.2017.03.010.

Charng, Y.-Y., Liu, H.-C., Liu, N.-Y., Chi, W.-T., Wang, C.-N., Chang, S.-H., Wang, T.-T., 2007. A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. Plant Physiol. 143 (1), 251–262. http://dx.doi.org/10.1104/pp.106.091322.

Chauhan, H., Khurana, N., Agarwal, P., Khurana, P., 2011. Heat shock factors in rice (Oryza sativa L.): genome-wide expression analysis during reproductive development and abiotic stress. Mol. Genet. Genomics 286 (2), 171–187. http://dx.doi.org/10.1007/S00438-011-0638-8.

Chauhan, H., Khurana, N., Nijhavan, A., Khurana, J.P., Khurana, P., 2012. The wheat chloroplastic small heat shock protein (sHSP26) is involved in seed maturation and germination and imparts tolerance to heat stress. Plant, Cell Environ. 35, 1912–1931.

Feder, M.E., Hofman, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243–282.

Frank, G., Pressman, E., Ophir, R., Althan, L., Shaked, R., Freedman, M., Shen, S., Firon, N., 2009. Transcriptional profiling of maturing tomato (Solanum lycopersicum L.) microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones, and sugars in the heat stress response. J. Exp. Bot. 60 (13), 3891–3908. http://dx.doi.org/10.1093/jxb/erp234.

Gechev, T.S., Van Breusegem, F., Stone, J.M., Denev, I., Laloi, C., 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. Bioessays 28 (11), 1091–1101. http://dx.doi.org/10.1002/bies.20493.

Giorno, F., Guerriero, G., Baric, S., Mariani, C., 2012. Heat shock transcriptional factors in Malus domestica: identification, classification and expression analysis. BMC Genomics 13, 639. http://dx.doi.org/10.1186/1471-2164-13-639.

Grover, A., Mittal, D., Negi, M., Lavania, D., 2013. Generating high temperature tolerant transgenic plants: achievements and challenges. Plant Sci. 206, 38–47. http://dx.doi.org/10.1016/j.plantsci.2013.01.005.

Guo, J., Wu, J., Ji, Q., Wang, C., Luo, L., Yuan, Y., Wang, Y., Wang, J., 2008. Genome-wide analysis of heat shock transcription factor families in rice and Arabidopsis. J. Genet Genomics 35 (2), 105–118. http://dx.doi.org/10.1016/S1673-8527(08)60016-8.

Hahn, A., Bublak, D., Schleiff, E., Scharf, K.-D., 2011. Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. Plant Cell. 23 (2), 741–755. http://dx.doi.org/10.1105/tpc.110.076018.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125 (1), 189–198. http://dx.doi.org/10.1016/0003-9861(68)90654-1.

Hu, X.-J., Chen, D., Lynne Mclntyre, C., Fernanda Dreccer, M., Zhang, Z.-B., Drenth, J., Kalaipandian, S., Chang, H., Xue, G.-P., 2018. Heat shock factor C2a serves as a proactive mechanism for heat protection in developing grains in wheat via an ABA-mediated regulatory pathway. Plant. Cell Environ. 41 (1), 79–98. http://dx.doi.org/10.1111/pce.12957.

Huang, Y.-C., Niu, C.-Y., Yang, C.-R., Jinn, T.-L., 2016. The heat-stress factor HSFA6b connects ABA signaling and ABA-mediated heat responses. Plant Physiol. 172 (2), 1182–1199. http://dx.doi.org/10.1104/pp.16.00860.

Hwang, S.M., Kim, D.W., Woo, M.S., Jeong, H.S., Son, Y.S., Akhter, S., Choi, G.J., Bahk, J.D., 2014. Functional characterization of Arabidopsis HsfA6a as a heat-shock transcription factor under high salinity and dehydration conditions. Plant. Cell Environ. 37 (5), 1202–1222. http://dx.doi.org/10.1111/pce.12228.

Jin, G.H., Gho, H.J., Jung, K.H., 2013. A systematic view of rice heat shock transcription factor family using phylogenomic analysis. J. Plant Physiol. 170 (3), 32132–32139. http://dx.doi.org/10.1016/j.jplph.2012.09.008.

Jung, H.-S., Crisp, P.A., Estavillo, G.M., Cole, B., Hong, F., Mockler, T.C., Pogson, B.J., Chory, J., 2013. Subset of heat-shock transcription factors required for the early response of Arabidopsis to excess light. Proc. Natl. Acad. Sci. 110 (35), 14474–14479. http://dx.doi.org/10.1073/pnas.1311632110.

Keleş, Y., Öncel, I., 2002. Response of antioxidative defence system to temperature and water stress combinations in wheat seedlings. Plant Sci. 163 (4), 783–790. http://dx.doi.org/10.1016/S0168-9452(02)00213-3.

