Improved thermotolerance in transgenic barley by overexpressing a heat shock factor gene (TaHsfA6b) from wheat

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May 5, 2020

Abstract

Temperature is one of the most important abiotic factors defining the yield potential of temperate cereal crops such as barley. The regulators of heat shock response (HSR); Heat shock factors (HSFs) modulate the transcription level of heat responsive genes in order to protect the plants against heat stress. In the present study, a heat shock factor from wheat (TaHSFA6b) is overexpressed in barley for providing thermotolerance. Transgenic barley lines overexpressing TaHSFA6b showed significant improvement in thermotolerance. The constitutive overexpression of TaHSFA6b gene upregulated the expression of major heat shock protein genes as well as other abiotic stress responsive genes. RNA-seq and qRT-PCR analysis showed upregulation of HSPs, chaperonins, DNAJ, LEA proteins and genes related to anti-oxidative enzymes in transgenic lines. Excessive generation and accumulation of ROS occurred in wild type plants during heat stress; however, the transgenic lines reflected improved ROS homeostasis mechanisms in the form of significantly low ROS accumulation under high temperature. There were no negative phenotypic changes in overexpression lines. The present study suggests that TaHSFA6b is one of the major regulators of HSR as it showed the capacity to alter the expression patterns of main defense related genes and enhance the thermotolerance of this cereal crops.

Introduction

Barley (*Hordeum vulgare* L.) is an important cereal crop used for malting, brewing industry and for animal feed worldwide. Barley belongs to Gramineae family and ranks fourth after wheat, rice and maize in worldwide economic importance. Being sessile organisms, plants cannot escape the deleterious effects of heat stress that affect plant growth, physiology and development (Lobel et al, 2007). Heat stress causes changes in various physiological and metabolic processes, such as the production of reactive oxygen species (ROS) leading to oxidative damage of DNA, lipids and degradation of proteins (Wahid et al, 2007). Heat stress is one of the main cause of decrease of agriculture production and yield globally by more than 50% (Wang et al, 2004). Being a temperate cereal, both yield and quality of produce of barley is decreased by heat stress (Lobel et al, 2007; Kalra et al, 2008).

Plants signal transduction pathway leading to thermotolerance is driven by heat shock transcription factors (HSFs) and heat shock proteins (HSPs). When the ambient temperature increases, an increase in fluidity of plasma membrane takes place allowing the influx of Ca^{2+}, which thereby binds to their downstream binding proteins, and leads to the activation of several kinases such as calcium dependent protein kinases, phosphatases, and cytoskeleton reorganization further fosters the upregulation of mitogen activated protein kinases etc. (Hirayama and Shinozaki, 2010, Kudla et al, 2010, Wahid et al, 2007). The activated kinases or phosphatases can phosphorylate or dephosphorylate particular transcription factors (TFs), due to which

the expression levels of stress-responsive genes have been regulated including *HSFs* and *HSPs* (Reddy et al, 2011, Kudla et al, 2010).

Transcription factors play an important role in regulation of gene expression with response to abiotic and biotic stresses. Heat stress transcription factors (HSFs) are the central components of responses to heat stress in plants (Nover and Scharf, 1997; Kotak et al, 2007). Hsfs constitute an important gene family involved in responses to abiotic and biotic stresses as well as in plant growth and development (von Koskull-Doring et al., 2007; Liu et al., 2011; Chauhan et al., 2011).*Arabidopsis* has 21 *HSF* genes (Scharf et al., 2012), tomato has 24 (Scharf et al., 2012; Fragkostefanakis et al., 2015), pepper and rice have 25 (Chauhan et al., 2009; Guo et al., 2015), soybean has 52 (Scharf et al., 2012) and wheat counts 56 HSF genes (Xue et al., 2014).