Kumar, M., Busch, W., Birke, H., Kemmerling, B., Nürnberger, T., Schöffl, F., 2009. Heat shock factors HsfB1 and HsfB2b are involved in the regulation of Pdf1.2 expression and pathogen resistance in Arabidopsis. Mol. Plant 2 (1), 152–165. http://dx.doi.org/10.1093/mp/ssn095.

Kumar, R.R., Goswami, S., Gupta, R., Verma, P., Singh, K., Singh, J.P., Kumar, M., Sharma, S.K., Pathak, H., Rai, R.D., 2015. The stress of suicide: temporal and spatial expression of putative heat shock protein 70 protect the cells from heat injury in wheat (Triticum aestivum). J. Plant Growth Regul. 1–18.

Kumar, R.R., Goswami, S., Sharma, S.K., Singh, K., Gadpayle, K.A., Kumar, N., Rai, G.K., Singh, M., Rai, R.D., 2012. Protection against heat stress in wheat involves change in cell membrane stability, antioxidant enzymes, osmolyte, H2O2 and transcript of heat shock protein. Int. J. Plant Physiol. Biochem. 4, 83–91. http://dx.doi.org/10.5897/IJPPB12.008.

Kumar, R.R., Goswami, S., Singh, K., Dubey, K., Singh, S., Sharma, R., Verma, N., Kala, Y.K., Rai, G.K., Grover, M., Mishra, D.C., Singh, B., Pathak, H., Chinnusamy, V., Rai, A., Praveen, S., 2016. Identification of Putative RuBisCo Activase (TaRca1)—The catalytic chaperone regulating carbon assimilatory pathway in wheat (Triticum aestivum) under the heat stress. Front. Plant Sci. 7, 986. http://dx.doi.org/10.3389/fpls.2016.00986.

Kumar, R.R., Sharma, S.K., Goswami, S., Singh, G.P., Singh, R., Singh, K., Pathak, H., Rai, R.D., 2013. Characterization of differentially expressed stress-associated proteins in starch granule development under heat stress in wheat (Triticum aestivum L.). Indian J. Biochem. Biophys. 50, 126–138.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227, 680–685.

Large, E.C., 1954. Growth stages in cereals illustration of the Feekes scale. Plant Pathol. 3 (4), 128–129. http://dx.doi.org/10.1111/j.1365-3059.1954.tb00716.x.

Lewandowski, C.M., Co-investigator, N., Lewandowski, C.M., 2015. Molecular Cloning. Sambrook. Maniatis Sambrookhttp://dx.doi.org/10.1017/CBO9781107415324.004.

Lin, B.L., Wang, J.S., Liu, H.C., Chen, R.W., Meyer, Y., Barakat, Delseny, M., 2001. Genomic analysis of the Hsp70 superfamily in Arabidopsis thaliana. Cell Stress Chaperones 6 (3), 201–208. http://dx.doi.org/10.1379/1466-1268(2001)006<0201:GAOTHS>2.0.CO;2.

Lin, Y.-X., Jiang, H.-Y., Chu, Z.-X., Tang, X.-L., Zhu, S.-W., Cheng, B.-J., 2011. Genome-wide identification, classification and analysis of heat shock transcription factor family in maize. BMC Genomics 12, 76. http://dx.doi.org/10.1186/1471-2164-12-76.

Liu, H., Charng, Y., 2012. Acquired thermotolerance independent of heat shock factor A1 (HsfA1), the master regulator of the heat stress response. Plant Signal. Behav. 7 (5), 547–550. http://dx.doi.org/10.4161/psb.19803.

Maehly, A.C., Chance, B., 1954. The assay of catalases and peroxidases. Methods Biochem. Anal. 1, 357–424. http://dx.doi.org/10.1002/9780470110171.ch14.

Meehl, G.A., Covey, C., Delworth, T., Latif, M., McAvaney, B., Mitchell, J.F.B., Stouffer, R.J., Taylor, K.E., 2007. The WCRP CMIP3 multimodel dataset: a new era in climatic change research. Bull. Am. Meteorol. Soc. http://dx.doi.org/10.1175/BAMS-88-9-1383.

Meiri, D., Breiman, A., 2009. Arabidopsis ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs. Plant J. 59 (3), 387–399. http://dx.doi.org/10.1111/j.1365-313X.2009.03878.x.

Mika, A., Lüthje, S., 2003. Properties of guaiacol peroxidase activities isolated from corn root plasma membranes. Plant Physiol. 132, 1489–1498.

Mishra, S.K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L., Scharf, K.D., 2002. In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. Genes Dev. 16 (12), 1555–1567. http://dx.doi.org/10.1101/gad.228802.

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11 (1), 15–19. http://dx.doi.org/10.1016/j.tplants.2005.11.002.