On the basis of structure, a typical Hsf consists of five conserved motifs, including a DNA- binding domain (DBD), which is connected to an oligomerization domain (OD) consisting of hydrophobic heptad repeats (HR-A and HR- B). Beside DBD and OD, a nuclear localization signal (NLS), a nuclear export signal (NES) and an activator peptide motif (AHA) (Mittal et al., 2009; Chauhan et al., 2011; Scharf et al., 2012) are also present. Based on the structural characteristics of their HR-A/B domain plant Hsf genes are divided into 3 classes: "A", "B", and "C" (Nover et al., 2001; Baniwal et al., 2004). The Class "A" and "C" Hsfs contain an extended HR-A/B with 21 and 7 amino acid residues between the HR-A and HR-B region, respectively, whereas, class B Hsf lack any such insertion in the HR-A/B region (Nover et al., 2001; Baniwal et al., 2004). In addition to this, class "A" Hsfs contain AHA activation domains (rich in (Aromatic, Hydrophobic, Acidic amino acids) that are absent in class "B" and "C" Hsfs (Doring et al., 2000). Thus, class A type Hsfs are involved in transcriptional activation (Shim et al., 2009), whereas Hsfs in class B serve either as repressors of gene expression (Ikeda et al., 2011) or transcriptional coactivators with class A Hsfs (Wang et al., 2014).

In plants, *HSFA1* is constitutively expressed and has a unique function as "master regulator" of heat shock response (Mishra et al., 2002). During normal conditions, HSFA1 is distributed in the cytoplasm (Scharf et al., 1998), and upon activation by heat stress, nuclear localization of HsfA1 starts which then leads to the expression of*HSFA2* and *HSFB1* and the formation of hetero oligomer (termed superactivator complexes) between HSFA1 and HSFA2 (Scharf et al., 1998; Heerklotz et al., 2001). The latter drives HSR by enhanced activation of heat stress response genes expression (Chan-Schaminet et al., 2009). HSFA2 becomes the most prominent HSF under heat stress conditions and recruited by HSFA1 upon heat shock (HS) exposures. Class B HSF members cannot activate themselves and function as either repressors of HS gene expression (Czarnecka-Verner et al., 2004; Ikeda and Ohme-Takagi, 2009; Kumar et al., 2009) or as a co-regulator enhancing the activity of class A HSFs and other housekeeping transcription factors in the context of the histone acetyl transferase–like protein1 (HAC1) (Bharti et al., 2004). *HSFA2* is also considered as an important linker between heat and oxidative stress responses (Chen et al, 2005) as *AtHSFA2* knockout mutants showed reduced response for basal and acquired thermotolerance as well as oxidative stress. On the other hand, overexpression leads to increased tolerance for both heat and oxidative stress. Additionally, overexpression of *HSFA2* in Arabidopsis, also resulted in enhanced tolerance to anoxia and submergence stress as well (Banti et al, 2010). Transgenic Arabidopsis overexpressing *OsHSFA2e* showed thermotolerance in various tissues such as cotyledons, rosette leaves, inflorescence stems and seeds (Yokotani et al, 2008). Charng et al, (2007) reported that *HSFA2* is not only essential for HSR upon heat stress but also in maintaining expression of *HSP* genes in continuous heat stress and recovery period.

In Arabidopsis, other HSFAs, such as HSFA4a and HSFA8, have been suggested to act as reactive oxygen species (ROS) sensors (Davletova et al., 2005), and HSFA4c is involved in root circumnutation, gravitropism and hormonal control of differentiation (Fortunati et al., 2008). On the other hand, HSFA5 is a negative regulator which is specific repressor of HSFA4 isoforms forming a HSFA4-HSFA5 complex (Baniwal et al., 2007).*HSFA7a* and *HSFA7b* are HSR factors (Liu et al., 2011) and*HSFA9* acts as a master regulator of the expression of *HSPs*during seed development (Kotak et al., 2007b). Consequently, it has been suggested that HSFs mediate a cross talk between HS and other abiotic stress-signaling cascades (Kotak et al., 2007a). *HSFA6a* and*HSFA6b* transcript levels are particularly induced in response to salt, osmotic, and cold stress (von Koskull-Döring et al., 2007; Hwang et al., 2014). Further, Hwang et al (2016) found that expression

of*HSFA6b* extensively increased with salinity, osmotic, and cold stresses, and also showed that it plays an important role in the response to ABA and in thermotolerance.

Previously, Chauhan et al (2013) showed that overexpression of a seed preferential HSF from wheat *TaHSFA2d* (later renamed as*TaHSFA6b* after classification of Scharff et al, 2012) in Arabidopsis provided increased heat and other abiotic stress tolerance. In the present study, we overexpressed *TaHSFA6b* in transgenic barley for enhancing thermotolerance observed a decrease in ROS under heat stress conditions, an improved stress tolerance and no negative phenotypical changes.

Materials and methods:

Plant material, growth conditions

Spring barley (*Hordeum vulgare L* .) cultivar Golden promise was grown in field condition to get the supply of immature embryos for barley transformation. Spikes were collected after 12-14 days after anthesis for immature embryos of about 1.5 to 2 mm in size. Immature embryos were dissected from young caryopsis and the embryonic axis was removed with a sterile surgical blade and put on the barley callus induction media with scutellum side up. For barley transformation methods described by Harwood and Smedly (2009) was followed.

Cloning of *TaHsfA6b* cDNA into binary vector for over-expression and sub-cellular localization

For transformation and over-expression studies, 1396 bp long full length cDNA of *TaHSFA6b* along 5' and 3' UTR (Gen Bank acc. No. KF061193, Chauhan et al 2013) was cloned in binary vector *pANIC6B* (Mann et al, 2011) through Gateway^{TM} methodology. For subcellular localization**,** the ORF of *TaHSFA6b* without its stop codon was PCR-amplified by using primer pairs*orfTaHSFA6b-F*-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGACCGGGTGCTGCTGC and*orfTaHSFA6b-R* -GGACCACTTTGTACAAGAAAGCTGGGTACGCGTCGACATATCTAGATCTCCTCC and cloned into *pDONR221* and subsequently into binary vector*pB7FWG2* by Gateway^{TM} cloning to construct*pB7FWG2:35S:TaHSFA6b-GFP* . The cloned sequence was verified by sequencing.

Transient expression in *N. benthamiana* and confocal laser scanning microscopy

The preparation of *Agrobacterium* and transient expression in *N. benthamiana* was performed as previously described (Li, 2011). The vector *pB7FWG2:35S:TaA6b-GFP* was transformed into *A. tumefaciens* GV3101. *A. tumefaciens* GV3101 harboring the binary vector was grown in LB-broth media containing 50 mg/L of spectinomycin, 30 mg/L gentamycin, 30 mg/L of rifampin to the stationary phase at 28 degC. After centrifugation, *A. tumefaciens* was resuspended in the infiltration medium (10 mM $MgCl_2$, 100 µM acetosyringone) to a final OD_{600} of 0.5. The suspension was infiltrated with a 1-mL needleless syringe into the abaxial surface of 4-week-old leaves of *N. benthamiana* . Protein localization was analyzed 24-48 hours after infiltration by confocal laser scanning microscope (TCS SP8, Leica). GFP was excited at 488 nm and the fluorescence detected between 498-525 nm.

Particle bombardment mediated transformation of barley

Particle bombardment transformation was conducted using the Particle Delivery system PDS-1000/He (Bio-Rad) as per the protocol described by Harwood and Smedly, 2009. For this the entire fragment representing selection, reporter and *TaHSFA6b* cassette was isolated by digesting the *pANIC6B:TaHSFA6b* plasmid by *Eco* RV and*Pme* I restriction enzymes (Supplementary Figure S1). One day after isolation, 25–35 immature embryos were placed keeping scutellum side up, on callus induction media containing 0.4M mannitol in the centre of each plate. Embryos were kept on the osmoticum medium for 4-6 hours before the bombardment and post bombardment the plates were kept at 8°C overnight. Next day, embryos were removed from the osmoticum plates and transferred to callus induction media containing 50 mg/L hygromycin for selection and kept at 22°C in the dark. After 14 days calli derived from transformed embryos were sub-cultured on fresh callus induction plates with 50 mg/L hygromycin. After four weeks, these calli were further, transferred to regeneration media (MS supplemented with 0.5mg/l 6-BAP) with reduced hygromycin concentration to 25

mg/L, and placed at 22°C in 16:8 light and dark. Well-developed plantlets with proper roots were carefully removed from the plates and transferred to Magenta boxes without any growth regulator further for 4-6 weeks. Well rooted plantlets were transferred to the pots with 1:1 mixture of farm soil and soilrite (Kel Perlite, India) for hardening and kept in green house.

After 5 days post bombardment and growth on callus induction media, histochemical assay for Gus activity was performed by dipping the transformed calli in Gus buffer solution for 24 hours at 37 °C to check transformation (Jefferson et al,1987).