Mohammed, A.R., Tarpley, L., 2011. High night temperature and plant growth regulator effects on spikelet sterility, grain characteristics and yield of rice (Oryza sativa L.) plants. Can. J. Plant Sci. 91 (2), 283–291. http://dx.doi.org/10.4141/CJPS10038.

Nishizawa, A., Yabuta, Y., Yoshida, E., Maruta, T., Yoshimura, K., Shigeoka, S., 2006. Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. Plant J. 48 (4), 535–547. http://dx.doi.org/10.1111/j.1365-313X.2006.02889.x.

Ohama, N., Sato, H., Shinozaki, K., Yamaguchi-Shinozaki, K., 2017. Transcriptional regulatory network of plant heat stress response. Trends Plant. Sci. 22 (1), 53–65. http://dx.doi.org/10.1016/j.tplants.2016.08.015.

Pelham, H.R.B., 1982. A regulatory upstream promoter element in the Drosophila Hsp 70 heat-shock gene. Cell 30 (2), 517–528. http://dx.doi.org/10.1016/0092-8674(82)90249-5.

Perez-Salamo, I., Papdi, C., Rigó, G., Zsigmond, L., Vilela, B., Lumbreras, V., Nagy, I., Horváth, B., Domoki, M., Darula, Z., Medzihradszky, K., Bögre, L., Koncz, C., Szabados, L., 2014. The heat shock factor A4A confers salt tolerance and is regulated by oxidative stress and the mitogen-activated protein kinases MPK3 and MPK6. Plant Physiol. 165 (1), 319–334. http://dx.doi.org/10.1104/pp.114.237891.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Prasad, K.V., Taiyab, A., Jyothi, D., Srinivas, U.K., Sreedhar, A.S., 2007. Heat shock transcription factors regulate heat induced cell death in a rat histiocytoma. J. Biosci. 32 (3), 585–593. http://dx.doi.org/10.1007/s12038-007-0058-4.

Ptashne, M., Gann, A., 1997. Transcriptional activation by recruitment. Nature 386 (6625), 569–577. http://dx.doi.org/10.1038/386569a0.

Richter, K., Haslbeck, M., Buchner, J., 2010. The heat shock response: life on the verge of death. Mol. Cell. 40 (2), 253–266. http://dx.doi.org/10.1016/j.molcel.2010.10.006.

Scharf, K.-D., Berberich, T., Ebersberger, I., Nover, L., 2012. The plant heat stress transcription factor (Hsf) family: structure, function and evolution. Biochim. Biophys. Acta - Gene Regul. Mech. 1819 (2), 104–119. http://dx.doi.org/10.1016/j.bbagrm.2011.10.002.

Schoffl, F., Prandl, R., Reindl, A., 1998. Update on Signal transduction regulation of the heat-shock response. Plant Physiol. 117, 1135–1141. http://dx.doi.org/10.1104/pp.117.4.1135.

Schramm, F., Ganguli, A., Kiehlmann, E., Englich, G., Walch, D., Von Koskull-Döring, P., 2006. The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in Arabidopsis. Plant Mol. Biol. 60 (5), 759–772. http://dx.doi.org/10.1007/s11103-005-5750-x.

Song, Y., Chen, Q., Ci, D., Shao, X., Zhang, D., 2014. Effects of high temperature on photosynthesis and related gene expression in poplar. BMC Plant Biol. 14, 111.

Tang, R., Zhu, W., Song, X., Lin, X., Cai, J., Wang, M., Yang, Q., 2016. Genome-wide identification and function analyses of heat shock transcription factors in potato. Front. Plant Sci. 7, 490. http://dx.doi.org/10.3389/fpls.2016.00490.

Wahid, A., Close, T.J., 2007. Expression of dehydrins under heat stress and their relationship with water relations of sugarcane leaves. Biol. Plant 51 (1), 104–109. http://dx.doi.org/10.1007/s10535-007-0021-0.

Xin, C., Wang, X., Cai, J., Zhou, Q., Liu, F., Dai, T., Cao, W., Jiang, D., 2016. Changes of transcriptome and proteome are associated with the enhanced post-anthesis high temperature tolerance induced by pre-anthesis heat priming in wheat. Plant Growth Regul. 79, 135–145. http://dx.doi.org/10.1007/s10725-015-0119-x.

Xue, G.-P., Sadat, S., Drenth, J., McIntyre, C.L., 2013. The heat shock factor family from triticum aestivum in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. J. Exp. Bot. 65 (2), 539–557. http://dx.doi.org/10.1093/jxb/ert399.

Yokotani, N., Ichikawa, T., Kondou, Y., Matsui, M., Hirochika, H., Iwabuchi, M., Oda, K., 2008. Expression of rice heat stress transcription factor OsHsfA2e enhances tolerance to environmental stresses in transgenic Arabidopsis. Planta 227 (5), 957–967. http://dx.doi.org/10.1007/s00425-007-0670-4.