Confirmation and transgene expression analysis of transgenic lines

Genomic DNA was isolated from the leaves of putative barley transgenic plants by using DNA express solution as per manufacturer's instructions (Himedia). Primers amplifying gene of interest *TaHSFA6b*(*TaHSFA6b Full F -R*), and selection marker gene hygromycin (*HPT Full F -R*) were used to confirm their integration in barley transgenic lines (Supplementary Table 1). Transgene expression of*TaHSFA6b* was analyzed by RT-PCR in T1 generation of progeny of transgenic lines.

Determination of antioxidant enzymes activities

Preparation of plant extract

For assessment of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) enzymes activities, 500 mg of leaf tissue of both transgenic and wild type barley plants was frozen in liquid nitrogen followed by grinding in 5 mL of one step extraction buffer containing 100 mM phosphate buffer (pH 7.5), 0.5 mM EDTA and 1% PVP. The homogenate was filtered through 4 layers of cheese cloth followed by centrifugation at 15000x g for 20 min at 4°C. The supernatant was collected and used for assays as described below. All the steps in enzymes preparation and activity assays were carried out at 4 °C.

Estimation of Superoxide dismutase activity

SOD activity was analyzed by monitoring the inhibition of the photochemical reaction to nitroblue tetrazolium photoreduction (NBT) as described by Dhindsa et al. (1981). The composition of reaction mixture contains 1.5 ml phosphate buffer (pH 7.8, 100 Mm), water 900µl, methionine 200µl of 200 µM, NBT 100 µl, EDTA 100 µl (3Mm), sodium carbonate 100 µl (1.5 M), enzyme extract 300 µl and the riboflavin was added lastly (100 µl, 60 µM). For proper mixing, tubes were inverted 2-3 times and kept under fluorescent light. The lights were turned off after 15 minutes to stop the reaction and the tubes were immediately placed in dark. Absorbance was measured by UV/VIS spectrophotometer at 560 nm, and one unit of SOD activity (U) was defined as the amount of enzyme required to cause the 50% inhibition of the NBT rate. The results were expressed in Umg^{-1} protein.

Estimation of Catalase activity

Catalase activity was measured according to Aebi et al. (1984). The composition of enzyme assay mixture consisted of 100 µL enzyme extract, 100 mM phosphate buffer (pH 7.0) and 30 mM H_2O_2 in a total volume of 1.5 ml. The H_2O_2 was added in the last and absorbance was measured by UV/VIS spectrophotometer at 240 nm. The disappearance of substrate (H_2O_2) by enzymatic breakdown was monitored by decrease in absorbance till 3 minutes.

Estimation of Ascorbate peroxidase activity

The activity of ascorbate peroxidase enzyme assay was measured according to Nakano and Asada (1981). The composition of enzyme assay mixture consisted of 150 µL enzyme extract, 50 mM phosphate buffer (pH 7.0), 0.2 µM EDTA, 0.2mM ascorbate, and 2.0 mM H_2O_2 in a total volume of 3 ml. Ascorbate oxidation was measured by taking the absorbance at 290 nm by UV/VIS spectrophotometer at the moment of H_2O_2 addition and 1 min later. The difference in absorbance (ΔA290) was divided by the ascorbate molar extinction coefficient (2.8 $Mm^{-1}.cm^{-1}$). The enzyme activity was expressed as µmol of $H_2O_2min^{-1}$ mg^{-1} protein, taking into account that 1.0 mol of ascorbate is required for the reduction of 1.0 mol of H_2O_2.

Expression analysis by RNA sequencing and qRT-PCR

RNA isolation and sequencing

Total RNA was isolated from leaf sample from five-week old *TaHSFA6b* overexpressing transgenic plants and wild type plants using the RNEasy plant RNA isolation kit with on column DNase digestion, as per manufacture's instruction (Qiagen, Germany). For this two overexpressing lines were used along with 2 replicates of wild type plants. High quality RNA samples were sent to Bionivid Technology Private Limited (Bengaluru, India) for RNA-sequencing by Illumina HiSeq platform with a read length of 150bp.

Quality control of the all the reads was done using NGSQC Tool kit (Patel et al. 2012) and reads having a Phred score >Q30 were selected for further analysis. For alignment of reads and transcript identification, the reference genome of barley was downloaded from ENSEMBLE (plants/release40/gff3/Hordeum-vulgare). For alignment of reads on the reference genome of barley TopHat pipeline was used (Trapnell et al. 2009). Cuffdiff and Cufflink pipelines were used for identification of differentially expressed transcripts in transgenic lines and wild type by using default parameters (Trapnell et al. 2009). Transcripts with fold change log2 ratio[?]2 were considered as differentially expressed transcripts. Unsubstantiated hierarchical clustering of differentially expressed genes was performed by using Cluster 3.0 and visualized by using Java Tree View. Gene ontology and KEGG pathways that contained expressed transcripts were identified by using DAVID Functional Annotation Tool (DAVID Bioinformatics Resources 6.8, NIAID/NIH) (Hua ng et al. 2009).

Results

Sub cellular localization and generation of overexpression lines in barley for *TaHSFA6b*

Chauhan et al. (2013) have shown that TaA6b has all the characteristic features of a class A Hsf such as DNA binding domain, HR A/B region, C-terminal AHA type transactivation domain and a predicted nuclear localization signal. To assess the subcellular localization of TaA6b, we made a C-terminal GFP fusion construct and transiently expressed it in *Nicotiana benthamiana* leaves. In Figure 1, it can be seen that the distribution of GFP is uniform in nucleus and cytoplasm in both control and heat stress (HS) conditions, suggesting that TaA6b protein is localized in both nucleus and cytoplasm regardless of the presence of HS, it should be noted that it is absent from the nucleolus.

Previously, *TaHSFA6b* has been shown to increase heat stress tolerance in Arabidopsis upon over expression. In the present investigation we have over expressed *TaHSFA6b* in barley by cloning *TaHSFA6b* under control of *ZmUbi* promotors. The transformation was done by biolistic method and a total of 40 lines were generated by using immature embryos of Golden promise as per the protocol described by Harwood and Smedly (2009). Transgenic lines were confirmed by PCR amplification of complete cDNA clone of *TaHSFA6b*and *HPT* gene (Supplementary Figure S2A and B). Further, PCR product of 4 different lines viz. 1, 2, 6 and 8 were sequenced and they all were found to have the wheat gene i.e *TaHSFA6b* . Over expression of *TaHSFA6b* was confirmed in T1 generation by RT-PCR. Most of the transgenic lines were found to constitutively express *TaHSFA6b* revealed by RT-PCR (Supplementary Figure S3). For all further analysis, plants were grown in controlled environment and other analysis were done on the plants of T2 generation in 3 different transgenic lines viz. AP2-1, AP2-2 and AP6-2.

Analysis of thermotolerance

To check if the *TaHSFA6b* overexpression plants are showing increased tolerance towards high temperature, we grow plants of 3 transgenic lines and WT in temperature controlled environment of 35 ^{o}C right from germination. We found that initially all seeds germinated and grow normally during first 5-6 days (1 leaf stage), however, afterwards WT plants started showing reduced growth and with flaccid leaves, while plants of transgenic lines keep growing and increasing in height and making new leaves. After 3 weeks of providing continuous heat stress environment, all the plants of WT either showed senescence or died, while those of 3 transgenic lines were healthier and greener, and making new leaves (Figure 2).

Since elevated temperature results in production of reactive oxygen species we checked the status of free

oxygen radicals in plant grown under constant temperature by NBT staining method (Wohlgemuth et al. 2002). We found marked difference in staining in leaf of WT and transgenic lines. While there is not much difference in the leaves of WT plants and transgenic lines under control conditions, however, marked difference is observed in NBT staining under heat stress condition. It was much less in the transgenic lines AP2-2 and AP6-2 (Figure 3A) compared to wild type. Further, to quantify the activity of Reactive Oxygen Species (ROS), we measured the activity of 3 enzymes: Ascorbate peroxidase, Catalase and Superoxide dismutase. We found the all these enzymes showed higher activity in transgenic plants as compared to WT plants. Ascorbate peroxidase and catalase showing significantly higher activity under heat stress conditions (Figure 3B).

It has been shown that HSFs and specially A type have profound effect on global transcriptome of plant. We proceeded for transcriptomics through RNA-sequencing of WT and plants from two transgenic lines (AP 2-1 and AP 2-2). A total of 103 million reads each were obtained from WT and trangenic plants. Principal component analysis (PCA) revealed a clear separation between replicates of WT and transgenic plants (Figure 4A). Analysis of differential gene expression revealed that a total of 1565 transcripts differentially expressed with a log2 fold change of$>$ 2 and p-value of 0.05, these represent 663 upregulated and 902 downregulated genes (Supplementary Table S2).

To get an idea what the top upregulated genes are involved in, we used signature search option in Genvestigator omics analysis tool (https://genevestigator.com/gv/doc/intro_plant.jsp) by choosing available RNA sequencing experiments for barley. As can be seen from supplementary figure S4, our upregulated genes are mostly involved in plants responses to abiotic stresses such as heat, drought and salinity. A careful analysis of upregulated genes revealed that these genes belong to heat shock proteins and other molecular chaperones, genes related to water and oxidative stress and biosynthesis of stress related metabolites (Supplementary Table S2). To confirm RNA sequencing data, we performed qRT-PCR expression analysis on some selected genes representing HSPs, ROS scavenging enzymes, drought response and stress signaling components. As can be seen in figure 5, all these genes showed significantly higher expression in terms of relative fold change in different transgenic lines as compared to wild type plants. Further, we checked the performance of wild type and transgenic plants from line AP2-2 under simulated water stress. For this, seeds were sown in nursery pots and water was given till filed capacity and the pots were kept in a greenhouse kept at 26°C temperature and 16:8 hour photoperiod. No water was given after first instance and the plants were observed for four weeks from date of sowing. All the seeds germinated well and start growing normally, however after three weeks, signs of water stress were visible as the leaves start rolling and limping. At four week stage, all the wild type plants showed severe limping of leaves, on the other hand plants from transgenic line AP2-2 were still showing erect and longer leaves (Figure 6A). We measured relative water content in last fully opened leaves of both wild type and transgenic plants and found that transgenic plants had around 50% more relative water content than wild type plants (Figure 6B).

Discussion:

A number of studies have been carried out to functionally characterize the HSF genes for their potential role in thermotolerance and its signaling (Chauhan et al., 2013, Wang et al., 2017, Zhu et al., 2018). *TaA6b* is mainly nucleo-cytosolic and heat stress does not affect its nucleocytoplasmic distribution (Figure 1). Huang et al. (2016) reported *HSFA6b* from Arabidopsis to be localized to both the nucleus and cytosol, with partial but not complete translocation to the nucleus which is in consistence with our results. Hu et al (2015) have also found that fourteen Hsfs in *Fragaria vesca* localized in nucleus, out of which 6 were also localized in cytosol. Baniwal et al., (2007) studied two Hsfs from tomato and found that *HsfA4b* was localized in the nucleus, whereas *HsfA5* was predominantly detected in the cytoplasm.

It has been demonstrated that transgenic plants with overexpression of HSF genes show improved thermotolerance, however, most of the studies were based on model plants like *Arabidopsis* and tobacco (Busch et al. 2005, Li et al., 2005, Zhu et al., 2009, Wu et al., 2018). Heat induced up-regulation of class A6 HSFs has been observed in wheat and *TaHSFA6b* was one of the predominantly expressed genes in the leaves during early heat stress conditions (Xue et al., 2013), which suggests that the constitutive overexpression of *TaHS-*

FA6b may enhance tolerance to high temperature conditions. Previously, it has been shown that *TaHSFA6b* provides tolerance against high temperature stress in transgenic *Arabidopsis* (Chauhan et al., 2013).

In the present study, the role of *TaHSFA6b* gene in enhanced thermotolerance of transgenic barley has been investigated. Transgenic lines of barley constitutively overexpressing the *TaHSFA6b* gene under the control of *ZmUbi* promoter showed notable thermotolerance (Figure 2). Enhanced thermotolerance of these plants can be explained by increased expression of abiotic stress responsive genes including antioxidative enzymes (catalase, glutathione S-transferase and peroxidase), HSPs, LEA protein and proteins involved in Ca^{2+} signaling pathway. Similarly, Xue et al., 2015 reported that *TaHSFA6f* regulates the transcription of heat responsive genes involved in heat stress signaling cascade including Hsps and Golgi anti-apoptotic protein (GAAP).

Excessive generation and accumulation of reactive oxygen species (ROS) severely affect the plant cell by increased levels of oxidative injuries leading to destruction of cellular structure and function (Schutzendubel and Polle, 2002). Barley transgenic lines showed significantly low levels of superoxide accumulation in comparison to wild type plants under high temperature conditions (Figure 3A). This difference in superoxide accumulation can be attributed by the quenching of excessive ROS levels by upregulated antioxidative defense system. Chloroplasts and mitochondria are the major sites for ROS production under heat stress (Suzuki and Mittler, 2006). Ascorbate peroxidases (APX) are the main enzymes which regulate the release of ROS from their generation site (Koussevitzky *et al.* , 2008); upregulated APXs ameliorate the oxidizing environment created by the high ROS accumulation (Badawi et al., 2004, De Pinto et al., 2015).

Deficient expression of catalase (CAT) results into increased levels of peroxide (H_2O_2), which gives rise to imbalance in ROS homeostasis; upregulation of CAT removes the excess H_2O_2 and maintains the ROS balance (Vandenabeele et al., 2004).

Transcriptome analysis revealed that *TaHSFA6b* regulated genes are mostly the genes which are heat, draught and oxidative stress responsive. In the present study it was observed that the transcription of HSPs was positively affected and higher expression of HSPs demonstrated in RNA-seq data and confirmed by qRT-PCR analysis (HSP70-2, HSP70-5, HSP90-2, and HSP18.1). Protein folding, intracellular localization and degradation are considered as the primary functions of Hsps (Qu et al., 2013), however, the important role of HSPs during heat and other abiotic stresses has been well studied in different plant systems (Hu et al., 2009, Jacob et al., 2017). In a heat tolerant cultivar of rice; N22, a significant upregulation of HSPs was observed demonstrating the important role of HSPs in plants (Jagadish et al., 2010).

The constitutive upregulation of chaperonins (Cpn60-1, Cpn60-2, and Cpn60-10) was also observed in *TaHvHSFA6b* overexpressing transgenic barley lines. Chaperonins are the high molecular weight complex proteins helping the chaperons in protein folding (Evstigneeva et al., 2001, Reddy et al., 2016). Chaperonins have been reported to function in folding the newly translated proteins as well as assisting the chaperons in refolding denatured proteins under stress conditions (Hill et al., 2001, Levy-Rimler et al., 2002, Wang et al., 2004). Increased expression of DNAJ proteins was also observed; DNAJ proteins work as co-chaperones with HSP70 and regulate the protein homeostasis (Pulido et al., 2017). Wang et al., 2019 reported that DNJ protein of*Solanum lycopersicum* (SlDnaJ20) showed heat inducibility and DNJ20 overexpressing transgenic tomato plants performed better under heat stress conditions in terms of fresh biomass, total chlorophyll, chlorophyll fluorescence and less accumulation of ROS.

Heat stress creates oxidizing environment inside the cell by over production of ROS, which leads to peroxidation of membrane lipids (Mansoor et al., 2013). The aldehyde dehydrogenase (ALDH) is known for removing the lipid peroxidation generated reactive aldehydes (Brocker et al., 2012). The constitutive upregulation of ALDH gene in*TaHSFA6b* overexpression lines suggests their superiority over wild type plants in terms of less availability of reactive aldehyde species, as these are the most cytotoxic substances produced downstream of the ROS (Mano et al., 2019).

Extreme environmental conditions like high temperature, desiccation, salinity, and freezing; result into decreased cellular water content in plants (Fahad et al., 2017). Under high temperature conditions, a steep

decrease in cellular water content was observed in tomato plants (Morales et al., 2003). Expression of LEA proteins is upregulated to protect the cell water content and to prevent the dehydration caused protein denaturation (Kovacs et al., 2008). Goyal et al., (2005) reported that LEA protein may also act as chaperons and showed that these proteins can prevent the heat induced inactivation and aggregation of different enzymes such as citrate synthase. In the present study, LEA proteins expression levels were remarkably high in transgenic lines in comparison to wild type plants, which contributes to explaining the better performance of transgenics over wild type plants.

Global climate change and increasing temperatures are major issues of international concern. High temperature severely affects the growth, production and final yield capacity of food crops, therefore, it is important to understand the plant responses towards heat stress at physiological, biochemical and molecular levels. HSF and HSP genes have been well studied and suggested as key players in plant heat stress response, however there is no sufficient knowledge about the roles and stress mitigation potentials of individual genes in cereal plants. Conclusively, the present study investigated and characterized a heat responsive HSF gene of wheat; *TaHSFA6b* in barley. *TaHSFA6b*plays regulatory role in heat shock response pathways. Transgenic lines of barley showed notable superiority over wild type under high temperature conditions. The positive alteration in thermotolerance may be attributed by the coordinated upregulation of defense mechanisms related genes in transgenics. Transcriptomic analysis of overexpression lines suggests that *TaHSFA6b* works as an activator of HSPs as well as other stress responsive genes. Therefore, we suggest that the *TaHSFA6b* gene may be used for molecular breeding to generate heat-tolerant cultivars of temperate cereal crops which are highly susceptible to heat stress.

Acknowledgements

This work is supported by an early career research award (ECR/2016/000084) by SERB-DST, Government of India to HC. AKP, SKM and PS thank MHRD and RC thanks UGC for research fellowships. PS also thanks research internship by FRQNT, University of Quebec, Trois-Rivières Quebec, Canada.

Conflict of interest

Authors declare no conflict of interest.

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Table 1: Transcriptomic analysis of barley transgenic lines by RNA sequencing: s ummary of total number of high quality reads and bases were obtained from RNA sequencing data of barley. The bases represent having > 30 phred score and reads represents> 70 percent high quality bases.

S.NO	Sample name	Total high quality reads	Total high quality bases
1	Line 2-1 and Line 2-2	63,227,3347	9,56,790,1,890
2	WT-1 and WT-2	40,140,844	2,978,890,272

Figure legends:

Figure 1. Subcellular localization of TaA6b Hsf. The ORF of *TaA6b*was cloned in *CaMV35S::TaA6b-GFP* vector in which GFP was fused at the C-terminus. The TaA6b-GFP fusion protein and GFP control were transiently expressed in *N. benthamiana* leaves and observed by fluorescence microscopy. The left column is the green fluorescence channel, the center column is the DIC and the right column is the merge of the two images.

Figure 2. Effect of constant heat stress on transgenic lines (Line 2-1, Line 2-2 and Line 6-2) and wild type control plants (WT) in greenhouse with constant temperature at 35 ^{o}C. Seeds were germinated in 2 inch nursery pots and kept in temperature controlled greenhouse from sowing and pictures were taken at specified time.

Figure 3.A. Detection of reactive oxygen species by NBT staining method in the leaves of barley plants from wild type (WT) and transgenic (AP2-2 and AP6-2) plants over-expressing wheat HSF (*TaHsfA6b*) under control and heat stress conditions.

B. The activity of three different ROS scavenging enzymes viz ascorbate peroxidase (APX), catalase and super oxide dismutase (SOD) in wild type (WT) and transgenic (AP2-2 and AP6-2) plants under heat stress conditions. Error bars SD (n=5), P values **= <0.05 and ***= <0.001

Figure 4.A. Principle component analysis of RNA sequencing in WT (RCWT) and transgenic plants of two lines AP 2-1 and AP 2-2, showing grouping of biological replicates of WT and transgenic lines.

B. Volcano plot showing comparison of differentially expressed genes (DEGs) in wild type and transgenic

barley plants. NS- Not significant , Log2 fold change- transcripts with fold change of >2 but non significant p-value, P-Transcript with p value<0.05, log2FC& P differentially expressed transcripts with log2FC >2 and p-value < 0.05.

Figure 5. qRT-PCR expression analysis of some upregulated genes in RNA-seq data (Supplementary table S2). Y-axis represent relative fold change calculated by 2ˆ-ddct method.

Figure 6. Analysis of transgenic and WT plants under simulated drought stress treatment.A. 4 weeks-old wild type and transgenic plants of overexpression line AP2-2, under limited water condition.

B. Relative water content of leaves of wild type and transgenic plants of line AP2-2 kept under water limitation condition.

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